Disease associations of primary bile acid diarrhoea and investigations into potential treatments and a possible metabolic syndrome of low fibroblast growth factor 19

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Dedication

For Claire, the love of my life, with whom I look forward to continuing the process of lifelong enlightenment for the rest of my days and my mother who started me on that path.

With my sincere gratitude to Julian for his support, understanding and mentorship that surpassed that expected of any supervisor, and probably many saints. Also to those that that made the day-to-day so enjoyable and prevented a multitude of laboratory catastrophes, particularly Jon and Fauza.

This work is dedicated to all those teachers, both formal and informal that encouraged and nurtured a young mind despite seemly inescapable odds and probable ingratitude at the time.

Finally I would like to acknowledge the undergraduates that have worked on these projects and I hope they will one day be writing a thesis dedication themselves; Gagan, Sima, Iman and Jenna.

Abstract

The endocrine growth factor FGF19 controls bile acid synthesis and contributes to lipid and glucose metabolism. Low levels are associated with bile acid diarrhoea (BAD), high triglycerides and obesity raising the possibility of metabolic syndrome incorporating chronic diarrhoea. The pathogenesis of low FGF19 in individuals is not known and treatments are currently not available. The only available treatment for BAD may lower FGF19 further.

Analysis of a retrospective database of 303 patients with BAD and showed an OR of 2.5 with NAFLD. Prospective studies of 127 patients with NAFLD showed an OR of 6.2 for BAD. This was associated with increased BA synthesis but with normal FGF19, possibly indicating hepatic FGF19 resistance. Experiments using human ileal explants showed that patients with BAD had lower median FGF19 mRNA fold change in response to chenodeoxycholic acid stimulation (16 (range 8-204) vs. 185 (73-416)). Incubation with 50mM cafestol showed a 3.4 fold increase in FGF19. Using the same model, the addition of 100mM ursodeoxycholic acid (UCDA) to 5mM obeticholic acid (OCA) increased FGF19 expression by 2.5 times that of OCA alone, which has therapeutic consequences. A placebo controlled trial showed that the FGF19 lowering effect of bile acid sequestrants can be ameliorated by administering them in colonic release capsules for BAD.

I conclude that although there are significant disease associations of primary BAD (NAFLD, gallstones, hypertriglyceridaemia), this association can't be explained by low FGF19 alone. Since all groups appear to have dysregulated BA metabolism, it appears that there are multiple pathophysiological mechanisms underlying BAD, including reduced ileal FGF19 expression and hepatic FGF19 resistance. The novel binding mechanism of UDCA to ileal BA-binding protein maybe used to increase the potency of OCA. Colonic release BA sequestrants are a viable treatment for patients with BAD and increase FGF19 compared to traditional sequestrants.

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List of abbreviations

5HT	5-hydroxytryptamine
ABX	Antibiotics
AE	Adverse event
ALT	Alanine transaminase
AMPK	AMP-activated protein kinase
ANOVA	Analysis of variance
APCI	Atmospheric pressure chemical ionisation
ASBT	Apical sodium linked bile acid transporter
AST	Aspartate transaminase
ATF4	Activating transcription factor 4
AU	Arbitrary units
BA	Bile acid
BAD	Bile acid diarrhoea
BAM	Bile acid malabsorption
BAS	Bile acid sequestrant
BD	*Bis en die*; twice daily
BM	Bowel movement
BMI	Body mass index
BSEP	Bile salt export protein
BSFS	Bristol stool form scale
C4	Hydroxy-4-cholesten-3-one
CA	Cholic acid
CAF	Cafestol
CAR	Constitutive androstane receptor
CCX	Cholecystectomy
CDCA	Chenodeoxycholic acid
cDNA	Complimentary deoxyribonucleic acid
CFTR	Cystic fibrosis transmembrane conductance regulator
CI	Confidence interval
CM	Complete medium
CRC	Colonic release cholestyramine
CT	Computerised tomography
Ct	Cycle threshold
CTGF	Connective tissue growth factor
CX	Cholecystectomy
CYP7A1	Cholesterol 7α-hydroxylase
DCA	Deoxycholic acid
DM	Diabetes mellitus
DMEM	Dulbecco's modified eagles medium
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EC50	Effective concentration 50%
eCRF	Electronic clinical research file
ELISA	Enzyme-linked immunosorbent assay
ERK 1/2	Extracellular signal-regulated kinase 1 / 2
FGF15/19	Fibroblast growth factor 15/ 19
FGFR1c	Fibroblast growth factor receptor 1c
FGFR4	Fibroblast growth factor receptor 4
FPR	Formyl-peptide receptor
FXR	Farnesoid X receptor
GAPDH	Glyceralderhyde-3-phophate dehydrogenase
GCDCA	Glyco-Chenodeoxycholic acid
GLP-1/2	Glucagon-like peptide -1 / 2
Glu	Glucose
GS	Gallstones
HCC	Hepatocellular carcinoma
HDL	High density lipoprotein
HNP-4α	Hepatocyte nuclear receptor factor 4-α
HOMA	Homeostatic model assessment
IBABP	Ileal bile acid binding protein

IBAT	Ileal bile acid transporter
IBS-D/C/A	Irritable bowel syndrome-diarrhoea / constipation/alternating predominant
IF	Intestinal failure
IFG	Impaired fasting glucose
IMP	Investigational medicinal product
IQR	Interquartile range
IR	Insulin resistance
ITT	Intention to treat
KLB	Klotho-β
KO	Knock out
LCA	Lithocholic acid
LDL	Low density lipoprotein
LRH-1	Liver receptor homolog-1
MAF	Minor allelic frequency
MC	Microscopic colitis
miRNA	Micro ribonucleic acid
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
n	Number
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NOS	Nitric oxide synthase
NTCP	Sodium taurocholate co-transporting polypeptide
OATT	Oral-anal transit time
OCA	Obeticholic acid
OR	Odds ratio
OSTα / β	Organic solute transporter α / β
pBAD	Primary bile acid diarrhoea
PBC	Primary biliary cirrhosis / cholangiopathy
PCR	Polymerase chain reaction
PGC1-α	Peroxisome proliferator-activated Receptor γ co-activator 1-α
PP	Per-protocol
PPARγ	Peroxisome proliferator-activated Receptor γ
PXR	Pregane X receptor
PYY	Peptide YY
qPCR	Quantitative PCR
REC	Regional ethics committee
RQ	Relative quotient
RSV	Resveratrol
rtPCR	Real time PCR
RXR	Retinoid X receptor
SAE	Serious adverse event
sBAD	Secondary bile acid diarrhoea
SD	Standard deviation
SE	Standard error
SeHCAT	^{75}Selenium–homocholic acid taurine
SGLT-2	Sodium-glucose co-transporter 1
SHP	Small hetrodimer partner
SIRT1	Sirtuin 1
SNP	Single nucleotide polymorphism
SREBP 1/2	Sterol regulatory element-binding protein 1/2
TC	Total cholesterol
TCDCA	Tauro-chenodeoxycholic acid
TG	Triglycerides
TGR5	G protein-coupled BA receptor
TNFα	Tumour necrosis factor α
UCDA	Ursodeoxycholic acid
US	Ultrasound
V1-5	Visit 1/2/3/4/5
W1-6	Week 1/2/3/4/5/6
WAT	White adipose tissue

Declaration of originality

The work contained within this thesis is my own and completely original. Where experiments have been performed by colleagues or collaborators and the data supplied to me for combination with my own, it has been clearly stated in the methods section. All analyses, interpretation and comment are my own.

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1 Introduction

1.1 Bile acid synthesis and the enterohepatic circulation

Bile acids (BAs) are formed from cholesterol within hepatocytes. Their synthesis from cholesterol involves 17 enzymes, of which the rate- limiting step is the 7α-hydroxylation of sterol precursors by the enzyme cholesterol 7α-hydroxylase (CYP7A1). The BAs are then conjugated with glycine or taurine. This classical pathway creates 95% of BAs.[1]

The conjugated BAs are secreted by the hepatocytes in to the biliary tract by the bile salt export protein (BSEP) and stored in the gallbladder.[2] When food enters the duodenum, cholecystokinin is released, stimulating gall bladder contraction and secretion of bile in to the small bowel. A small

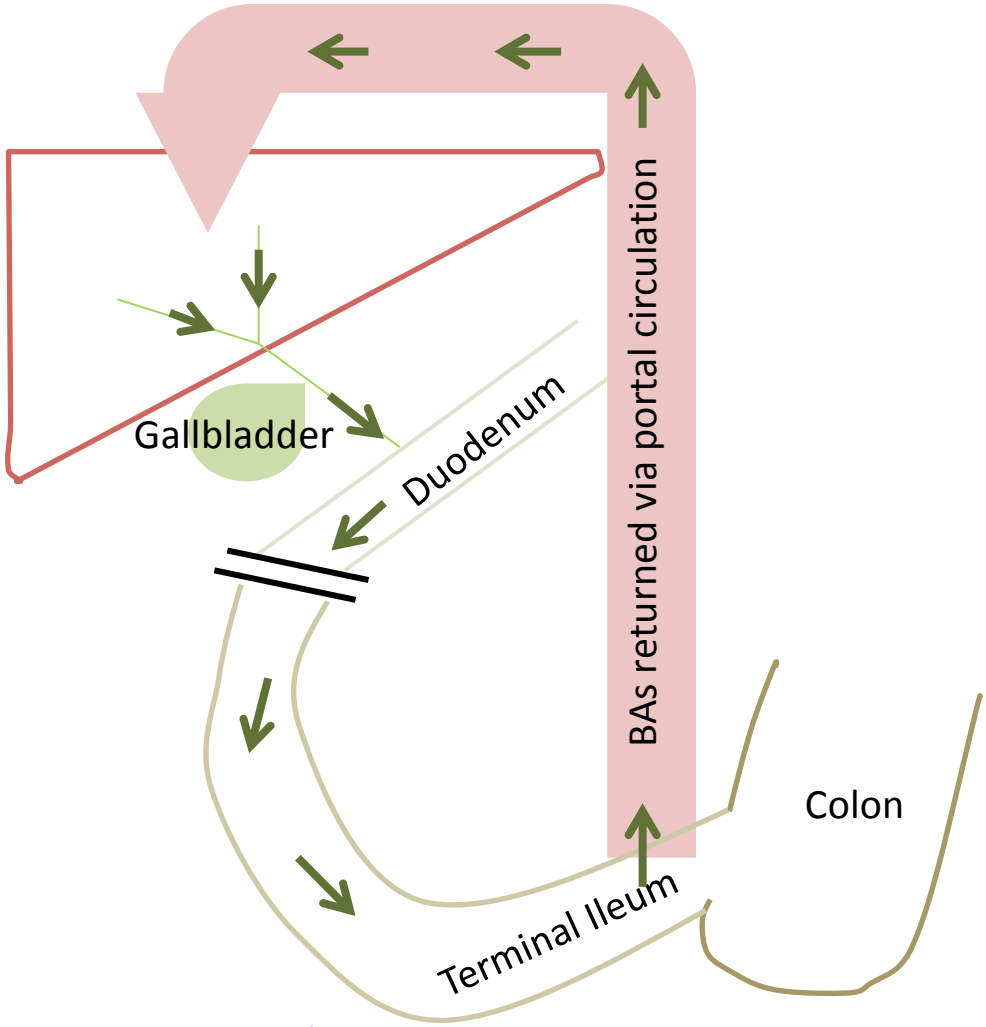


Figure 1:1 Enterohepatic circulation of bile acids.

amount of bile acid is absorbed in the proximal jejunum.[3] When bile reaches the terminal ileum, over 90% is absorbed and returned to the liver via the portal circulation.[4] 600mg of BA is produced daily, with the entire bile acid pool cycling 6-7 times per day.[5]

The liver synthesises 2 primary BAs: chenodeoxycholic acid (CDCA) and cholic acid (CA), these can be dehydroxylated by intestinal bacteria to the secondary BAs; lithocholic acid (LCA) or deoxycholic acid (DCA). All 4 of these BAs can be absorbed in the ileum and then conjugated and re-excreted by the liver.[6] Once in the colon the bile acids can undergo further bacterial modification changing the bile acid structure through oxidisation or modification of their side-chains.[7]

Bile acids perform many functions; they are vital for intestinal lipid absorption, cholesterol homeostasis, excretion of lipid soluble waste and heavy metals. Within the biliary tract and intestine they form mixed micelles with fatty acids and monoglycerides that in turn incorporate fat soluble vitamins A, D, E and K that allow intestinal absorption of these substances.[8] Disturbance in bile acid homeostasis can lead to liver disease, gallstones and chronic diarrhoea or constipation.

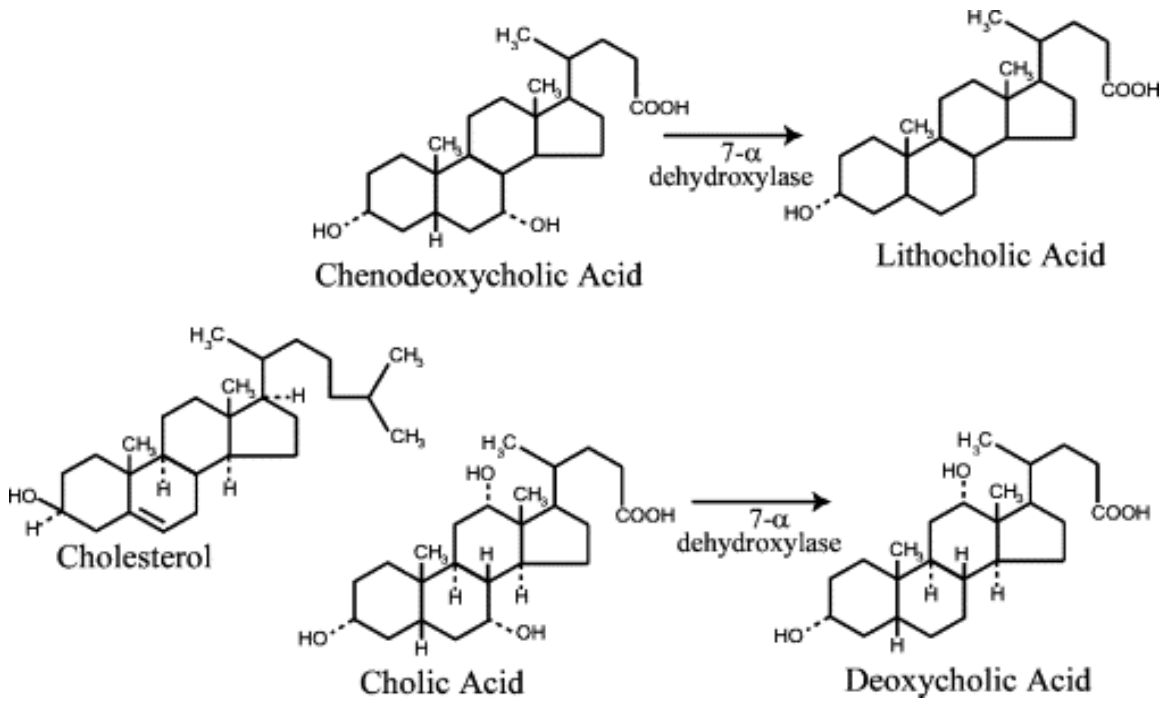


Figure 1.2 Chemical structure of cholesterol, the 2 primary bile acids, chenodeoxycholic and cholic acid and their respective deoxyfied structures as secondary bile acids lithocholic and deoxycholic acid.[9]

1.2 Bile acid transport in liver and intestine

1.2.1 Intestinal bile acid transport

A small amount of conjugated BAs are absorbed passively throughout the length of the small intestine through paracellular junctions and thorough carrier mediated transport, but the vast majority is thorough active uptake at the terminal ileum.[3]

Uptake of BAs at the apical surface of the ileal mucosa is by active transport. This is performed by the apical sodium linked bile acid transporter; ASBT (also known as the ileal bile acid transporter, IBAT). BAs are then bound to the ileal bile acid binding protein (IBABP) within the cytoplasm and transported across the basolateral membrane by the heterodimer organic solute transporter α and β (OSTα and OSTβ).[9]

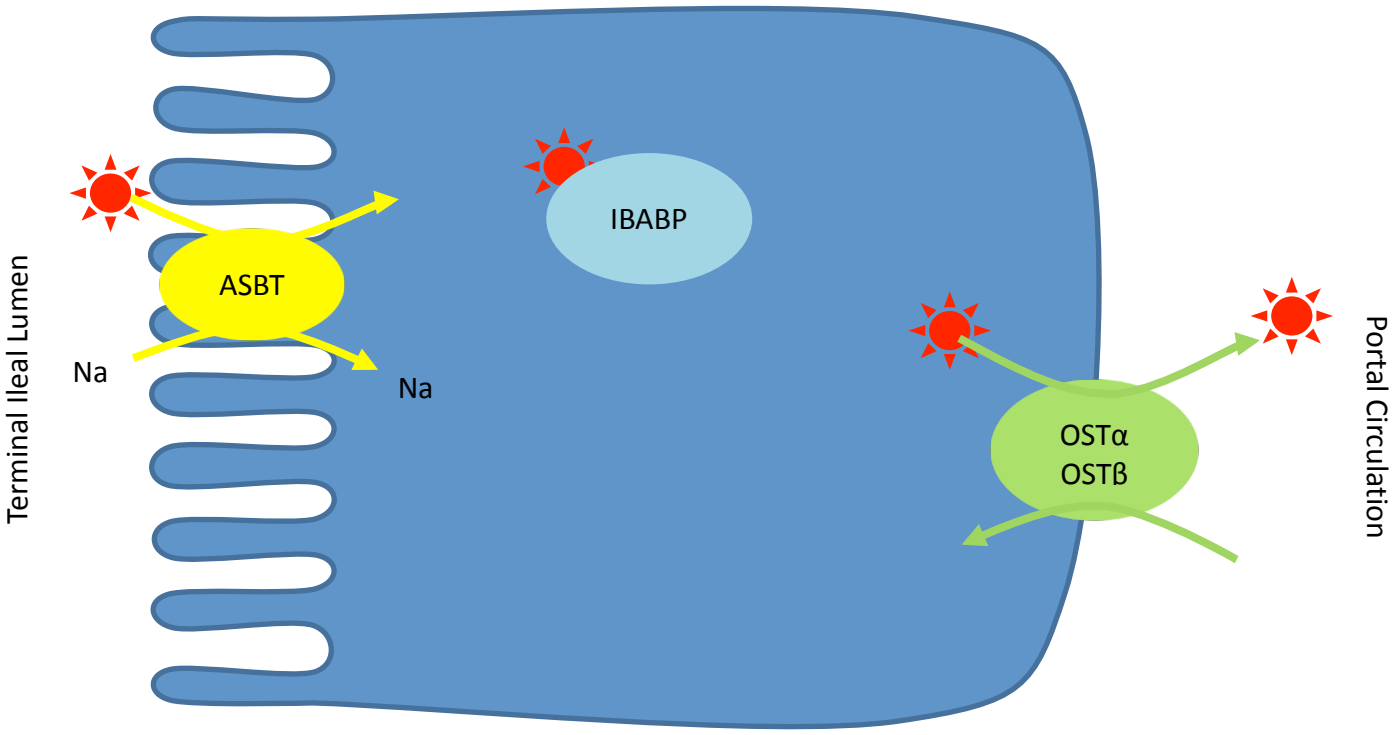


Figure 1:3 Bile acid transport in the enterocyte. ASBT: apical sodium bile acid transporter. IBABP: Ileal bile acid binding protein. OSTα / β: organic solute transporter. Red stars denote bile acids.

1.2.2 Hepatic bile acid transport

97% of bile acids are recirculated via the enterohepatic circulation. The bile acids are primarily absorbed from the portal circulation by periportal hepatocytes that secrete them in to the bile canaliculus. New bile formation is preferentially performed by perivenous hepatocytes.[10]

Conjugated BAs are transported from the portal circulation, across the sinusoidal membrane by sodium taurocholate co-transporting polypeptide (NTCP), which is structurally similar to ASBT found on enterocytes. Once in the hepatocyte the BAs are involved in feedback and signalling mechanisms that are described in the next section. Transport out of the hepatocyte into the bile canaliculus is performed by the bile salt export pump; BSEP. The essential role of BSEP is exhibited by the observation that mutations cause progressive familial intrahepatic cholestasis type 2.[11]

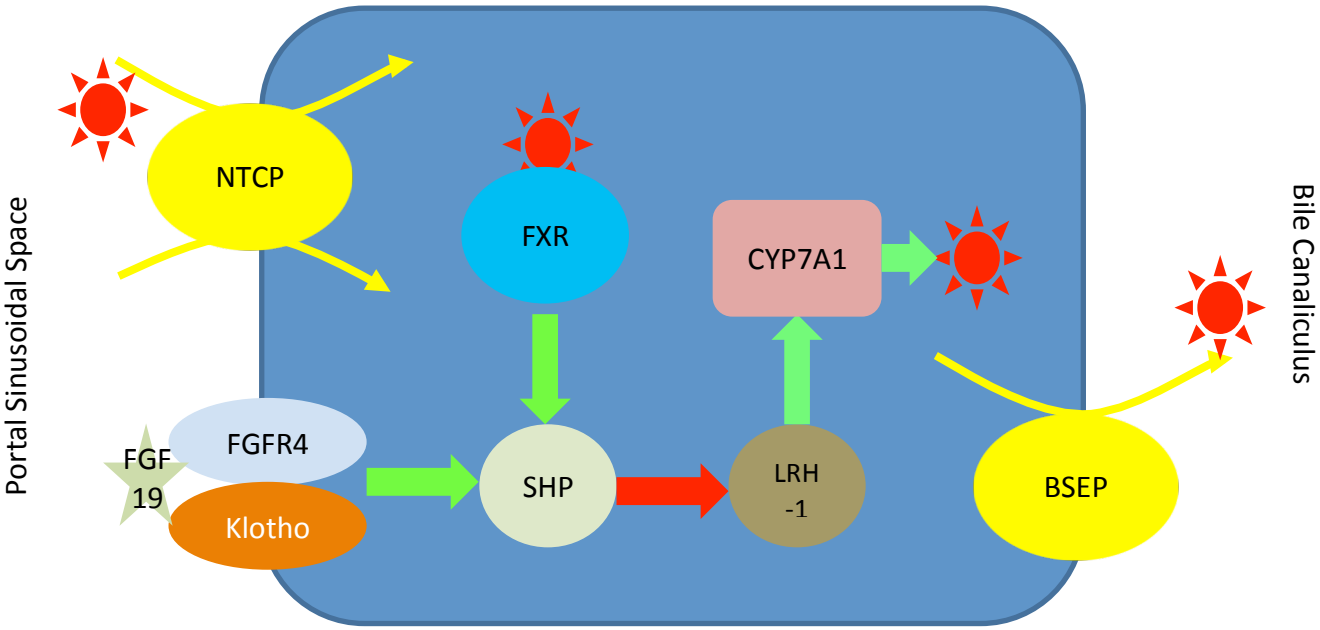


Figure 1:4: Schematic diagram of bile acid transport and signalling in the hepatocyte. NTCP: sodium taurocholate co-transporting polypeptide. FGFR4: fibroblast growth factor receptor 4. SHP: small hetrodimer partner. FXR: farnesoid X receptor. LRH-1: liver receptor homolog-1. CYP7A1: cholesterol 7 alpha-hydroxylase. BSEP: bile salt export pump. FGF19: fibroblast growth factor 19. Red stars denote bile acids.

1.3 Regulation of Bile Acid Synthesis

The size of the BA pool is maintained within limits by control of BA synthesis by positive and negative feedback mechanisms. Central to the control of BA synthesis is the farnesoid X receptor (FXR) that binds BAs and activates expression of genes involved in BA metabolism.[12] These include the genes encoding IBABP, OSTα and OSTβ, ASBT and fibroblast growth factor 19 (FGF19) in the intestine. In human adults, FXR is expressed in high levels in the liver, intestine, kidney and adrenal glands.[13]

There are other nuclear receptors for which BAs are ligands, these include pregane X receptor (PXR), vitamin D_3 receptor and constitutive androstane receptor (CAR).[14-16] Together with FXR and other factors, these form a complex network of transcription factors.

Within hepatocytes FXR inhibits transcription of the rate-limiting enzyme in bile acid synthesis, CYP7A1 through short heterodimer partner (SHP).[17] SHP represses gene expression by interfering with the function of transcription factors such as liver receptor homologue 1 (LRH1), hepatocyte nuclear receptor factor 4-α (HNF-4α) and peroxisome proliferator-activated receptor γ co-activator 1-α (PGC1-α).[18] FXR also up-regulates the bile acid conjugating enzyme bile-acid-CoA:amino acid *N*-acetyltransferase and BSEP.[19, 20]

BAs vary in their potency as FXR agonists. Previously it was thought that FXR had different affinities for the 2 major BAs; CDCA and CA, but our group has shown that their potency for FXR stimulation in ileal explants is similar. However, less abundant BAs such as lithocholic acid (LCA) do show much lower potency as FXR agonists.[21] SHP inhibits CYP7A1 along with liver receptor homolog in order to reduce bile acid synthesis, but a second pathway of negative feedback was suspected after it was found that removing elements of the SHP pathway did abolish the ability of BAs to self-regulate.[1]

1.4 Fibroblast Growth Factor 15 / 19

In 2005 it was shown that fibroblast growth factor 15 (FGF15, a mouse orthologue of FGF19 in humans) was released by FXR stimulation in the small intestine and inhibited CYP7A1 through FGF receptor 4 (FGFR4) in the hepatocyte.[22] Action of FGF15/19 on FGFR4 is further modulated by a protein Klothoβ (KLB).[23] FGF15 can also be induced by vitamin D via its intestinal receptor and vitamin A induces FGF15 via FXR through its heterodimer retinoid X receptor (RXR).[24]

FXR and FGF19 are highly expressed ileal enterocytes and gallbladder cholangiocytes and to a lesser extent in the kidney, testes and thalamus.[25] The exact contribution of FGF19 to CYP7A1 inhibition in humans is unknown, since BAs in the portal circulation also have a contribution, but studies in mice suggest at least half, and probably the majority of CYP7A1 inhibition is via FGF19.[25]

1.5 TGR5

There are only 2 receptors that have bile acids as major ligands, FXR and the G protein-coupled BA receptor (TGR5). While FXR expression is mostly in the terminal ileum in adults, TGR5 in ubiquitous in human tissue. It has shown to have functions in energy metabolism, glucose homeostasis, bile composition, secretion and inflammation.[26-28] Within the intestine TGR5 is expressed within the enterochromograffin cells and enteric neurons and DCA has been shown to act on these cells to release 5-hydroxytryptamine (5HT).[29]

1.6 Bacterial metabolism of bile acids

Bile acids are directly metabolised by bacteria in the bowel lumen. This change of the BA structure can influence the BAs absorption, excretion and affinity for FXR or TGR5 through the reactions described below. In addition, different bacteria have the ability to oxidise and epimerise the 3,7 or 12 hydroxy groups of BAs through hydrosteroid dehydrogenases.[30]

1.6.1 Deconjugation

BAs are conjugated with either glycine or taurine in the liver prior to excretion into bile. Conjugated BAs are highly hydrophobic and are poorly absorbed in the bowel lumen. Deconjugation by bacterial bile salt hydrolases (BSH) is a prerequisite to further bacterial modification.[31] Bacteria with high activity of BSH include *Bacteroides, Bifidobacterium, Clostridium, Lactobacillus and Listeria.[32]* Modification of the microbiome with the probiotic VSL#3 has been shown to increase the proportion of faecal conjugated BAs and increase FGF15 in mice.[33]

1.6.2 7-Dehydroxylation

Dehydroxylation of the 7α group of primary bile acids results in production of secondary bile acids and is probably the most physiologically significant bacterial modification in humans. Bacteria with 7α-dehydroxylation activity include *Clostridium* and *Eubacterium sp.[32]*

1.6.3 Desulphonation

Sulphonation of BAs is performed in the liver by the sulphotransferase-2 (SULT2) enzyme and is a protective mechanism, preventing cytotoxic effect of LCA and promoting excretion in the urine.[34] Genera with high sulphatase activity include *Clostridium, Fusobacterium, Peptococcus* and *Pseudomonas.*[32] High proportions of faecal sulphated BAs are reported to be a cause of functional constipation in children.[35]

1.7 Bile acid diarrhoea

1.7.1 Discovery of bile acid diarrhoea

Alan Hofmann first described the syndrome of cholerheic enteropathy following ileocaecal resection in 1967.[36] This form of bile acid malabsorption (BAM) is now termed secondary or type 1 and commonly seen in patients with terminal ileal Crohn's disease, either active or resected. Shortly afterwards diarrhoea due to colonic BAs was described in patients with histologically normal terminal ileum, this was termed idiopathic, primary or type 2 BAM.[37] Type 3 BAM is associated with a heterogeneous range of gastrointestinal conditions including coeliac disease and post-cholecystectomy. For reasons explained below, we prefer to call type 2 BAM; primary bile acid diarrhoea (BAD), and types 1 and 3 secondary BAD.

When bile acids reach the colon in concentrations exceeding 3mmol/L, changes to the physiology of the colon occur.[38] Multiple mechanisms appear to mediate secretion in the colon secondary to bile acids. CDCA and DCA have been shown to decrease net sodium absorption and increase chloride secretion in human ileal tissue.[39] However, this effect has not been seen with CA.[40] In fact, the pro-secretory effects of bile acids have been shown to be highly structure-specific and susceptible to bacterial metabolism, such as the conversion of the pro-secretory CA to the less secretory DCA.[41] Bacteria also have the ability to convert non-secretory bile acids to pro-secretory forms.[35] Rectal administration of DCA has been shown to increase proximal colonic propagation waves and decrease pain threshold to rectal balloon insufflation in healthy volunteers.[42] A recent study using TGR5 knockout mice showed that the colonic motor response to BAs is mediated by TGR5 that in turn releases 5-HT and calcitonin gene-related peptide. This study found a 2.2 fold decrease in colonic transit time and a 2.6-fold increase in defecation frequency in mice that over-expressed TGR5 compared to knockout. Administration of DCA stimulated colonic motility in wildtype, but not TGR5 knockout mice, proving that BAs have direct effects on colonic motility.[43] This pro-motility effect was not seen in the small bowel, confirming previous work that showed gastric emptying and small

bowel transit are inhibited by TGR5 stimulation.[44] The same mouse model has shown that TGR5 mediates the itching sensation in cholestatic disease.[45] These findings taken together tell us that BAs decrease rectal pain threshold and TGR5 modulates pain and is co-located with enterochromograffin cells and intrinsic primary afferent neurons in the bowel. It seems likely that BAs and TGR5 are important component in pain perception in functional bowel disease.

1.7.2 Effect of bile acids on the colon

It has been postulated that bile acids are endogenous laxatives.[46] Therefore a reduction of the concentration of BAs delivered to the colon may result in constipation. CDCA and CA are the only BAs have pro-secretory effects in the colon, but they are vulnerable to bacterial metabolism in the colon and lose their pro-secretory effects. This hypothesis was tested in a pediatric population of 73 children with functional constipation. These children had higher fecal levels of monosulfated CA and CDCA when compared to non-constipated controls (5.5% vs. 3.4%). This increase was mostly attributable to 6 outliers whose major fecal bile acid was 3-sulphate-CDCA. Sulphation of CDCA abolishes its laxative effect, leading the authors to conclude, that in a subset of functional constipation, lack of bacterial desulphation may be a causative factor.[46] Although this study has not been repeated in an adult population, a recent study has shown that patients with IBS-C have a lower total amount of total BAs in feces compared to healthy volunteers. The composition of their faecal bile acids were different with a reduction of the relatively pro-secretory DCA and an increase in the non-secretory LCA. This study also found a negative correlation between the colonic transit time and the percentage of LCA of the fecal bile acid composition.[47]

As well as undergoing bacterial modification, there is evidence that bile acid production is altered in constipation. A Swedish study of 26 female subjects with IBS-C or functional constipation by Rome II criteria found that 11 (42%) had markedly prolonged oral-anal transit times (OATT) of over 4.3 days.[48] This subgroup were found to lack the 3-4 fold increase in serum hydroxy-4-cholesten-3-one (C4) at lunchtime that has been shown to be part of a normal diurnal variation in healthy

volunteers.[48] Three patients with severely delayed OATT were found to have high baseline levels of serum C4 indicating increased bile acid synthesis, whether this translated into increased faecal bile acids is not known, but it is possible that these patients had bowel flora that was not desulphating secretory BAs. Oral administration of bile acids has been shown to shorten colonic transit in females with IBS-C. This placebo-controlled study of 36 subjects compared colonic release CDCA at doses of 500mg and 1000mg and found that CDCA improved stool form, frequency and ease of passage in a dose dependent manor.[49] CDCA reduced colonic transit time but retarded gastric emptying as seen in other studies. They also found that patients with IBS-C had reduced fasting serum C4 compared to healthy volunteers, suggesting decreased BA synthesis. A summary of the known actions of BAs in the colon is shown in table 1.1.

Effect	Method	Mechanism
Laxative	Water secretion	Activation of CFTR via adenylate cyclase.[50, 51] Inhibition of apical Cl-/OH- exchange by tauro-CDCA.[52]
	Mucus Secretion	Increased mucus secretion, direct effect on goblet cells.[53, 54]
	Accelerated colonic motility	Likely via TGR5 stimulation of myenteric ganglionic neurons and Nitric oxide synthase (NOS).[55] Possible cholinergic agonism by tauro-LCA.[56]
Metabolic	GLP-1, GLP-2 and PYY release	Via TGR5 from enteroendocrine L-cells[57]
	Increased BA transport and decreased synthesis	Increased transcription of FGF19, ASBP, IBABP, OSTα/OSTβ through FXR stimulation.[58]
Inflammatory	Increased mucosal permeability to bacteria	Via muscarinic and nicotinic afferent nerves.[59] Tight junction expression decreased in TGR5 null mice.[60]
	Enteroprotection in inflammation	Multiple mechanisms through FXR activation.[61, 62]
	Inhibition of leukocyte chemotaxis	Formyl-peptide receptor (FPR) antagonism by CDCA.[63]
Cell turnover	Epithelial proliferation	Activation of epidermal growth factor receptors.[64] Activation of mitogen-activated protein kinase (MAPK) signalling pathways.[65]
	Apoptosis Resistance	Cause apoptosis in super-physiological concentrations.[66] Induce apoptosis resistance in chronic exposure.[67]
	DNA / Mitotic Damage	Oxidative DNA damage and spindle formation disruption.[68]

Table 1.1: Effects of bile acids in the colon

1.8 Clinical aspects of BAD

1.8.1 Prevalence of BAD

The most widely used diagnostic test for BAD is the ^{75}Selenium–homocholic acid taurine retention test, with 7 day retention <15% being diagnostic. In a systematic review of 18 studies including 1223 subjects with IBS-D type symptoms, 32% were found to have SeHCAT retention values of <10% and hence would benefit from bile acid sequestrates. This figure of around a third of patients with chronic diarrhoea was surprisingly consistent across all the studies and populations included. Based upon the relative prevalence of IBS-D in the UK, this review suggested that the prevalence of primary BAD in the general population of the UK may be as high as 1%.[69] A prospective trial of 152 patients with diarrhoea undergoing SeHCAT found that 54 (36%) had primary BAD, 26 (17%) had secondary BAD (types 1 and 3) and 72 patients had diarrhoea with normal SeHCAT. Patients with primary BAD were as likely to report pain and bloating (59% and 73% respectively) as the diarrhoea controls.[70]

1.8.2 Mechanisms of BAD

1.8.2.1 Historical theories

Using the old nomenclature of bile acid malabsorption, a defect of the ASBT seemed a likely explanation. In 1991 it was shown that ileal biopsies showed the same activity of Sodium/bile acid co-transport than healthy controls.[71] It was concluded that genetic defects of bile acid transportation must be rare, and in 1997 the SLC10A2 mutation in ASBT gene was described in a rare congenital form of diarrhoea.[72] This mutation has not been found in the adult population of primary BAD.[73] The search for the cause of primary BAD moved into the cell, focusing on the cytoplasmic BA binding protein, ileal bile acid-binding protein (IBABP) and the basolateral membrane transporters OSTα and OSTβ. Our group found no significant differences in polymorphisms or expression of these proteins between patients with diarrhoea and controls.[74] However this study was the first to describe high levels of FGF19 in the human ileum.

1.8.2.2 Ileal disease and secondary BAD

Surgical resection of the ileum will almost always cause BA malabsorption, and is the first described cause of BAD.[75] This surgery is most commonly associated with Crohn's disease, but ileal inflammatory disease without resection can also reduce BA absorption and cause diarrhoea. Crohn's disease patients with low activity score have been found to have diarrhoea with increased faecal bile acid excretion and low FGF19.[76, 77]

Other malabsorptive small bowel diseases can cause true bile acid malabsorption, such as coeliac disease, but the prevalence of BAD in these conditions is not known.

Cholecystectomy is known risk factor for BAD, up to 85% of patients experience chronic diarrhoea post-cholecystectomy.[78] In a series of 373 patients undergoing SeHCAT scanning, cholecystectomy had an odds ratio of 2.51 for a SeHCAT <15%, ileal resection for Crohn's disease conferred an OR of 12.4. [79] Despite this high prevalence, the cause of BAD post-cholecystectomy is not known.

1.8.2.3 Defective FGF19 feedback

In 2009 a completely new mechanism for primary BAD was proposed by Walters et al.[80] They found that 17 patients with primary BAD, 13 confirmed with SeHCAT scanning had significantly lower fasting serum FGF19 and raised C4 compared to controls. This negative correlation between C4 and FGF19 suggested an increased bile acid pool in primary BAD and a defective negative feedback mechanism using FGF19. This was collaborated by previous work that described a 90% increase in the bile acid pool in patients with primary BAD, despite their excessive faecal losses.[81] These findings have caused a paradigm shift from malabsorption to overproduction of bile acids due to defective negative feedback by the hormone FGF19. It is for this reason it is suggested that bile acid malabsorption (BAM) is re-termed as bile acid diarrhoea (BAD).

A prospective study comparing FGF19 and C4 with SeHCAT found the same inverse relationship with FGF19 and C4 and that a FGF19 <145pg/mL was predictive of a positive SeHCAT. A modified score adjusted for C4, age and BMI (all factors that change FGF19 levels) showed a negative predictive

value of 86% and positive predictive value of 61% for a SeHCAT <10%. Furthermore, a FGF19 of <145pg/mL was highly predictive of having a full response to bile acid sequestrants in 94% of patients.[70] In a retrospective trial of patients undergoing C4 measurement for diarrhoea, serum FGF19 <145pg/ml was found to have a 58% sensitivity and 79% specificity for detecting a serum C4 >28ng/ml, This increased to 74% and 72% for a C4 >60ng/ml.[82] FGF19 is relatively stable in stored serum and is measured by a commercially available ELISA kit, it may be the simple diagnostic test primary BAD requires.

An interesting observation has been made by many BAD studies and all FGF19 studies. Patients with IBS-D have a higher Body Mass Index (BMI) than controls with IBS-C or healthy volunteers.[70, 83] FGF15 transgenic mice have lower body masses despite increased food uptake, these mice were also protected from diet induced obesity and had lower serum, glucose and lipid levels.[84] The reverse is also true, FGFR4 and FGF15 knockout mice show characteristics of the metabolic syndrome.[85] Development of FGF19 pathway modulators in diabetes and metabolic syndrome is underway.

1.8.2.4 Genetic Aspects

The identification of the FGF19 pathway in bile acid homeostasis has led to renewed interest in finding single nucleotide polymorphisms (SNPs) associated with low FGF19 and diarrhoea. In 2010, Wong and coauthors analyzed 15 SNPs associated bile acid transport and regulation in 435 subjects with IBS.[86] They found that a SNP for Klothoβ (KLB, rs17618244) was associated with faster colonic transit and was prevalent in patients with the IBS-D subtype, but not within other IBS subtypes or healthy volunteers. This SNP coded for an unstable variant of KLB that has significantly shorter half-life. KLB binds to FGFR4 in the endoplasmic reticulum of hepatocytes to permit FGF19 mediated inhibition of bile acid production by CYP7A1 making this a biological plausible cause of primary BAD.[87] A study by the same group used a smaller population of 26 patients with IBS-D and compared total faecal BAs, C4, FGF19 and polymorphisms in KLB and FGFR4 to healthy controls and IBS-C [88]. They found increased fasting C4 and faecal BAs in the IBS-D group, but this was not

associated with the same polymorphism in KLB or fasting FGF19. There was a small association with 3 polymorphisms in FGFR4 (rs1966265, rs351855 and rs1768244) with stool BA excretion, perhaps indicating that primary BAD is a heterogeneous group of different polymorphisms and associations. Both the KLB rs497501 and the FGFR4 rs351855 SNPs are predictors of a good response to the bile acid sequestrant colesevelam.[89] A recently published exome sequencing of 16 patients with IBS-D (further subgrouped by FGF19, and C4 and increased colonic transit) revealed 2 further SNPs of FGFR4 and 1 of KLB that appeared predictive of BAD.[90] Two FGFR4 variants were also predictive of IBS phenotype and increased colonic transit time in a cohort of 405 IBS patients and 228 healthy controls.

A summary of SNPs associated with BAD is provided in Table 1.2. Apart from one TGR5 SNP, all of the described associations are for FGFR4 and its modulator KLB. Both of these have the potential to increase BA production and increase FGF19 release. No FXR polymorphisms associated with primary BAD have been identified.[91]

Protein Affected	SNP	Associations
Klothoβ	Rs17618244	Accelerated colonic transit within IBS-D. [86] Accelerated colonic transit in response to lower dose CDCA in IBS-C [49]
Klothoβ	Rs1015450	Increased fecal bile acids.[92]
Klothoβ	Rs4975017	Reduced colonic transit in response to Colesevelam. [89]
FGFR4	Rs1966265	Modulates rs17618244 effect. [86] IBS-D with low FGF19 and raised C4.[92]
FGFR4	Rs351855	Modulates rs17618244 effect. [86] Accelerated colonic transit.[92] IBS-D with low FGF19 and raised C4.[92] Reduced colonic transit in response to Colesevelam.[49]
FGFR4	Rs434434	Accelerated colonic transit.[92] IBS-D with low FGF19 and raised C4.[92] IBS-D phenotype.[92]
FGFR4	Rs376618	Accelerated colonic transit in response to CDCA. [49]
TGR5	Rs11554825	Accelerated small bowel transit.[93]

Table 1.2: A selection of significant single nucleotide polymorphisms (SNP) and the their associations with functional gastrointestinal disease. MAF: minor allelic frequency

1.8.3 Diagnosis of BAD

1.8.3.1 Faecal bile acid measurement

Diagnosis of BAM/BAD previously required measurement of faecal acids which is time consuming and not available outside research institutions. An advance on this is faecal ^{14}C taurocholate, in which radiolabeled bile acids are measured in stool, but this still relatively time consuming and requires a 72 hour stool collection.[94]

1.8.3.2 SeHCAT

The most common diagnostic test is the ^{75}Selenium–homocholic acid taurine (SeHCAT) retention scan. This test requires the oral administration of the gamma-emitting synthetic bile acid; ^{75}SeHCAT that enters the enterohepatic circulation, and is measured using a gamma camera at 3 hours and 7 days. Whole body retention of less that 15% at day 7 is abnormal, with values of less that 10% highly predictive of a response to bile acid sequestrants.[95] SeHCAT has been shown to have a sensitivity of 100% and a specificity of 94% for BAD, [96] and it is able discriminate BAD from other causes of diarrhoea, proving that bile acid excretion is not an inevitable consequence of increased transit.[97] Despite being in use in Europe for over 20 years, SeHCAT is not available in North America, leading to under-recognition of BAD in these countries.

1.8.3.3 Trial of bile acid sequestrants

The pragmatic approach of a therapeutic trial of bile acid sequestrants has never been validated against faecal bile acid measurement or SeHCAT and usually poorly tolerated by patients.[98]

1.8.3.4 Serum C4 / FGF19

Despite the reliability of SeHCAT, there is still a need for a simplified test that can be made widely available in North America. In 1993 raised Serum C4 has been shown to correlate to low SeHCAT results.[99] In Edinburgh serum C4 has been used as the preferential test for BAD for many years, their series of 1846 patients with diarrhoea compared serum C4 to response with bile acid sequestrants (in the absence of other pathology, this was taken to be diagnostic for primary BAD),

they identified 35 patients with primary BAD in this manner and reported a sensitivity and specificity of 97% and 74% respectively. The median C4 level was 54ng/ml, compared to a median of 20ng/ml for diarrhoea controls. The test was accurate for defining primary BAD from the IBS population with a cut off of 30ng/ml producing a likelihood ratio of 10.6.[100] In a study comparing serum C4 to SeHCAT in 23 patients with diarrhoea, a sensitivity and specificity of 90 and 78% respectively was observed for C4 predicting a positive SeHCAT.[101] This study included only 5 patients that we would define as primary BAD and these patients were not found to have significantly raised C4 using a relatively high cut-off value of 48ng/ml. Apart from the high cut-off, another reason for this may the diurnal variation in C4, more recently it is recommended that serum C4 be taken in the morning in the fasting state. The C4 assay requires high-performance liquid chromatography, which is technically difficult for lab staff. Our own experience is that C4 is unstable in serum at room temperature, and requires either immediate processing or freezing at -80^{o}C. Despite these limitations, the ability to perform a single, serological test means that serum C4 measurement remains an attractive diagnostic test for BAD.

1.8.3.5 Measuring volatile organic compounds

Experimental tests that are in development include detecting differences in volatile organic compounds in urine using an 'electronic nose'. A recent study of 110 subjects including 45 healthy controls, 42 with ulcerative colitis and 23 with BAD diagnosed by SeHCAT found a distinct chemical signature in the urine of patients with BAD that could be developed into an instant, portable test for BAD.[102] The fact that this hardware will differentiate between and diagnose multiple conditions increases its appeal.

1.8.4 Current treatment of BAD

1.8.4.1 Bile acid sequestrants

Bile acid sequestrants (BAS) remain the mainstay of treatment for bile acid diarrhoea. Cholestyramine is a bile acid bind resin and has been used since the 1970s. A study of 9 patients with a median SeHCAT retention of 8% showed a marked reduction in stool frequency from 5 to 2/day with cholestyramine.[103] A larger study of 68 patients with diarrhoea and a SeHCAT scan <15% with no other cause (primary or type II BAD), 75% had a positive effect on bowel habit with cholestyramine, although this positive effect is not quantified.[104] However, adverse effects such as abdominal pain, bloating and constipation are common, cholestyramine reduces the intestinal absorption of other medications and its formation as a non-soluble powder is unpalatable to most patients. These factors result in a 40-70% discontinuation rate despite its efficacy.[105] Colesevelam is a newer bile acid sequestrant that specific bile acids and does not appear to interfere with drug absorption, it is also taken in tablet form.[106] Colesevelam improved diarrhoea in 88% patients and was tolerated for up to 4 years in 67%. Of the cohort of 45 patients only 18% discontinued because of adverse effects or tablet burden, the remainder were non responders, or symptoms ceased.[98]

With low FGF19 emerging as a pathogenic factor for pBAD and metabolic syndrome, preventing FXR stimulation in the ileum by binding BAs may have unappreciated adverse effects. Mice fed colesevalam have undetectable levels of ileal FGF15 mRNA.[107] *Lundasen et al.* showed that administration of cholestyramine to healthy volunteers reduced FGF19 by 87% and increased C4 18 fold.[108] Since FGF19 has beneficial metabolic effects on hepatic gluconeogenesis and lipid storage, these effects may be undesirable. Unpublished work from our own group showed significant depression of FGF19 in 2 healthy volunteers taking cholestyramine for 2 weeks. This resolved on cessation, see fig 1.5. [109]

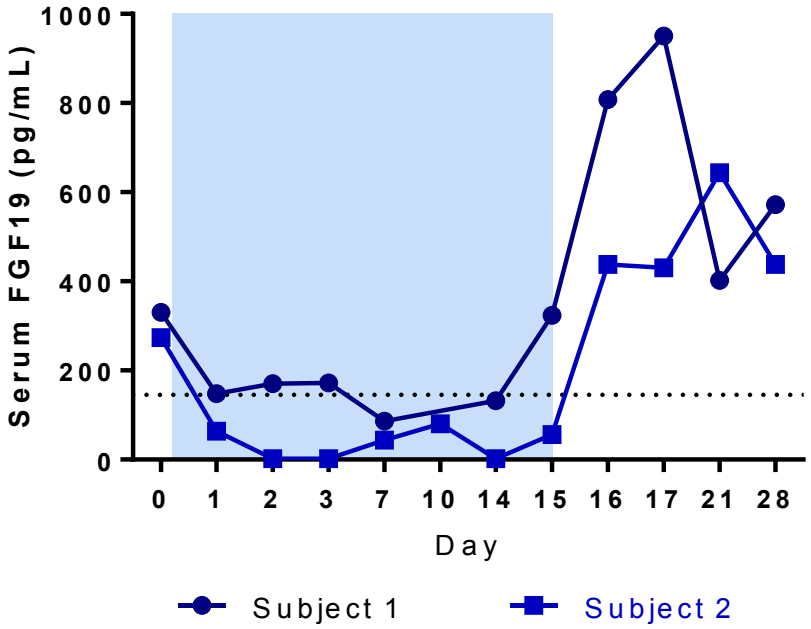


Figure 1.5: Effect of 2 weeks of 4g daily cholestyramine treatment on serum FGF19 in 2 healthy volunteer, treatment started on day 0 until day 14, denoted by shaded area. Dashed line represents 145pg/mL the lower limit of normal.[110]

1.8.4.2 Opioid receptor agonists

Loperamide and codeine are widely used in chronic diarrhoea of all causes and act by slowing bowel motility. Their efficacy has not been studied in BAD and the prevalence of their use is uncertain, since they are widely available without prescription, but anecdotally they are used on an as required basis as an adjunct to bile acid sequestrant therapy by up to 50% of patients.

1.8.4.3 FXR agonists

Obeticholic acid (OCA) is a synthetic bile acid and a potent FXR agonist that is currently in phase 3 trials for primary biliary cirrhosis. When our group cultured ileal biopsies in 20µM of OCA for 6 hours there was a 10 fold increase in FGF19 mRNA expression compared to 50µM of CDCA, the most potent endogenous BA (that in turn, increased expression by 300 fold from baseline).[21] In a phase II trial in patients with type 2 diabetes and non-alcoholic fatty liver disease, OCA was shown to improve insulin sensitivity, promote weight loss and reduce liver enzymes during a 6 week course.

Stimulation of FGF19 was shown in a dose dependant manor, as was a reduction in serum C4. It is worth noting that the most common adverse effect was constipation (5 out of 21 subjects on the higher dose of 50mg).[110] A proof of concept study in 10 patients with primary BAD showed that a low dose (25mg) of OCA increased fasting FGF19 from 133 to 237pg/mL and reduced stool frequency and form. There was also a tendency towards improvement in abdominal pain, urgency and bloating.[111]

1.9 Effects of fibroblast growth factor 15 /19

1.9.1 Glucose homeostasis

Transgenic mice expressing FGF19 have lower body weights despite increased food intake.[112] Direct effects of FGF19 on carbohydrate metabolism are mediated through fibroblast growth factor receptor 1c (FGFR1c) that is preferentially expressed in white adipose tissue (WAT). Administration of FGF19 to WAT induces extracellular signal-regulated kinase 1 / 2 (ERK1/2) signalling in a Klothoβ dependant manner, increasing glucose uptake.[113] Intracereberal injection of FGF19 increases metabolic rate, suggesting direct central nervous system effects.[114]

FGF15 KO mice are glucose intolerant and store 50% less hepatic glycogen than wild type mice, this effect was reversed by FGF15 replacement.[115] FGF15 represses gluconeogenesis by inhibiting the peroxisome proliferator-activated receptor γ (PPARγ) pathway.[116] In humans, higher FGF19 after Roux-en-Y surgery is associated with remission of type 2 diabetes.[117] Treatment with OCA improved the homeostatic model assessment-insulin resistance (HOMA-IR), a measure of insulin resistance in patients with NAFLD.[118]

1.9.2 Lipid homeostasis

As bile acid synthesis is a key excretion pathway for cholesterol, repression of CYP7A1 results in elevation of cholesterol. Therefore FGF15 KO and FGFR4 mice show characteristics of metabolic syndrome and particularly hypercholestrolaemia.[119] Re-expression of FGFR4 in the liver improves serum lipid and cholesterol levels, but not glucose tolerance and insulin sensitivity suggesting that these effects are medicated through FGFR1c.[119] In humans high C4 is associated with higher triglycerides (TG), but this association was not found with FGF19.[120] In our own series of 162 patients with diarrhoea, FGF19 correlated significantly with total cholesterol, LDL and TG, however SeHCAT had a negative correlation with TG.[121] Treatment with obeticholic acid raised serum cholesterol, that reversed after treatment withdrawal.[118]

1.9.3 Gallbladder filling

FGF19 is abundantly expressed in the gallbladder, where it is secreted into the bile and has an exocrine function.[122] FGF15 knock out (KO) mice have empty gallbladders, even in the fasted state.[123]

1.9.4 Tumour promotion

FGF19 and FGFR4 are co-expressed in a proportion of human liver, lung and colon tumours.[124] Expression of FGF19 was associated with recurrence and a relatively poor prognosis in hepatocellular carcinoma.[125] In a transgenic mouse model, overexpression of human FGF19 in skeletal muscle (to levels many hundred times those normally found) resulted in increased hepatocyte proliferation and hepatocellular carcinomas, and increased proliferation also occurred after injection of supraphysiological amounts (30µg/mouse) of recombinant FGF19.[126] Any concerns about FGF19 promoting hepatocellular carcinoma in humans may not turn out to be realistic at physiological levels. The relationships between FXR and hepatocarcinogenesis are complex,[127] and the critical interactions of FGF19 with FGFR4 appear different in humans to those in mice.[128]

1.9.5 Disease associations in humans

1.9.5.1 The Metabolic Syndrome

A diagnosis of metabolic syndrome requires 3 out of 5 metabolic abnormalities to be present; abdominal obesity, hypertriglyceridaemia, low HDL, hypertension or hyperglycaemia.[129] No study as yet has examined the association of all of these risk factors together with FGF19. However individual risk factors have been studied. A study in a Chinese population found that lower serum FGF19 levels were independently associated with the deterioration of metabolic status from normal to impaired fasting glucose to diabetes.[130] Patients with IBS-D have a higher body mass index (BMI) than controls with IBS-C or healthy volunteers; most of these can be expected to have low FGF19.[88, 131] It is possible that FGF19 contributes to the metabolic syndrome, or there is a separate 'metabolic syndrome of low FGF19'.

1.9.5.2 Non-alcoholic fatty liver disease (NAFLD)

NAFLD affect 30-40% of the Western population and its incidence is rising. NAFLD comprises of the spectrum of fatty liver disease from benign hepatic steatosis of whom 30% may progress to inflammatory non-alcoholic steatohepatitis (NASH) and 3% progress to fibrosis and cirrhosis.[132] Insulin resistance and dyslipidaemia and obesity are important risk factors for the development of NAFLD.[133] Since these risk factors are associated with low FGF19, it is possible that low FGF19 is involved in its pathogenesis. A study of 15 adults with NAFLD reported normal serum FGF19 levels, but reduced C4, indicating hepatic resistance as a possible factor.[134] Lower FGF19 was associated with biopsy proven NAFLD in 91 patients and a more severe histology ballooning score. [135]

The causes of obesity and NAFLD in the adult population are very heterogeneous, but studies in the paediatric population have shown stronger association with low FGF19. A study of 23 obese adolescents showed that low FGF19 was associated with insulin resistance, and inversely correlated with ALT and triglycerides.[136] In a study of 84 children with biopsy proven NAFLD and 23 controls. Low FGF19 was associated with NALFD (median FGF19 81 v 201pg/mL in controls) and progression to NASH (54pg/mL). Hepatic steatosis is also a problem in patients with intestinal failure (IF) requiring parental nutrition. A study of 52 paediatric patients with IF showed an association with low FGF19, hepatic steatosis, portal inflammation and fibrosis staging on liver biopsy and serum TNF-α.[137]

The FXR-FGF19 axis shows promise as a therapeutic target in NAFLD. The FLINT trial was a multicentre, double blind trial of OCA 25mg daily v placebo for 72 weeks. 229 patients completed the trial, but it was stopped early after interim analysis showed a clear benefit of OCA with improved steatosis, lobular inflammation and fibrosis scores on repeat biopsy in 45% of patients on OCA v 21% on placebo.[118]

1.9.5.3 Gallstones

Polymorphisms of FXR are associated with increased risk of gallstones in some populations, although it is unclear whether this is related to variation in FGF19.[138] However reduced ileal mRNA expression of FGF19 is associated with gallstones in non-obese females compared to controls.[139]

1.9.6 Modifiers of the FGF15/19 response

1.9.6.1 DIET1

DIET1 is a 236kD protein expressed almost exclusively in epithelial ileal cells. A polymorphism in mice reduced FGF15 excretion and increase faecal bile acid loss.[140] DIET1 co-localises in vesicle-like structures suggesting that the two proteins have a functional interaction and maybe involved in extracellular transport of FGF19.[141] A similar polymorphism has been found in humans, and is associated with lower serum FGF19 in patients with a SeHCAT<10%.(unpublished data)

1.9.6.2 Sterol regulatory element-binding protein-2 (SREBP2)

SREBP2 is a transcription factor that positively regulates transcription of target genes involved in cholesterol metabolism.[142] Overexpression of SREBP2 decreased FGF19 mRNA expression in human colonic cells by interfering with binding of FXR to the IR-1 containing region of the FGF19 gene.[143]

1.9.6.3 Sirtuin1 (SIRT1)

SIRT1 senses intracellular nutrient uptake and affects multiple metabolic functions through deacetylation, including bile acid homeostasis.[144] SIRT1 directly acts on FXR by deacetylation. Acetylation of FXR increases its stability, but prevents binding to RXR and subsequent binding to DNA.[145] Transgenic SIRT1 expressing mice also have increased hepatic CYP7A1 expression, indicating that the deacetylation of FXR has to balanced with its availability for hetrodimerisation with RXR.[146]

Intestine specific *Sirt1* knockout mice have reduced FXR signalling and increased hepatic BA synthesis.[147] Leptin deficient mice have reduced SIRT1 and FXR signalling, presumably due to the inhibitory effects of constant nutrient intake.[145] This offers an alternative explanation for the association of low FGF19 and overweight.

1.10 Future therapeutic targets for BAD and low FGF19

1.10.1 Modified bile acid sequestrants

Bile acid sequestrants are effective, safe and inexpensive. However, they are poorly tolerated and may exacerbate FGF19 deficiency as explained in section 1.8.4.1. One solution to improve tolerability may be to administer in a delayed release formulation. The only trial to date of this approach was a double blind placebo cross over trial of cholestyramine capsules in a cellulose acetate phthalate coating in 14 patients with ileal resections for Crohn's disease.[148] The trial showed a median reduction in stool frequency of 35% but no data on tolerability was published. Albireo Pharmaceuticals have developed colonic release cholestyramine capsules in a preparation called A3384.

1.10.2 FXR agonists

1.10.2.1 Obeticholic Acid

The potential use of this synthetic bile acid with a high affinity for FXR in BAD has already been discussed in section 1.8.4.4.

1.10.2.2 GW4064

GW4064 is synthetic FXR agonist that has widely been used in vitro. In ASBT KO mice it has been shown to reduce faecal BA loses and C4.[149] However, it is relatively non-selective and is poorly absorbed in vivo, limiting its clinical application. Its non-steroidal structure has been used to develop other patented compounds, FXR-450, WAY-362450 and PX20350 that are in phase I trials for NAFLD and cholestatic diseases.[150]

1.10.2.3 Fexaramine

Fexaramine is a non-absorbable FXR agonist. Mice fed fexaramine for 3 days exhibited increased ileal FGF15 mRNA expression, a reduced hepatic CYP7A1 mRNA expression and increase ileal and serum FGF19 protein.[151] This study also demonstrated increased insulin sensitivity.

1.10.2.4 Cafestol

Cafestol (CAF) is a diterpene from coffee beans that has been shown to be responsible for the cholesterol raising effects of unfiltered coffee.[152] In a mouse model of dyslipidaemia, cafestol has been shown to down regulate CYP7A1.[153] In human CV-1 and HepG2 cells, a 20µM solution of CAF was found to activate FXR at 40-50% of the potency of CDCA.[154] In same study, mice fed CAF for 14 hours were shown to increase ileal FGF15 mRNA expression by 4-5 fold, a corresponding decrease in hepatic CYP7A1 mRNA was also noted. Increased coffee consumption is associated with reduced hepatic fibrosis in NAFLD and Hepatitis C.[155, 156] Although whether this effect is mediated by CAF or another component on coffee is unknown. As FXR agonists have been shown to be of benefit in both pBAD and sBAD, Cafestol may have similar effects.

1.10.2.5 Ursodeoxycholic Acid

Ursodeoxycholic acid (UCDA) varies in structure from CDCA by only an epimerisation of the 7-OH group. The net effect of UCDA on FXR is controversial. No effect on FXR target genes has been reported in Alexander and CV-1 cell lines.[58, 157, 158] Furthermore, Parks et al reported that bile acids with lesser FXR agonist potential disrupt CDCA-FXR binding in a dose dependant fashion, although they only proved this with the secondary bile acids, LCA and DCA.[157] These effects may be specific to hepatic cell lines, since in ileal Caco-2 cells UDCA acts as a weak FXR agonist, inducing transcription of *IBABP* at a concentration of 50µM at 70% of that seen with the same concentration of CDCA.[159] Co-incubation of UCDA with CDCA halved the effect of CDCA on *IBABP* transcription at 50µM, but paradoxically increased concentrations of UCDA with the same CDCA concentration increased *IBABP* transcription. The same experiment in Hep-G2 cells showed no effect of UDCA, as shown before, but a dose dependant reduction in CDCA mediated FXR activity.

In humans, administration of UCDA before and after cholecystectomy and withdrawal in patients with PBC did not affect serum FGF19.[160, 161] Understanding the differential effects of UCDA in the

liver and the ileum when co-administered with other bile acids will be important since OCA is likely to be extensively used with UDCA when it becomes licenced for the treatment of PBC.

1.10.3 FGF19 replacement

A theoretical concern with systemic FGF19 therapy is tumourgenesis, particularly hepatocellular carcinoma (HCC).[127] A modified FGF19, known as M70, has a five amino-acid deletion and three amino-acids substituted at the N-terminal region. M70 showed none of the liver tumour-inducing activity of FGF19 when these forms were expressed in mice via an adenoviral vector for 24 weeks.[162]

M70 retained the ability of FGF19 to inhibit bile acid synthesis through decreased activity of CYP7A1. This was confirmed over a range of doses, both in human primary hepatocytes and in mice injected with either protein. It was shown in two mouse models of cholestasis (one extrahepatic and one intrahepatic) to be protective when it was given for five days before bile duct ligation and for four additional days. Necrotic areas in the liver and markers of damage were reduced by both FGF19 and M70. M70 was shown to normalize hepatic transporter transcripts which were deranged in the cholestasis models.[162]

A randomized, double-blind, placebo-controlled trial of subcutaneous injections of M70 (3mg/d) or placebo for 7 days was reported in the same paper. M70 suppressed serum levels of 7α-hydroxy-4-cholesten-3-one (C4) by over 95%, indicating marked suppression of new bile acid synthesis and this effect persisted for 24h after dosing. Post-prandial serum bile acid levels were decreased. No serious adverse events or laboratory abnormalities occurred.

These findings suggest that injections of M70 (and perhaps native FGF19) could have a therapeutic role in BAD, particularly in secondary disease where ileum has been resected or is diseased. In these conditions FXR agonists such as obeticholic acid may not be able to stimulate ileal FGF19 production sufficiently and injected replacement therapy may be preferable.

1.10.4 SIRT1 agonists / SREBP2 antagonists

1.10.4.1 Resveratrol

Resveratrol (RSV) is a naturally occurring polyphenol found in the skin of black grapes. It is widely believed to be an important contributory element to the protective cardiovascular effects of red wine.[163] Resveratrol exerts it effects through a number of actions. It is a cyclooxygenase-2 (COX-2) inhibitor with anti-inflammatory and anti-tumour effects.[164] But most of its effects are thought to be through agonism of SIRT1.[165] In HepG2 cells 50-100µM concentrations of RSV reduced intracellular fat and carbohydrate accumulation in a SIRT1 dependant manor.[166] This dosage also increased FXR protein by 30% over 24 hours, and increased mRNA expression of SHP and CYP7A1.

Studies of mutated human and murine SIRT1 have suggested that resveratrol is not a direct activator, but binds to the steady-state-enzyme-substrate-complex in a substrate dependant manor.[167]

In mice resveratrol supplementation protects against the metabolic effects of a high fat diet, as well as protecting against cataracts, vascular dysfunction and age-related decline in motor-function.[168] Mice fed Resveratrol for 30 days showed reduced adipocyte size,[169] serum triglycerides,[170] and aproprotein-B-100 and Apo-B-48.[171] In mice fed a high fat diet for 60 days resveratrol reduced serum triglycerides, hepatic steatosis, *Srebp1* mRNA expression and increased *Sirt1* mRNA expression.[172]

In humans, trials of resveratrol have concentrated on the anti-inflammatory, anti-tumour or metabolic effects. No symptomatic benefits have been found in these small studies, a dose ranging study of forty healthy volunteers found that daily resveratrol for 21 days reduced IGF-1 and improved insulin sensitivity.[173] 20-30% of those on the highest doses (2.5 or 5g) experienced gastrointestinal side effects, including diarrhoea. Diarrhoea was also noted in another study of 8 healthy volunteers [174] and in obese women.[175] This effect may be a SIRT1 independent effect of RSV since it has been shown to inhibit ASBT protein expression via the ubiquitin-proteasome pathway. [176] In the

same study ASBT mRNA expression was increased in Caco-2 cells, but decreased in COS-2 cells treated with RSV, so the anticipated effects in vivo are unclear.

Due to its known effects on steatohepatitis in mice and insulin sensitivity in humans, RSV is of interest in NAFLD. In a randomised double-blind study of 60 patients with NAFLD, 3 months of 150mg BD of RSV reduced AST, ALT, LDL, TC, TNFα and FGF21.[177] FGF19 was not measured.

The role of SIRT1 in FXR-FGF19 regulation is unclear, but the knowing effects of resveratrol on FGF19 and ASBT expression would aid anticipation of its effect on patients with BAD if its use in NAFLD increases.

2 Hypothesis and Aims

2.1 Hypothesis

The research hypothesis is in two parts:

Low FGF19 and, by association, primary bile acid diarrhoea may be contributory to several metabolic diseases such as hypertriglyceridaemia, reduced insulin sensitivity, non-alcoholic fatty liver disease, gallstones and overweight. These conditions together with primary bile acid diarrhoea will comprise a 'metabolic syndrome of low FGF19'. Low serum FGF19 and the rs12256835 DIET1 polymorphism will be more prevalent in these disease populations.

FGF19 expression can be modulated in ileal explants using novel compounds that have already been proven to be safe in humans. Colonic release cholestyramine will have beneficial effects patients with diarrhoea and cause less reduction in serum FGF19 than currently available formulations.

2.2 Aims

The aims of this research are covered in 3 chapters:

2.2.1 Chapter 3: Disease associations of BAD

- To examine disease associations with primary bile acid diarrhoea and low FGF19.
- To determine the prevalence of primary bile acid diarrhoea in patients with non-alcoholic fatty liver disease.
- To determine the prevalence of the rs12256835 DIET1 polymorphism in conditions associated with low FGF19.

2.2.2 Chapter 4: Regulation of ileal FGF19 expression

- To examine the association of SIRT1 and SREBP2 with ileal FGF19 expression.
- To examine the effect of cafestol, resveratrol and ursodeoxycholic acid on ileal FGF19 expression.

2.2.3 Chapter 5: Effects of conventional and colonic release cholestyramine

- To assess the effects of colonic release cholestyramine and conventional bile acid sequestrants in healthy volunteers and patients with primary BAD.

3 Disease associations of BAD

3.1 Methods: Disease associations of BAD

3.1.1 Analysis of Database for Associations of BAD

3.1.1.1 Identification of subjects and data collection

Results for patients undergoing SeHCAT tests at Imperial Healthcare NHS Trust have been collected on a database since 2009. Patients that have had a SeHCAT elsewhere and been referred to Imperial Healthcare NHS Trust as a tertiary centre were also added to the database, as were patients who had SeHCAT tests before this time but were seen in our clinic during this period. There have been small breaks in the collection of data over the years, but up to November 2014, the list of SeHCAT retention values was fairly comprehensive. Information on the following conditions were collected retrospectively:

- Colorectal cancer / colorectal adenoma
- Inflammatory bowel disease
- Microscopic colitis
- Non-specific histological inflammation on colonic, terminal ileum or duodenal biopsy.
- Dyslipidaemia
- Elevated liver enzymes
- Fatty liver on imaging
- Gallstones / cholescystectomy

This information was drawn from a variety of trust clinical systems, listed below. Information of significant co-morbidities, especially liver disease was also recorded if the information available, but the clinical notes were not interrogated.

- Scorpio (Endoscopy reporting software)

- Date of last colonoscopy
- Total number of polyps (for all colonoscopies reported)
- Size of largest polyp
- Presence of colorectal cancer (or reporting of a previous resected cancer)

- Pi (Pathology reporting software) and PACS (radiology reporting software) Before March 2014, when it was upgraded to Cerner (integrated pathology and radiology reporting software).
  - Histology (specimens taken at anytime)
    - Abnormal Colonic biopsies
    - Abnormal ileal biopsies
    - Abnormal duodenal biopsies
  - Clinical biochemistry (only the samples taken at the time nearest to date of the SeHCAT day 7 were recorded)
    - Alanine transaminase (mg/ml)
    - Total Cholesterol (mmol/L)
    - HDL/Total Cholesterol ratio (mmol/L)
    - Faecal Calprotectin (ng/ml)
  - Clinical imaging (at any time)
    - Presence of fatty liver on ultrasound, MRI or CT scan
    - Presence of gallstones or cholecystectomy on ultrasound, MRI or CT scan. Patients known to have a cholecystectomy, but not with available imaging were also included in this category.

After collection the cohort was split into those with BAD (SeHCAT<15%) and controls (SeHCAT>15%).

3.1.1.2 Categorisation of BAD

Patients with a SeHCAT <15% were categorized as type 1,2 or 3 BAD. This was performed both at the time of addition to the database and retrospectively at the time of study analysis by examination of outpatient clinic letters, clinical imaging, endoscopy and histology reports during the period 2009-2014. Letters and reports that quoted findings that predated this period were also included. Patients with terminal ileal resection or ileal Crohn's Disease were recorded as type 1. The presence of any other gastrointestinal disease or surgery distal to the pylorus, including cholecystectomy was recorded at type 3. Any patient with no evidence of another GI disease was categorized as type 2 (or primary BAD, pBAD).

3.1.1.3 Statistical methods

Continuous data was compared to SeHCAT 7 day retention value using Spearman's rank correlation. Median SeHCAT values were described for categorical data. Odd ratios for each disease were calculated for SeHCAT <15% and analysed using Fisher's exact test. Further subgroup analysis was performed for those with low FGF19 (<145pg/ml) against those with normal FGF19 and the 3 subtypes of BAD. Groups were compared for significant associations using Mann-Whitney-U tests. A p value of <0.05 was deemed as significant. All statistical analysis was performed using Prism 6 software (Graphpad Software Inc, La Jolla, CA, USA).

3.1.2 Incidence of low FGF19 and Diarrhoea in NAFLD

Building on the findings from the retrospective database, a prospective observational study was designed with the primary objective of discovering the incidence of chronic diarrhoea in non-alcoholic fatty liver disease? In addition to the primary objective, 7 secondary objectives were also targeted:

- What is the prevalence of primary bile acid diarrhoea in non-alcoholic fatty liver disease?
- What is the prevalence of primary bile acid diarrhoea in non-alcoholic steatohepatitis?
- What is the prevalence of chronic diarrhoea in non-alcoholic steatohepatitis?

- What is the prevalence of low FGF19 in non-alcoholic fatty liver disease?
- What is the prevalence of low FGF19 in non-alcoholic steatohepatitis?
- What is the correlation of FGF19 with hepatic transient elastography score?
- What is the prevalence of *DIET1* polymorphisms in non-alcoholic fatty liver disease?

3.1.2.1 Subjects

Patients with NAFLD were be recruited from the NAFLD clinic at St Mary's Hospital between February and September 2015. These were patients with an existing diagnosis of NAFLD referred to tertiary care by GPs or secondary care specialists. All patients in the clinic are measured for height and weight and routinely have fasting blood tests and a Fibroscan (transient elastography). If the Fibroscan reveals potential hepatic fibrosis (Fibroscan >6kPa), the patients are offered a liver biopsy. Approximately 20-30% of patients under go biopsy.

All patients aged 18-80 were recruited, only those with other potential causes of liver disease or chronic diarrhoea or were unable to consent were excluded. See appendix 2.1 for the full protocol with exclusion criteria.

The study aimed to recruit 124 patients with a power 80% to detect an incidence of pBAD of 2.5% with an α of 0.05 (as predicted by the retrospective study).

3.1.2.2 Data collection

After consenting to participate the patient was questioned for their ethnicity, alcohol consumption and a brief medical and drug history. A single blood test was performed for FGF19, C4 and genomic DNA. If the patient was not fasted they were scheduled for a morning blood test at another time. In addition the following data was collected from the medical notes:

- Age
- BMI
- Platelet count

- Albumin
- AST/ALT ratio
- Fasting lipid profile
- Fasting blood glucose
- Fibroscan score
- Medical and medication history (cross-checked with the patient's given history)
- Viral hepatitis markers, autoantibodies, serum ferritin/ total iron binding capacity

3.1.2.3 Identification of patients with diarrhoea

On the initial visit each patient was asked the question: *'have you experienced diarrhoea for more than the last 3 months?*' If the patient said yes, they were given a daily symptom diary to record for 7 consecutive days. The symptom diary recorded the frequency and Bristol stool form scale of each stool (a validated surrogate of GI transit time).[178] As well as 3 1-5 Likert scale questions on abdominal pain, bloating and urgency which are (part of the Frequency of Digestive Symptoms Questionnaire which has been validated in healthy volunteers and individuals with IBS).[179, 180] After 7 days the diaries were collected, if the total number of stools was ≥21 stools/week with 50% ≥type 5 the patient was asked to attend the gastroenterology outpatients where investigations appropriate to the patient's presentation as determined by the clinician were performed, including a SeHCAT test.

3.1.2.4 Analysis

3.1.2.4.1 Blood sample processing and storage

Blood samples were placed immediately on ice and sera for FGF19 and C4 were centrifuged at >8000rpm for 10 mins, the separated sera was stored at -80C within 2 hour of collection until transfer to King College (protocol in appendix 1.1). Blood for DNA was frozen at -20C until extraction (method described in section 4.1.3.2 and protocol in appendix 1.2)

3.1.2.4.2 FGF19/C4

3.1.2.4.2.1 FGF19

Sera were transferred to the biochemistry lab at Kings College Hospital in batches. FGF19 was measured in triplicate by a commercially available ELISA kit (Quantikine ELISA, R&D systems Europe, Abingdon, UK) and the mean value reported in pg/mL.

3.1.2.4.2.2 C4

C4 was measured by Tandem (liquid/gas phase) chromatography (LC-MS/MS). The assay is locally calibrated by Kings College laboratory between the values of 2.5-1000 nmol/L and uses the following specifications: A Supelco Analytical, Ascentis Express C18 fused core column (15 cm x 4.6 mm, 2.7 µm, SigmaAldrich) on a ThermoScientific TLX-2 system using water and methanol mobile phases (each with 0.1% (v/v) formic acid). A ThermoScientific Hot Pocket is used to maintain the column at 40°C. The TLX-2 system is coupled to a ThermoScientific Vantage TSQ triple stage quadruple mass spectrometer (ThermoScientific, UK) operated with atmospheric pressure chemical ionisation (APCI) source in positive ionisation mode.

3.1.2.4.3 NALFD severity assessment

3.1.2.4.3.1 NAFLD Fibrosis Score

The NAFLD fibrosis score will be calculated for each patient using the equation:

-1.675 + 0.037 × age (years) + 0.094 × BMI (kg/m2) + 1.13 × diabetes (yes = 1, no = 0) + 0.99 × AST/ALT ratio – 0.013 × platelet (×109/l) – 0.66 × albumin (g/dl)

A score of < -1.455 has a negative predictive value of 93% for fibrosis and >0.675 has a positive predictive value of 90% for fibrosis.[181]

3.1.2.4.3.2 Fibroscan

Transient hepatic elastography was measured by fibroscan 502 with medium or XL probes if BMI>30 (Echosens, Paris, France) for all patients prior to enrolment and recorded at the first visit. Fibroscans ere performed by specialist nurses who have performed over 50 procedures. Individual scores were

reported in kPa and were the median of 10 valid measurements, with a 60% success rate and an interquartile range of less than 30%. A Fibroscan score >7.9kPa has been shown to predict fibrosis score on histology with a sensitivity and specificity of 91% and 75% in NAFLD. [182]

3.1.2.4.3.3 Liver histology

Liver histology reports were collected for those patients who had undergone liver biopsy previously. The report was categorised into ascending levels of severity according to the wording: steatosis, steatohepatitis, fibrosis or cirrhosis.

3.1.2.5 Statistical analysis

The odds ratio of chronic diarrhoea in patients with NAFLD will be calculated and the variance assessed by fishers exact test. Demographic data will be assessed for variance using ANOVA. Non-parametric data will be assessed for variance using a Mann-Witney U test. Correlations will be calculated for SeHCAT and FGF19 values against NAFLD fibrosis score, ALT and fibroscan score.

3.1.3 Incidence of Diet1 Polymorphisms in NAFLD

3.1.3.1 Subjects

Venous blood was taken from subjects with NAFLD enrolled on the BAD in NAFLD study (3mls in EDTA) and frozen at -20°C awaiting DNA extraction.

3.1.3.2 DNA Extraction

Genomic DNA was extracted from whole blood using a commercially available DNA extraction kit (QIAmp DNA Blood mini kit, Applied Biosystems, Foster City, CA). The kit uses silica membranes in spin columns to bind DNA which then undergoes 4 washing steps.

The DNA extraction protocol is provided in appendix 1.2. Briefly, the procedure involved addition of 20µl protease and 200µl of lysis buffer to 200µl of blood, which was then vortexed for 15 seconds and incubated on a heat block at 56^{o}C for 10 minutes, after which 200µl of ethanol was added and vortexed again. The mixture was then transferred to a QIAmp spin column, centrifuged at 8000rpm

for 60 seconds. The follow through was discarded and 500µl wash buffer added to the column and centrifuged at 600rpm for 60 seconds, this step was repeated at the higher rpm of 14000. The column was then transferred to a clean tube and 200µl of elution buffer added. The spin column was incubated for 5 minutes at room temperature and centrifuged for 1 minute at 8000rpm. The resulting follow through contained the purified DNA.

DNA quantity and purity was analysed using a UV-VIS Microplate Spectrophotometer (Thermo Fisher Scientific, Waltham, MA). The instrument measures light absorption at 2 wavelengths, 260 and 280nm. The ratio between these is an indication of the purity of the sample. Ratios of 1.7-1.9 were acceptable, any sample outside of this range was extracted again. The instrument is also able to estimate the concentration, if the samples were over 25ng/µl they were diluted with elution buffer to achieve a concentration of between 3-25ng/µl. The samples were then frozen at -20^{o}C until use in PCR.

3.1.3.3 PCR

SNP genotyping was performed using commercially available TaqMan SNP assays (Applied Biosystems, Foster City, CA). The TaqMan probes are specific for each allele of the SNP and are labelled with 2 different chromophore reporter dyes (VIC or FAM) at the 3' end. The oligonucleotide probe is also attached to a non-fluorescent quencher at the 5' end. Binding to the correct nucleotide sequence releases the quencher and the probe emits a fluorescent signal that is detectable by the StepOne real-time PCR machine (Applied Biosystems). The instrument can differentiate between VIC and FAM signals. Included in the TaqMan 2x Genotyping Mastermix (Applied Biosystems) is the AmpliTaq DNA polymerase. After a 10 minute hold at 95^{o}C to activate the enzyme, the PCR machine cycles between 15 seconds of denaturation at 95^{o}C and 60 seconds of annealing/extension at 60^{o}C 40 times. On each cycle the number of amplicons and probe binding doubles creating an increasing fluorescent signal that reaches an exponential phase and eventually plateaus, as the regents are exhausted. After 40 cycles, the ratio of VIC:FAM signal for each well is calculated and plotted on an

allelic discrimination plot (figure 3.1) which can discriminate between homozygotes for allele 1 and allele 2 and heterozygotes.

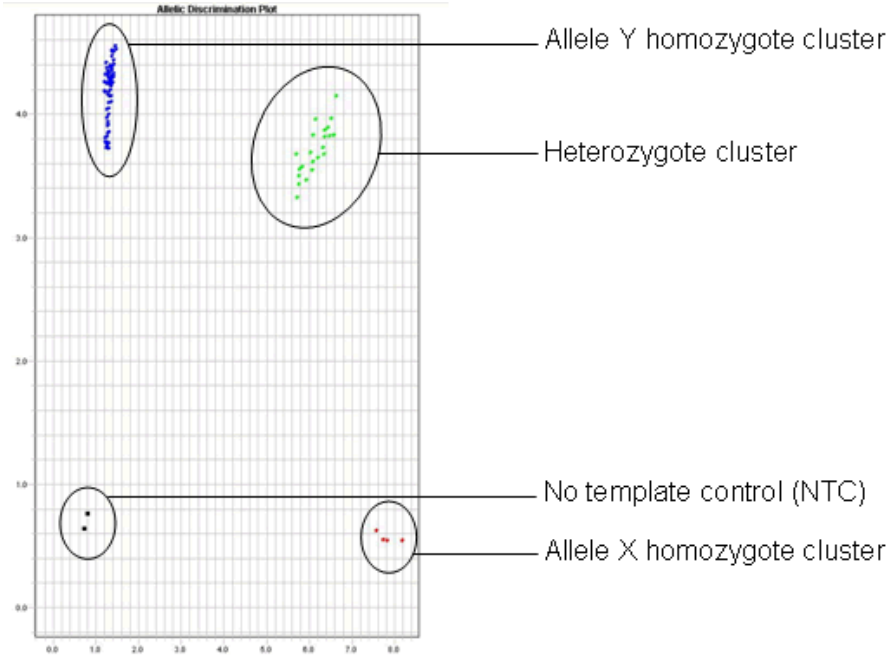


Figure 3.1 Example of an allelic discrimination plot.[183]

The full protocol used is available in appendix 1.3, but briefly the procedure was as follows. A master mix was prepared using 7.5µl 2x TaqMan genotyping mastermix (Applied Biosystems), 0.375µl of 40x Taqman SNP assay (Applied Biosystems) and 6.125µl of DNAase free water. 14µl were added to each well of a 96 well fast PCR plate. 1µl of purified DNA was added per well. The plate was briefly centrifuged and placed in a StepOne real time PCR machine. Where the sample was heated according to the following standard protocol:

- 10 minutes hold at 92^{o}C
- 40 cycles of:
    - 15 seconds at 92^{o}C
    - 60 seconds at 60^{o}C

Technical data of the primers are the property of the assay manufacturers, but the sequence used for the probe is: TTAATGTTTTTGCTTCCCCAGCACA[G/T]TATACAAGCACAACAGGAAGCTGCA. Results were plotted automatically by the StepOne software according to the fluorescent signal detected.

3.1.3.4 Statistical Analysis

Minor allelic frequency (MAF) was calculated for subjects with patients with diarrhoea, pBAD and NAFLD. The MAF was compared between disease groups and that stated on the 100 genomes. Fishers Exact test was used to compare MAFs between groups. A p value <0.05 was deemed significant. Genetic dominance effect was assessed by comparing the number of homozygotes with heterozygotes and mutant homozygotes. SeHCAT, FGF19, NALFD fibrosis score, and Fibroscan score were compared between subjects of different genotypes and significant assessed using Mann-Whitney U tests.

3.2 Results: Associations of BAD

3.2.1 Associations of primary bile acid diarrhoea (retrospective database)

578 SeHCAT values were identified on the database, 303 (52%) were positive with a value <15% and 179 (31%) were pBAD. Demographics are shown in table 3.1.

	Controls (SeHCAT≥15%)	**BAD (SeHCAT<15%)**	**SeHCAT 15-10%**	**SeHCAT 10-5%**	**SeHCAT <5%**
N	275	303	83	92	128
Type (n) 1/2/3	N/A	39/196/68	6/60/17	6/66/20	27/70/31
% female	64	60.7	59	59.8	62.5
Mean Age (years)	48.7	52.1	55.3	50.8	51
Mean FGF19 (pg/ml)	302.1 (54)	166.6 (72)	193.6 (16)	185.9 (30)	127.6 (26)

Table 3.1: Demographics of database, displayed by SeHCAT result and severity

3.2.1.1 Dyslipidaemia

242 patients had a total cholesterol recorded and 209 had a complete lipid profile. 239 patients had serum triglycerides (TG) measured. SeHCAT value correlated negatively with TG (rs=-0.33 P<0.0001). Primary BAD was significantly associated with a higher TG (mean 1.87 (96%CI 1.58-2.16) v 1.29 (1.12-1.47) mmol/L p<0.001).). The odds ratios for a patient with a positive SeHCAT having a TG ≥1.7 or ≥2.4mmol/L were 3.3 (p<0.001) and 3.1 (p<0.01) respectively. 209 patients had low density lipoprotein (LDL) recorded, that correlated positively with SeHCAT result, rs 0.14, p<0.05. There was no significant association with FGF19.

	ALL	Controls (>15%)	SeHCAT<15	PBAD	T1	T3
TC (n)	4.78 (242)	4.82 (111)	4.72 (131)	4.78 (81)	4.41 (16)	4.72 (34)
LDL	2.72 (209)	2.85 (100)	2.60 (109)	2.65 (69)	2.68 (14)	2.43 (26)
TG	1.57 (239)	1.29 (111)	1.82 (128)	1.87 (80)	1.22 (16)	1.97 (32)
TG>1.7 (n)	69	18	51	35	3	13
TG>2.4 (n)	37	9	28	20	0	8

Table 3.2: Lipid profile displayed by SeHCAT result and BAD type

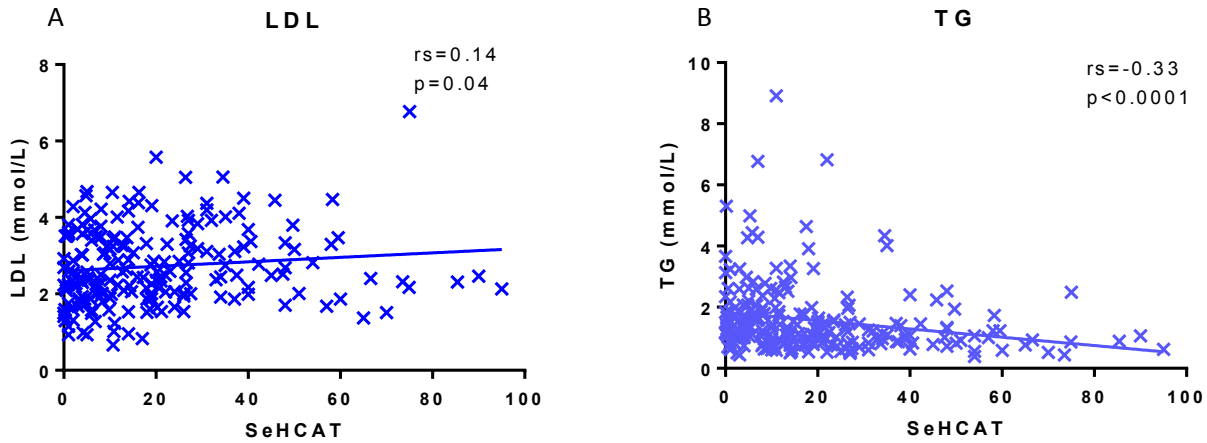


Figure 3.2: Correlation of LDL cholesterol (A) and triglycerides (TG) (B) with SeHCAT result

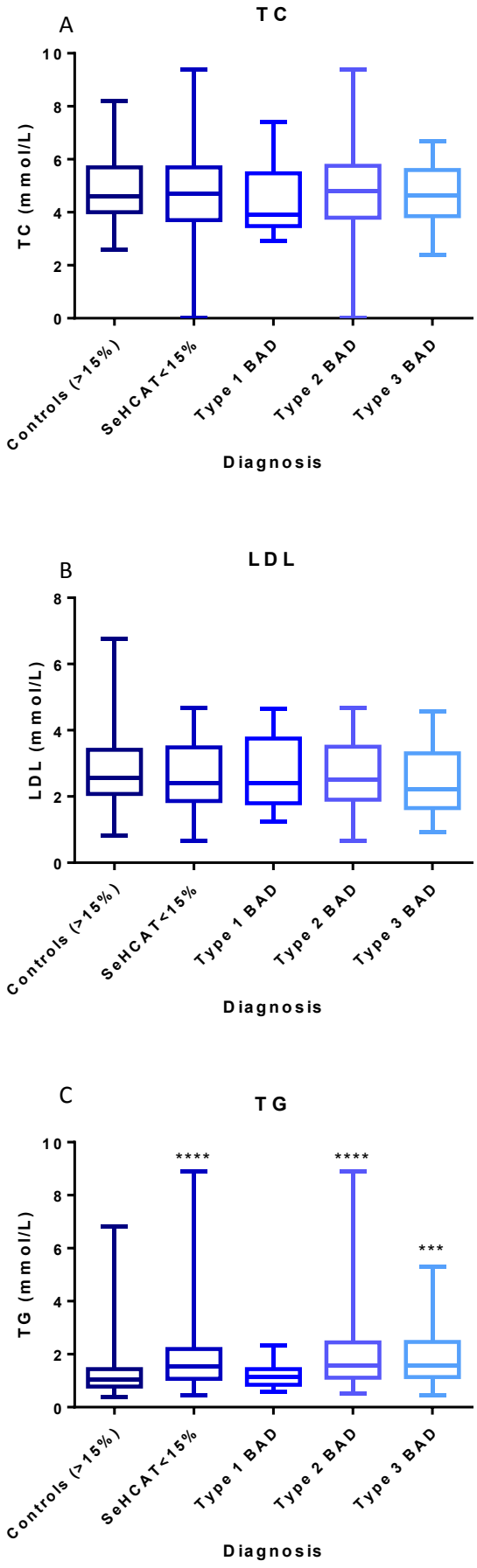


*Figure 3.3: Serum total cholesterol (A), LDL cholesterol (B) and triglycerides (C) by SeHCAT results and BAD type. ***p<0.001 ****p<0.0001*

3.2.1.2 NAFLD

425 patients had an ALT recorded, 184 had liver imaging and 176 had both. Demographics are shown in table 3.3. Patients with positive SeHCAT were more likely to be female and have a lower FGF19.

	Controls (SeHCAT >15%)	**BAD (SeHCAT<15%)**	**P value**
Number	208	217	
Female (%)	110 (53%)	153 (70%)	<0.005
Age (years)	50.7	50.0	0.5
Total Cholesterol (n)	4.87 (91)	4.78 (99)	0.6
FGF19 (pg/mL)	308.7 (51)	167.7 (72)	<0.005

Table 3.3: Demographics of patients with ALT recorded displayed by SeHCAT result

SeHCAT values correlated negatively with ALT (rs= -0.19, p<0.0001). A SeHCAT <15% predicted an ALT>31IU/L (36% v 21%, p<0.001), OR 1.72 (95%CI 1.14-2.61, p=0.01) and a fatty liver on imaging with ALT>31 IU/L (21% v 7%p<0.05), OR 3.05 (95%CI 1.1-8.45 p<0.05).

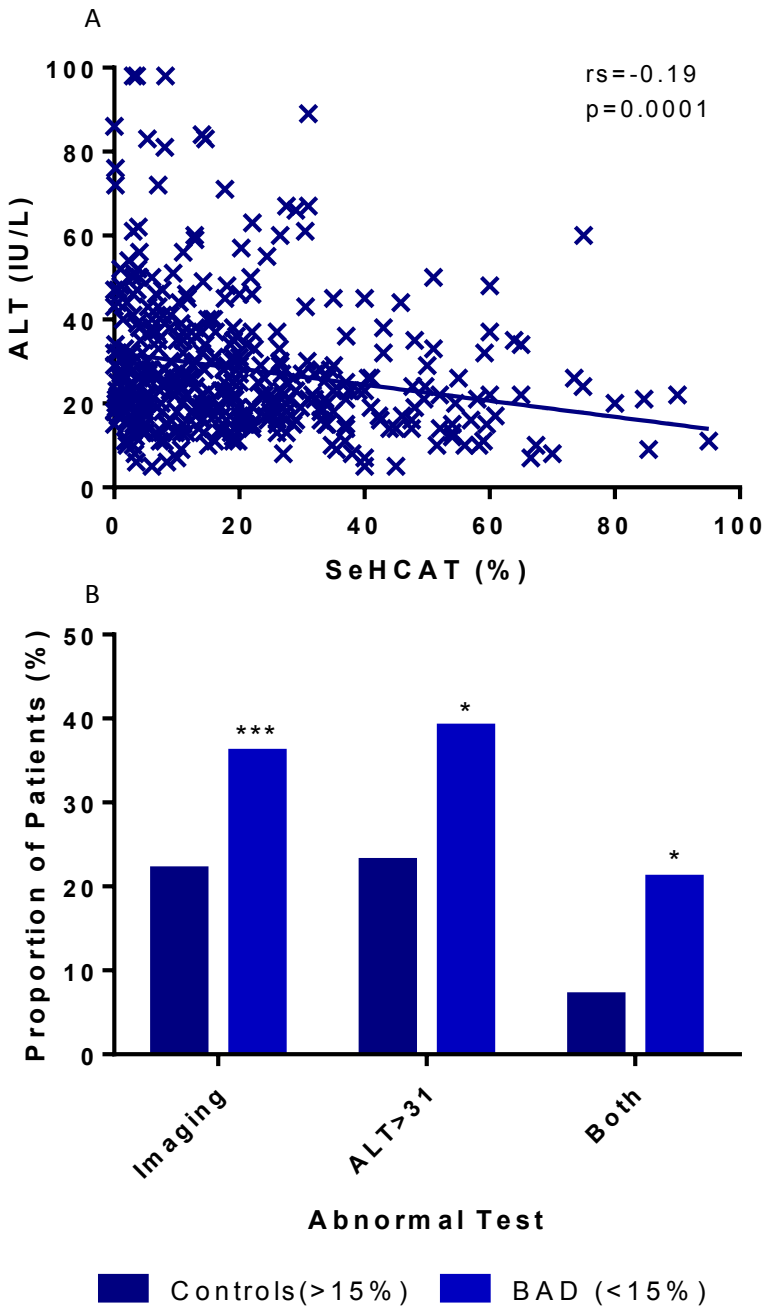


*Figure 3.4: Correlation of ALT with SeHCAT Value (A) and proportion of patients with abnormal tests by SeHCAT % retention (B). *p<0.05, ***p<0.0001*

128 of the pBAD patients had an ALT recorded and 57 had imaging. With secondary BAD excluded, SeHCAT value correlated negatively with ALT (rs=-0.23, p<0.0001). pBAD was associated with ALT>31IU/L (43% v 22%, p<0.001), OR of 2.64 (95%CI 1.63-4.26, p<0.001) and positive imaging with ALT>31IU/L (23% v 7%, p<0.05), OR 2.5 (95%CI 1.36-12.23, p<0.05).

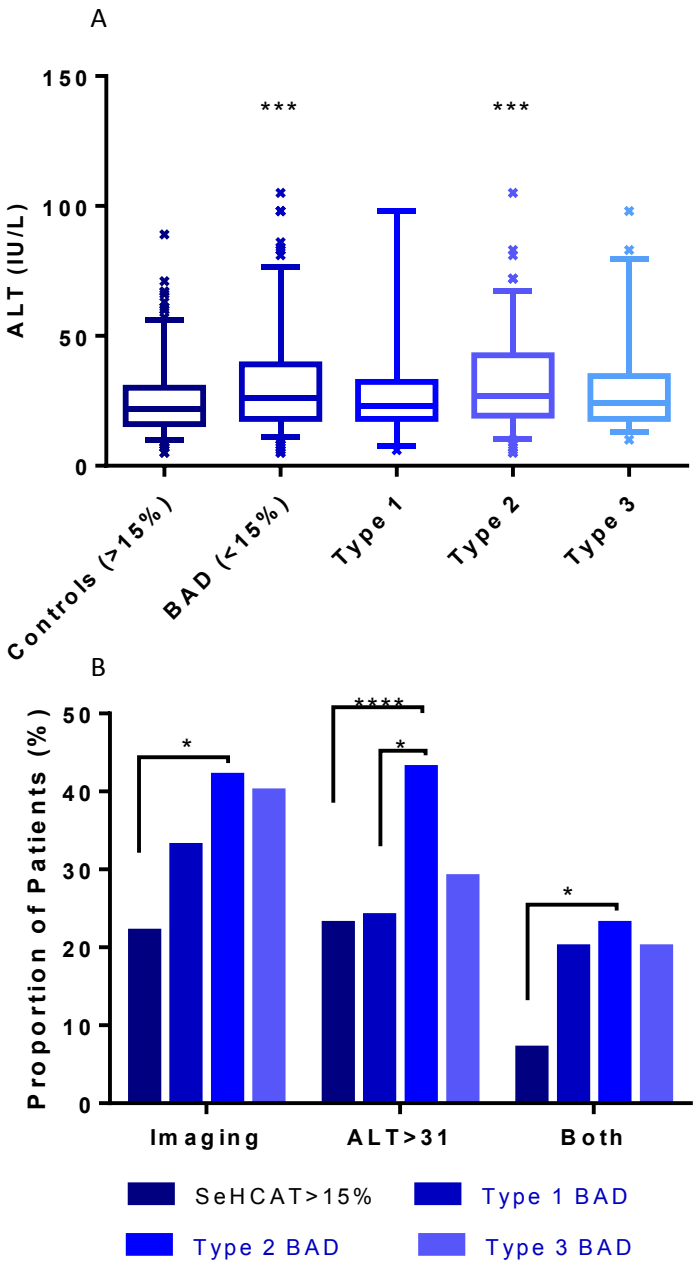


*Figure 3.5: Box and whisker plot (median, IQR, 5-95% CI) of ALT by BAD type (A) and proportion of abnormal tests by BAD type (B) *p<0.05, ***p<0.001, ****p<0.0001.*

123 patients had a fasting FGF19 and ALT recorded, no significant correlation was found (rs= -0.08, P=0.2), however FGF19 <70ng/ml was predictive of an ALT >40 IU/ml (40% vs12%, p<0.05), OR 5.13 (95%CI 1.28-20.61 p<0.05).

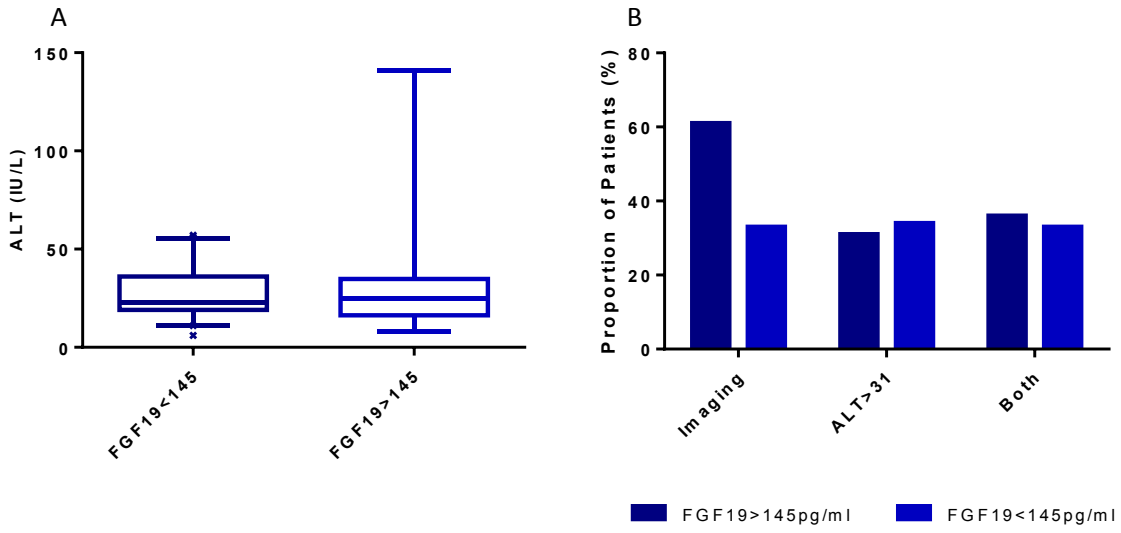


Figure 3.6: Box and whisker plot (median, IQR, 5-95% CI) of ALT displayed by FGF19 </> 145pg/ml (A). Proportion of patients with abnormal tests displayed by FGF19 </> 145pg/ml (B).

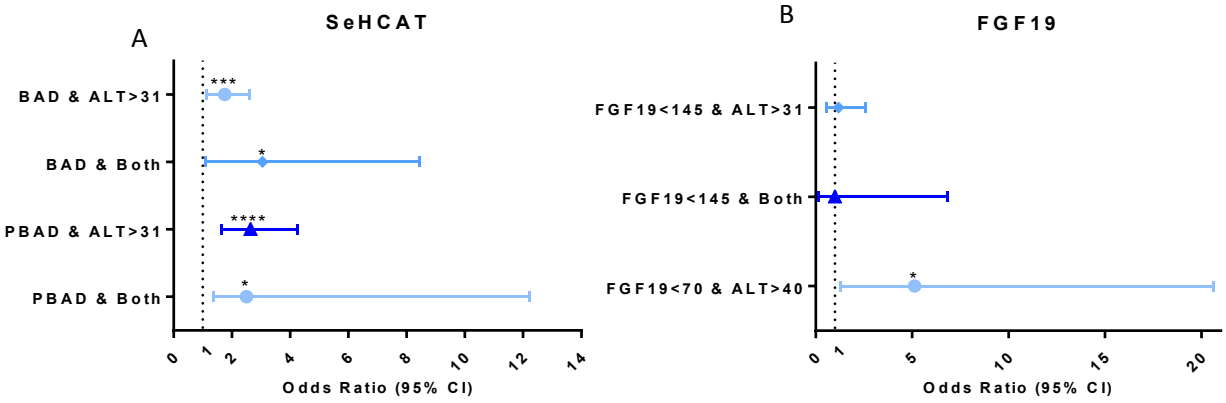


*Figure 3.7: Forrest plots of odds ratio with 95% CI. BAD and pBAD compared to SeHCAT negative controls (A) and FGF19<145 and <70 compared to FGF19>145 and >70pgml (B). *p<0.05, ***p<0.001, ****p<0.0001*

3.2.1.3 Gallstones

183 patients had imaging that reported upon the gallbladder, of which 9 were excluded due to the presence of polyps, leaving 176. Of these 103 (59%) had a positive SeHCAT scan, 47 (27%) had gallstones, 12 (7%) had a cholecystectomy, 59 (34%) had either gallstones or cholecystectomy. There was no significant difference in the rate of gallstones or cholecystectomy by gender (64% v 72%, p=0.2), but mean age in the gallstones or cholecystectomy group was higher (50 v 57, p<0.005). Median SeHCAT for the whole cohort was 11.7%.

	Controls (SeHCAT >15%)	**Type 1 BAD**	**Type 2 BAD**	**Type 3 BAD**	**All BAD**
n	73	13	52	38	103
% female	67	62	61	76	67
Mean age	55.3	54.8	51.5	52.3	52
Median SeHCAT	33	3.66	10.69	4.75	10.25
Median FGF19 (n)	251 (55)	187.5 (2)	177 (10)	174 (19)	176 (21)
Cholecystectomy	12	0	0	24	24
Gallstones	6	7	10	0	17

Table 3.4: Demographics and number of patients with gallstones or cholecystectomy displayed by SeHCAT value and BAD type.

The median SeHCAT for those with gallstones or cholecystectomy was significantly lower than those without (7.2%v14%, p<0.001). With cholecystectomy excluded, the median SeHCAT was still lower for those with gallstones (3.8%v14%, p<0.0001).

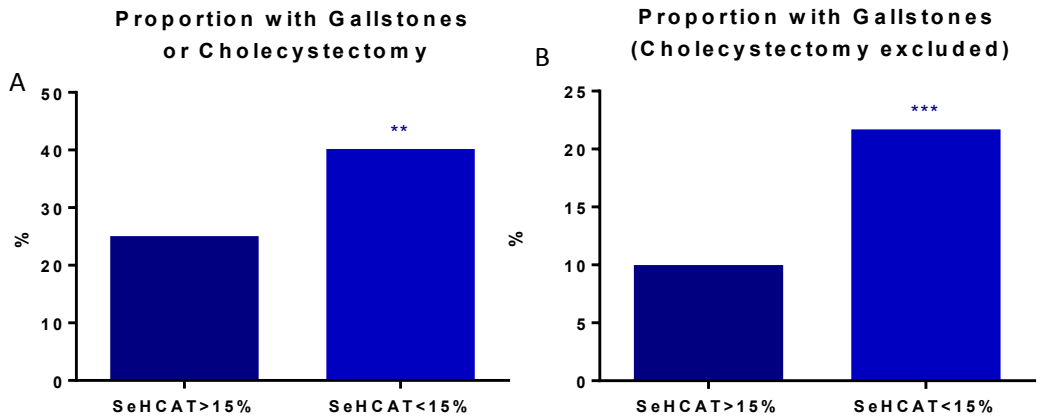


*Figure 3:8: Proportion of patients with gallstones and cholecystectomy (A) and with gallstones with without cholecystectomy (B) displayed by SeHCAT value. **p<0.01, ***p<0.001.*

Overall, a SeHCAT value of <15% conferred an increased risk of gallstones or cholecystectomy (OR=2.0, 95%CI 1.04-3.919, p<0.05) and the presence of primary bile acid diarrhoea was associated with gallstones, although this did not reach statistical significance (OR=1.98, 95%CI 0.65-5.99, p=0.23). FGF19 was measured in 60 patients with imaging, of whom 15 had gallstones or cholecystectomy. There was no significant difference in the median serum FGF19 between those with gallstones or cholecystectomy and those without (224v227pg/ml, p=0.7).

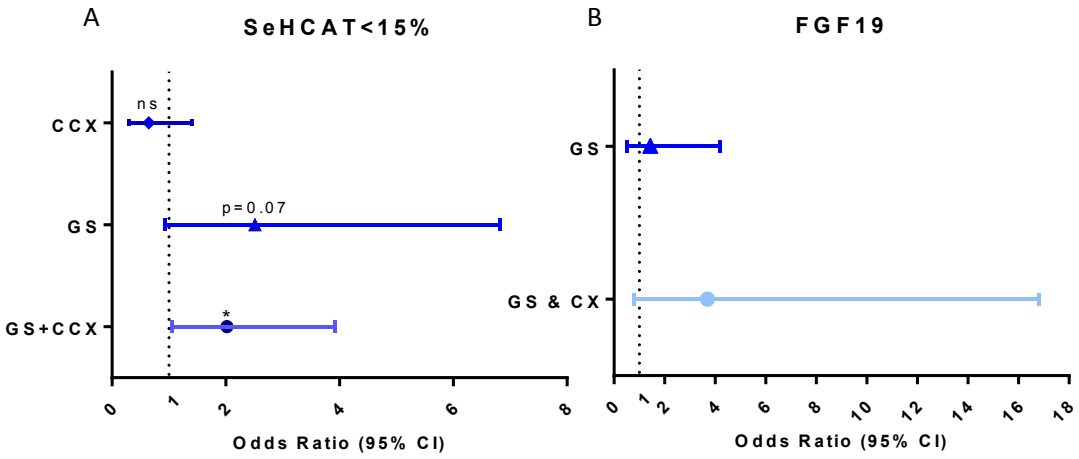


*Figure 3.9: Forrest plots of odds ratios of SeHCAT<15% (A) and FGF19<145pg/ml (B) with cholecystectomy (CCX), Gallstones without cholecystectomy (GS) gallstones with cholecystectomy (GS & CX). *p<0.05.*

3.2.1.4 Colorectal Polyps / Cancer

328 patients without IBD had a completed colonoscopy, of which 172 had a SeHCAT <15% and 156 had a SeHCAT >15%. SeHCAT 7d value did not correlate with number of polyps, the number of polyps>1cm or the number with colorectal cancer (results in table 3.5).

	SeHCAT<15%	SeHCAT>15%	P
n	172	156	
Age	49.7	50.9	0.62
Gender (n=male)	79	49	0.007
Number with polyps	43	34	0.51
Total number of polyps	88	83	0.53
N with polyps>1cm	3	5	0.39
Total N polyps>1cm	3	5	0.39
N with Cancer	1	1	0.95

Table 3.5: Demographics and numbers of patients with colorectal cancer and polyps. P values calculated by fishers exact test.

3.2.1.5 Histological Inflammation

246 patients had GI histology recorded, 32 had proven IBD and were excluded from the analysis, leaving 214 (107 had a SeHCAT <15%). 200 had colonic biopsies, 74 had ileal biopsies (72 had both). 70 had duodenal biopsies, of which 56 had colonic biopsies and 26 had ileal biopsies. 99 patients with colonic biopsies had a SeHCAT diagnostic of BAD (<15%) and 5 of these were positive for microscopic colitis. Therefore there was no significant difference in the incidence of microscopic colitis in patients with SeHCAT<15%. There were no significant associations of abnormal histology from any area between controls and those with a SeHCAT<15% (results in table 3.6).

	Duodenal		**Ileal**		**Colonic**		**Microscopic colitis**		**ANY**	
	Con	BAD	Con	BAD	Con	BAD	Con	BAD	Con	BAD
N (n abnormal)	34(6)	36(5)	34(2)	40(5)	101(10)	99(14)	4	5	19	25
Age	51	48	44.8	49	48.6	50.1	49.3	56	52	52
% female	85	64	79	63	75	55	50	80	74	48
Median FGF19 (n)	na	182(3)	na	202(4)	124 (12)	206 (1)	na	na	na	209

Table 3.6: Demographics and numbers of patients with abnormal histology displayed by SeHCAT value (Controls (con) >15%, BAD<15%)). ANY is the number of patients with any abnormal GI histology from duodenum, ileum or colon.

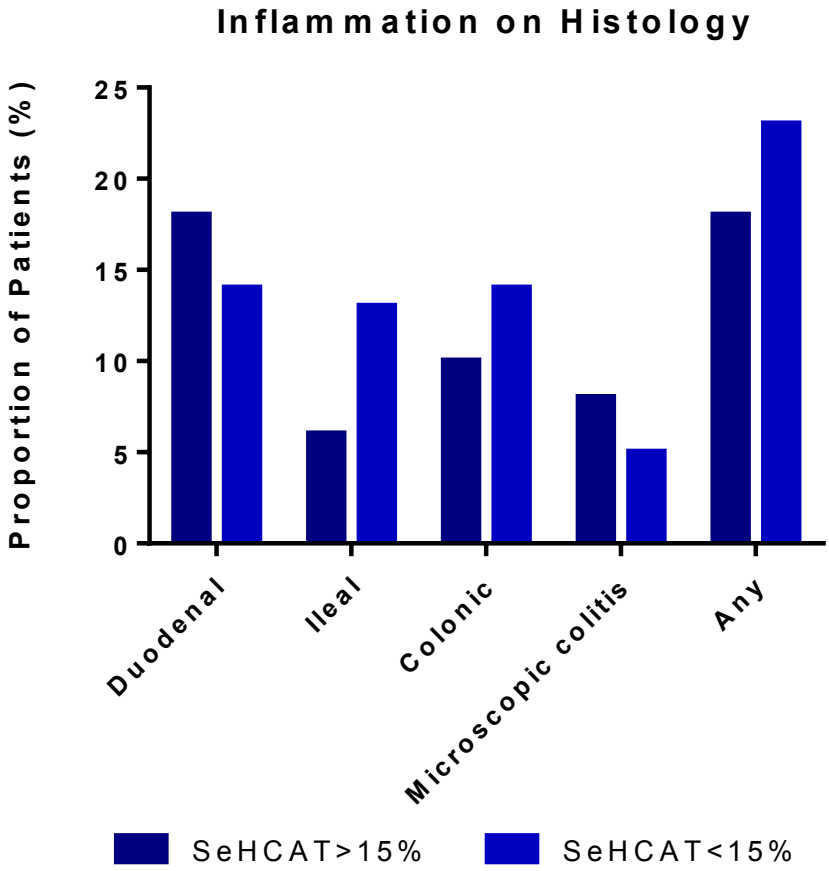


Figure 3.10: Proportion of patients with inflammation or microscopic colitis on endoscopic biopsy. ANY is the proportion of patients with any abnormal GI histology from duodenum, ileum or colon.

3.2.1.6 Gallstones, NAFLD and hypertriglyceridaemia occurring together

104 patients had ALT, TG and gallbladder imaging recorded. 63 patients had a SeHCAT<15%, 41 >15%. The frequency of these conditions occurring together is shown in table 3.7.

	Controls (SeHCAT>15%)	BAD (SeHCAT<15%)	P (Fishers)
n	41	63	
ALT>31	12	12	0.20
ALT>31 & TG>1.7	4	13	0.18
ALT>31 & TG>1.7 & Gallstones	1	4	0.65

Table 3.7: Number of patient with combination of ALT>31IU/L, triglycerides>1.8mmol/L and gallstones or cholecystectomy on imaging

The odds ratio of those 3 diseases occurring together is 2.7 (95% CI 0.29-25.01) with patients with BAD compared to controls with a SeHCAT>15%. The fisher's exact test for this is 0.65 (non-significant).

Only 19 patients with ALT, TG and gallstone data had FGF19 recorded, of these 7 were <145pg/ml. There were no incidences of hypertriglyceridaemia in the 3 patients with ALT>31IU/L and FGF<145mg/L making comparison of the groups impossible.

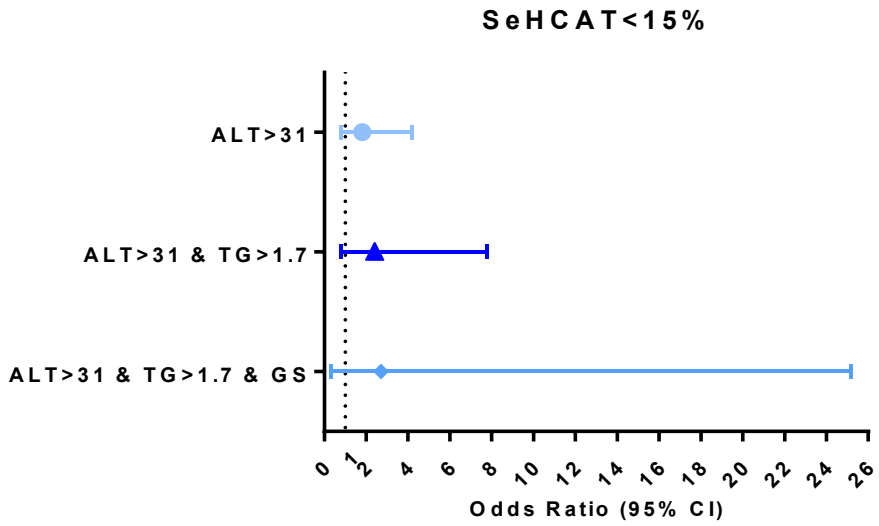


Figure 3.11: Forrest plot of odds ratios of SeHCAT<15% and combinations of ALT>31IU/L, triglyceridaemia>1.7mmol/L and gallstones or cholecystectomy on imaging.

3.2.2 Incidence of bile acid diarrhoea in non-alcoholic liver disease

127 patients were recruited off whom 32 (25%) reported that they experienced diarrhoea. Demographics for these groups are shown in table 3.8 and 3.9. Demographic factors that increased the likelihood of diarrhoea were female sex, higher BMI, white ethnic group, diabetic and on metformin (tables 3.8 and 3.9).

	All (n=127)	No Diarrhoea (n=95)	Diarrhoea (n=32)	p
Age (years)	52.51	52.28	53.18	0.74
Female (%)	42 (33)	17 (18)	25 (78)	0.0086
BMI (>25/>30(%))	30.84	29.9 (91/54)	33.5(97/66)	0.0007
White ethnic group (%)	66/123 (54)	44/94 (51)	22/29 (76)	0.0198
Asian ethnic group (%)	35/129(27)	31/94 (33)	4/29 (14)	0.059
Black ethnic group (%)	8/129 (6)	6/94 (9)	2/29 (7)	1
Far Eastern ethnic group	3/129 (2)	3/94 (3)	0/29 (0)	1
DM (%)	57 (45)	37 (39)	20 (63)	0.0246
FGF19	131.3	138.7	110.4	0.32
C4	53.8	43.9	82	<0.0001
Cholecystectomy	6	3	3	0.14
Alcohol (units/week)	2.26	2.47	1.63	0.41

Table 3.8 :Demographics by presence of diarrhoea.

	All (n=127)	No Diarrhoea (n=95)	Diarrhoea (n=32)	p
Metformin (%)	42 (33)	36 (38)	20 (63)	0.023
Insulin	14 (11)	8 (9)	6(19)	0.12
GLP-1 Agonist	5 (4)	2 (2)	3 (9)	0.10
Sulfonylurea	17	13	4	1
Glitazone	5	4	1	1
SGLT-2 Inhibitor	2	1	1	0.44
DDP Inhibitor	8	5	3	0.42
Orlistat (%)	6 (5)	5 (5)	1 (3)	0.58
Statin (%)	65 (51)	50 (56)	15 (47)	0.68
Fibrate	6	6	0	0.34
Exetimibe	2	1	1	0.44

Table 3.9: Demographics by presence of diarrhoea

3.2.2.1 Incidence of chronic diarrhoea

32 (25%) of patients reported subjective diarrhoea. Of these, 3 had a cholecystectomy, one a Roux-en-Y gastric bypass, one had a gastric neuroendocrine tumour, one had Bechet's disease, but without known bowel involvement. 25 of these patients had testing for FGF19 and C4. Of these 11 (44% of those with diarrhoea, 12% of all patients with NAFLD) had a FGF19 <145pg/mL and C4 >70nmol/L, which is diagnostic for BAD. 8 (10%) of those patients without diarrhoea had a FGF19 <145pg/mL and C4 >70nmol/L (p=0.001, OR 6.2 (95%CI 2.1-18.2). Metformin and diabetes were significant risk factors for diarrhoea, OR 2.7 (1.19-6.25) and 2.6 (1.1-5.97) respectively.

71 patients were not taking metformin, 12 of these had diarrhoea. With metformin excluded, 3 patients with diarrhoea had an FGF19<145pg/mL and C4>70nmol/L compared to 2 in the non-diarrhoea group (p=0.026 OR 11.7 (1.6-87.97). With metformin excluded, DM was not a risk factor

for diarrhoea although the numbers of this sub cohort were low (2/12 vs 4/69 patients, OR 2.75 (0.44-17.1) p=0.27).

3 patients reported >21 BMs per week. One of these patients had a Roux-en-Y gastric bypass and was empirically started on cholestyramine with good effect. A SeHCAT test was not necessary. The other 2 were offered SeHCAT testing, one declined, the other was positive at 12%. This lady had previously had a cholecystectomy, her FGF19 was 72.5 pg/mL and C4 25.9 nmol/L. The other 2 patients both had FGF19 of 43 and 67.5 pg/mL and C4 of 85.7 and 88.6nmol/L.

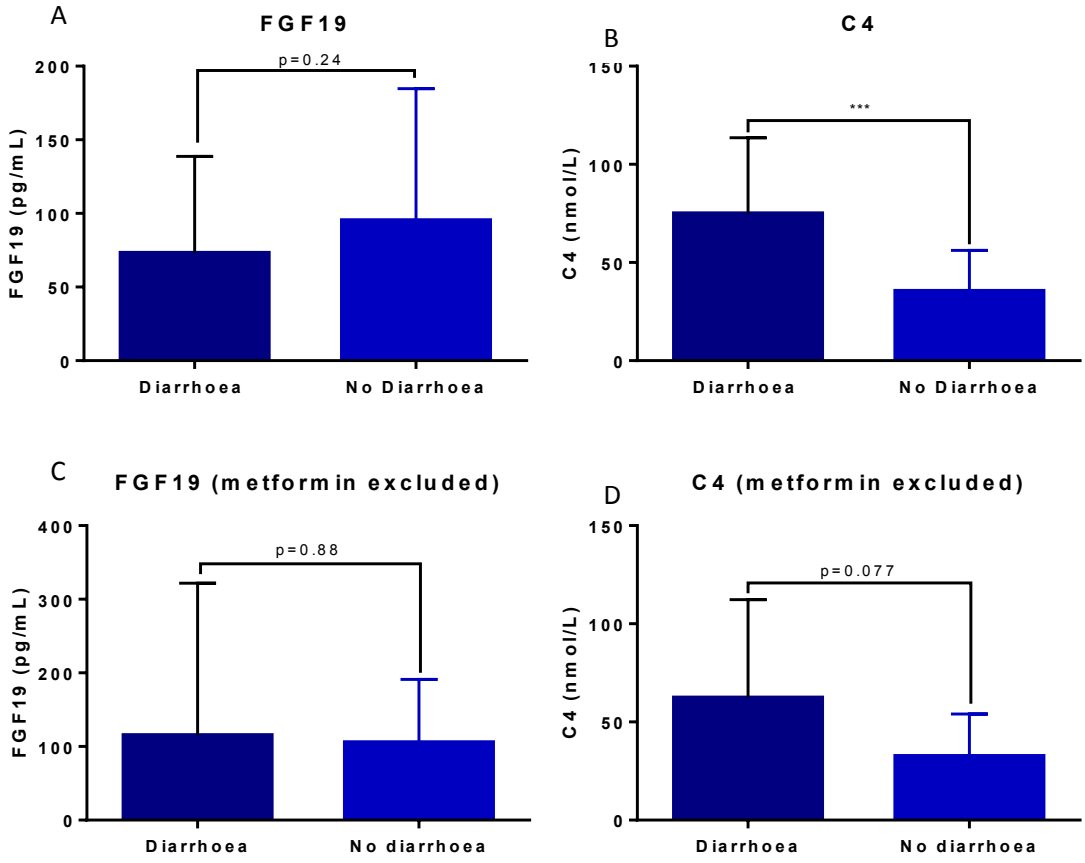


*Figure 3.12: Median (IQR) FGF19 and C4 in patients with diarrhoea compared to those without diarrhoea, all patients (A, B) and patients not taking metformin(C,D). ***p<0.001*

3.2.2.2 Predictors of severity of NALFD

3.2.2.2.1 Diarrhoea

The presence of diarrhoea was associated with a higher NAFLD fibrosis score (median -0.49 vs -1.12, n=25/70, p=0.0023), but not with higher Fibroscan score (n=20/58, 7.05 v 7.05, p=0.87) or presence of fibrosis or cirrhosis on biopsy (n=19/44 OR 1.35 (0.35-5.18, p=0.75). The number of BMs per day (n=12) did not correlate with any markers of NAFLD severity.

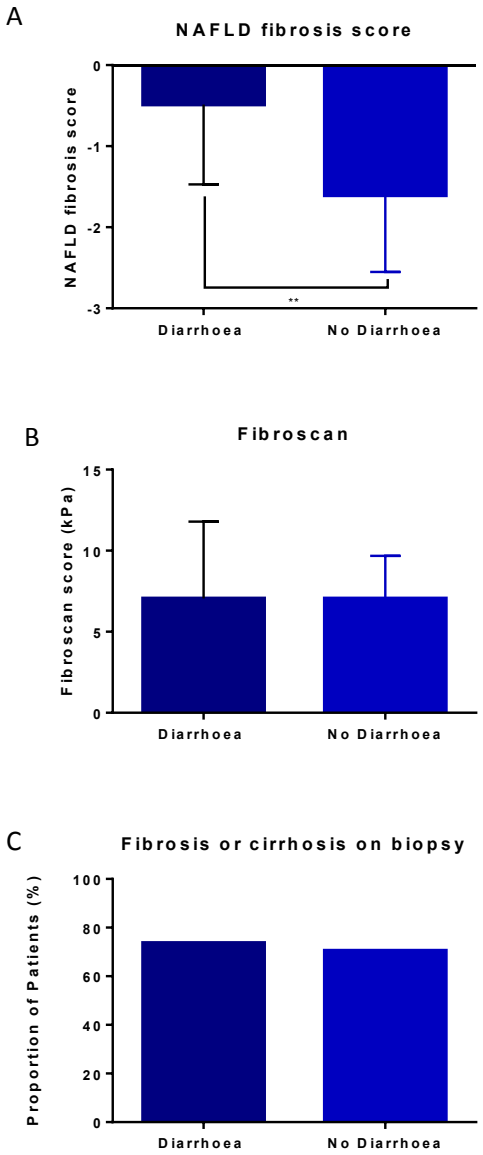


Figure 3.13 Diarrhoea as an indicator of severity of NAFLD. (A) median (IQR) NAFLD severity score, (B) Median (IQR) Fibroscan, (C) proportion of patients with either fibrosis or cirrhosis on liver biopsy.

3.2.2.2.2 FGF19 and C4

There were no differences in median NAFLD fibrosis score or Fibroscan stiffness when separated by the FGF19 above and below 145pg/mL (Table 3.10 and fig 3.14). Median NAFLD fibrosis score was higher for patients with a C4 >70nmol/L (-0.5 vs -1.55, p=0.02), but there were no significant difference in Fibroscan stiffness. 51 patients with FGF19/C4 had liver biopsy results available. Serum Median FGF19 and C4 was comparable across all severities of NAFLD by biopsy.

	FGF19<145	**FGF19>145**	**C4<70**	**C4>70**
NAFLD Fibrosis score	-1.16(-2.24--0.025)	-1.64 (-2.46—0.42)	-1.55 (-2.46—0.64)	-0.5 (-1.9-0.16)
Fibroscan	7.15 (5.48-10.7)	6.5 (5-8.23)	6.85 (5.4-8.95)	7.9 (5.9-12.2)

Table 3.10: Median (IQR) NAFLD Fibrosis score and Fibroscan stiffness by FGF19 and C4

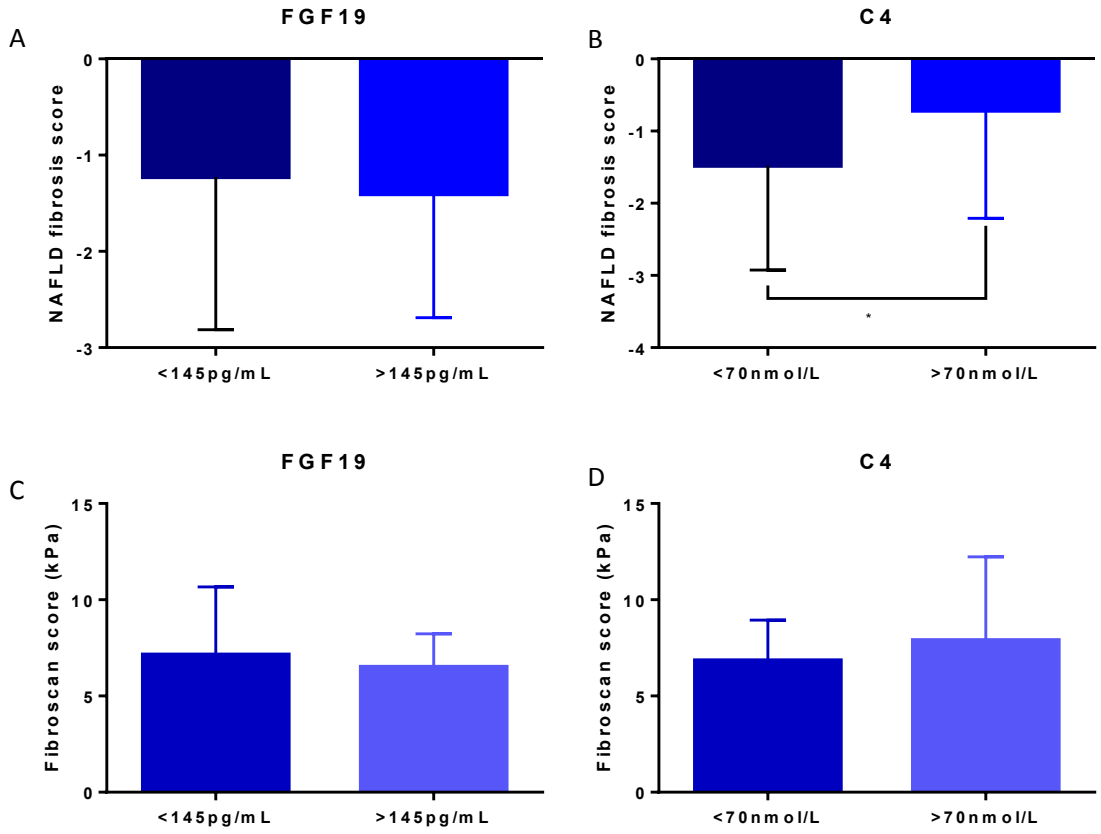


Figure 3.14: Median (IQR) NAFLD fibrosis score (A, B) and Fibroscan stiffness (C, D) by FGF19 and C4.

When patients were characterised by their FGF19 and C4 values together there were significant differences in the NAFLD fibrosis score, but not in fibroscan score. Patients with a FGF19<145pg/mL and a C4>70nmol/L had a higher median NAFLD fibrosis score than all other groups (-0.49 (-1.19-0.23).

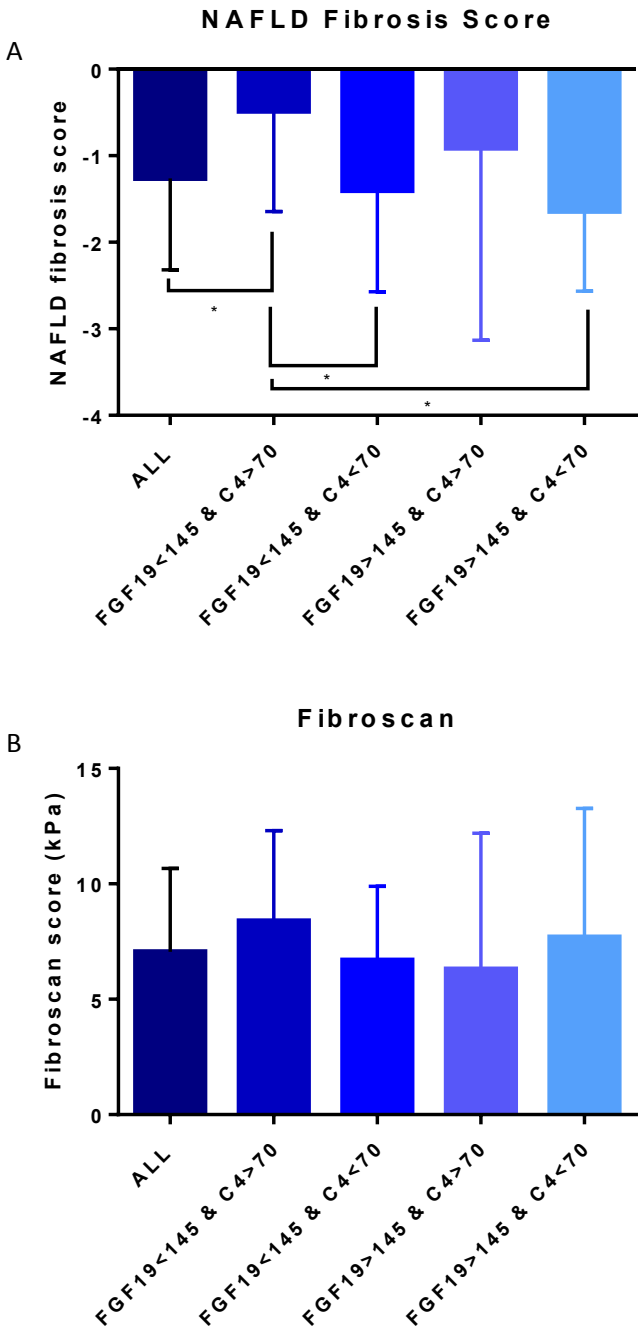


Figure 3.15: Median (IQR) NAFLD fibrosis score (A) and Fibroscan (B) by FGF19 and C4 together

51 patients with FGF19/C4 had liver biopsy results available. Serum Median FGF19 and C4 were comparable across all severities of NAFLD by biopsy.

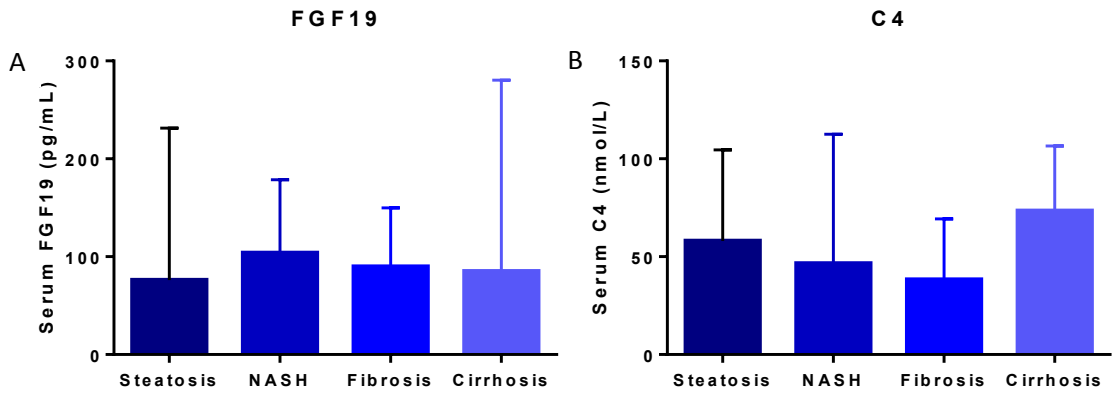


Figure 3.16: Median (IQR) FGF19 (A) and C4 (B) by liver biopsy results.

FGF19 correlated significantly with C4 and ALT (table 3.11 and figure 3.17), but not with the NAFLD fibrosis score or Fibroscan stiffness.

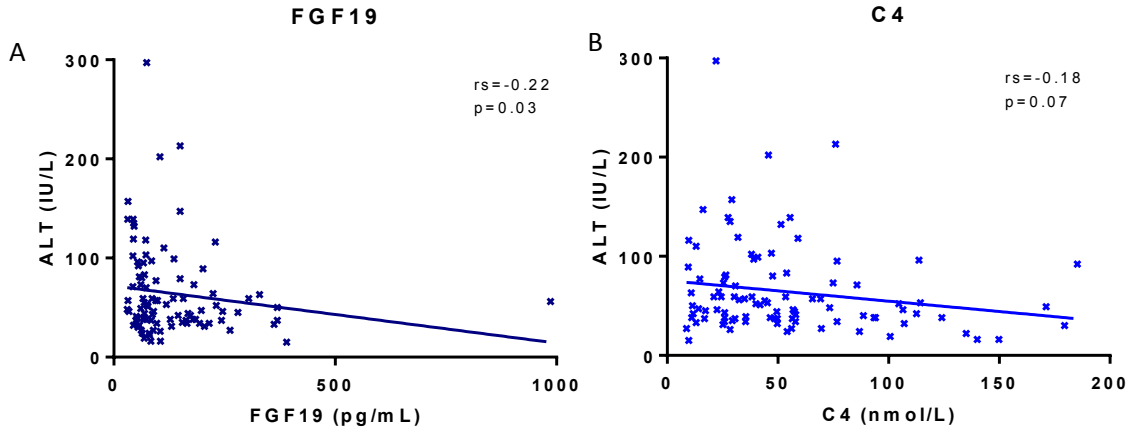


Figure 3.17: Correlations of ALT with FGF19 (A) and C4 (B).

		NAFLD Fibrosis Score	Fibroscan	ALT	AST	FGF19
C4	rs	0.14	0.04	-0.18	-0.18	-0.43
	P	0.17	0.73	0.07	0.09	****
NAFLD Fibrosis Score	rs		0.25	-0.37	-0.10	0.03
	p		0.03	***	0.34	0.76
Fibroscan	rs			0.10	0.25	-0.14
	p			0.38	0.03	0.24
ALT	rs				0.78	-0.22
	p				****	0.03
AST	rs					-0.18
	p					0.08

Table 3.11: Correlation matrix (Spearman's Rank, rs) for predictors of NAFLD severity with significant values in red.

3.2.2.3 *Effect of Metformin and Diabetes on diarrhoea and FGF19*

Being on metformin gave an OR of 2.73 (95%CI 1.19-6.25) for diarrhoea (p=0.02) and diabetes gave an OR for diarrhoea of 2.6 (95%CI 1.14-5.97, P=0.025). Only 3 patients were taking metformin who weren't diabetic, 1 of these had diarrhoea (OR 2.79 (0.22-35.05 p=0.42). 3 patients were diabetic without taking metformin. 1 of these patients had diarrhoea OR 1.13 (0.07-10.43 p=1).

Median FGF19 was lower and C4 higher in patients who were diabetic, or who were taking metformin, see figure 3.18. Median serum FGF19 was lower by 25.9% and 30% in diabetics and those taking metformin respectively. Median C4 was increased by 42.5% and 26.1% in diabetics and those taking metformin respectively.

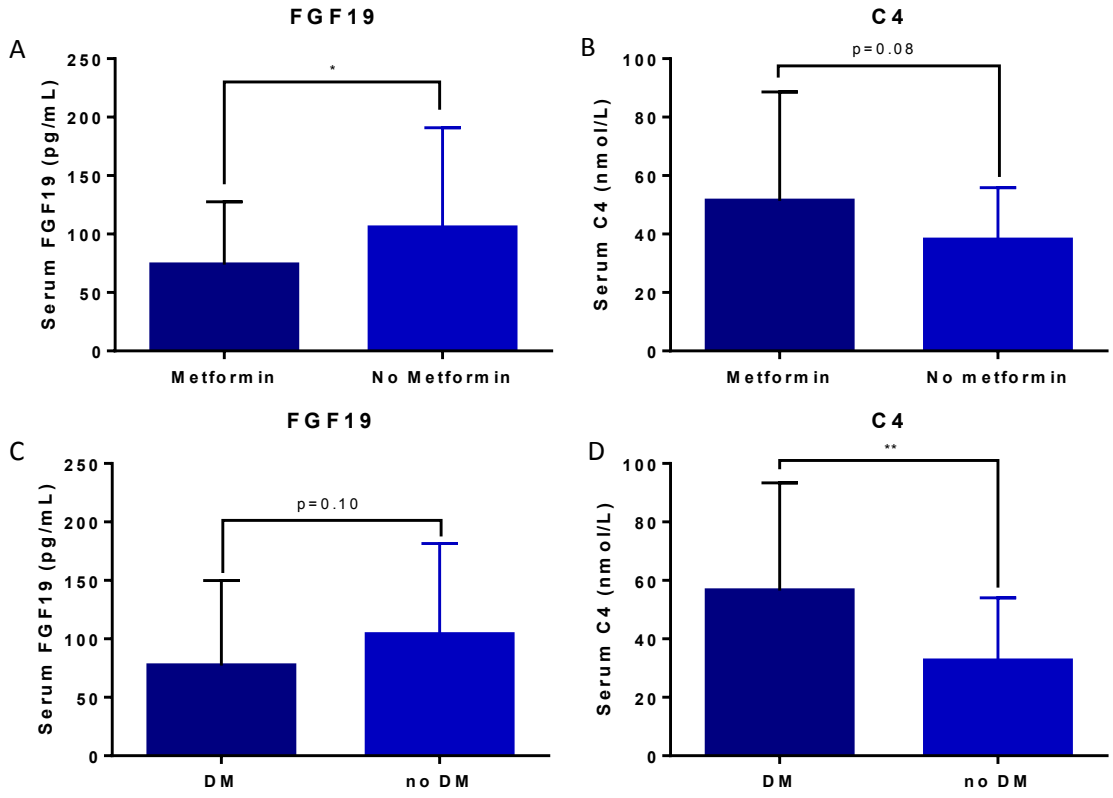


*Figure 3.18: Median (IQR) FGF19 and C4 of patients taking metformin (A, B) or diabetic patients (C,D). *p<0.05, **p<0.01.*

3.2.2.4 FGF19 and C4 and metabolic profile

FGF19 correlated negatively with serum fasting glucose (rs=-0.2, p=0.05) and C4 as could be expected. C4 did not correlate with glucose or any lipid fraction.

	TG		LDL		Glu		TC		HDL		FGF19	
	rs	p	rs	p	rs	p	rs	p	rs	p	rs	p
C4	0.05	0.60	-0.12	0.24	0.13	0.22	-0.12	0.24	0.06	0.53	-0.43	****
TG			-0.12	0.24	0.05	0.65	0.21	0.04	-0.24	0.02	0.13	0.22
LDL					-0.46	****	0.83	****	0.15	0.14	0.07	0.49
Glu							-0.46	****	-0.05	0.60	-0.20	0.05
TC									0.22	0.03	0.12	0.24
HDL											-0.03	0.75

*Table 3.12: Correlation matrix of FGF19, C4, glucose and lipid profile. Spearman's rank correlation (rs), significant values in red. ****p<0.001*

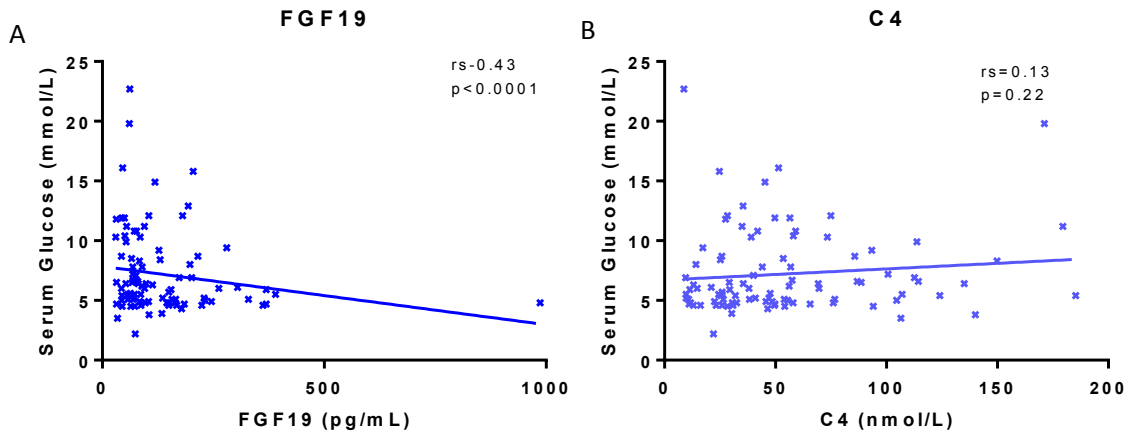


Figure 3.19: Correlation of FGF19 (A) and C4 (B) with glucose.

3.2.3 DIET1 polymorphism in NAFLD

94 patients were tested for the rs12256835 genotype. Demographics are as those in the BAD in NAFLD study (table 3.13). The minor allelic frequency (MAF) in all patients with NAFLD was 0.43.

3.2.3.1 Diarrhoea

The MAF was similar between patients with diarrhoea and those without (0.41 v 44, p=0.81). Homozygotes for the minor allele (GG) were twice as likely to have diarrhoea, although this did not reach statistical significance (6 v 12%, p=0.38). Only 3 patients had >21 BMs/week, one of these had a Roux-en-Y bypass (genotype TT), the other 2 were genotype TG.

	All (n=94)	**No Diarrhoea (n=69)**	**Diarrhoea (n=25)**	**BM>21/week (n=3)**	**p**
GG	7 (8)	4 (6)	3 (12)	0	0.38
TT	33 (58)	41 (59)	13 (52)	1	0.64
TG	54 (35)	24 (35)	9 (36)	2	1
T	87 (93)	65 (94)	22 (88)	3	0.38
G	40 (42)	28 (41)	12(44)	1	0.82

Table 3.13: Allelic frequency of DIET1 polymorphism in patients with NAFLD and diarrhoea

3.2.3.2 FGF19 and C4

The presence of one minor allele G decreased serum FGF19 from a median FGF19 of 112.1 pg/mL (IQR; 71.4-198.3) to 72.5 pg/mL (57.3-120.4), this decrease of 45.9% was statistically significant (figure 3.19). The addition of another G allele reduced FGF19 further (Median for GG 61.1 pg/L (32-76)) which was significantly different to the TT, but not the heterozygous genotype. ANOVA (Kruskal-Wallis) of the 3 genotype groups showed statistically significant differences in FGF19 (p=0.005).

Genotype did not significantly affect serum C4 levels. Median C4 for TT, GG, TG genotypes were 4.1 (25.1-61.7), 40 (25.4-65.6) and 47.6 nmol/L (25.9-90.1) respectively.

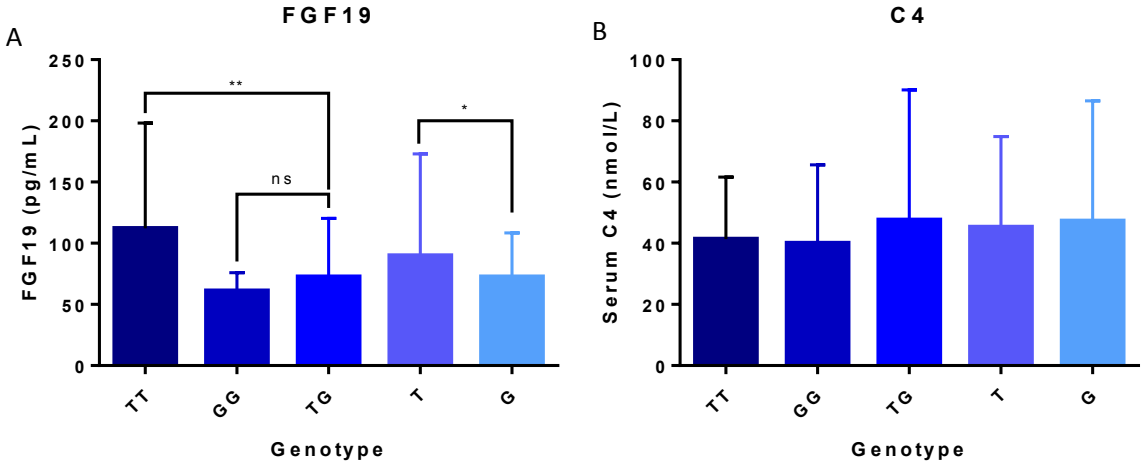


*Figure 3.20: Median (IQR) serum FGF19 (A) and C4 (B) by genotype. *p<0.05, **p<0.01, ns=non-significant.*

3.2.3.3 *Severity of NAFLD*

44 patients had a liver biopsy. There was no significant difference in the frequency of fibrosis or cirrhosis with the minor G allele (57% v 73%, p=0.65).

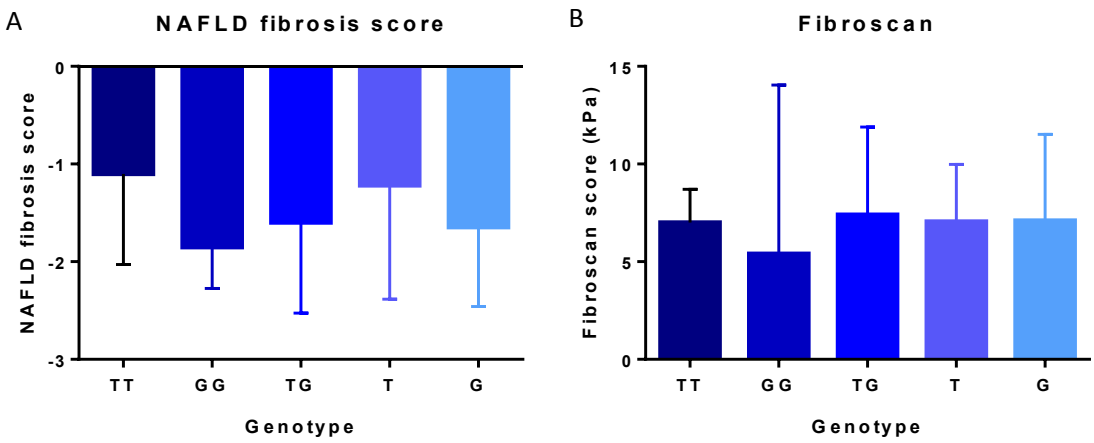


Figure 3.21: Median (IQR) NAFLD fibrosis score (A) and Fibroscan (B) by genotype.

	TT	**GG**	**TG**	**T**	**G**
Fibroscan	7 (5.4-8.7)	5.4 (5.5-14.1)	7.4 (5.5-11.9)	7.1 (5.4-10)	7.1 (5.4-11.5)
NAFLD Fibrosis score	-1.1 (-2.3— 0.36)	-1.9 (-2.3— 0.37)	-1.6(-2.5— 0.25)	-1.2 (-2.38— 0.27)	-1.7 (-2.46— 0.28)
Biopsy (Steatosis/NASH/ Fibrosis/cirrhosis)	(5/2/18/1)	(1/0/0/0)	(1/4/6/6)	(6/6/24/7)	(2/4/6/6)

Table 3.14: Markers of NALFD severity by genotype. Median Fibroscan and NAFLD fibrosis score (IQR) and total numbers of biopsies shown.

3.2.3.4 *Effect on glucose and lipid metabolism*

There were no significant differences in the incidence of DM, median BMI, fasting glucose, total cholesterol or LDL cholesterol based on genotype. There was a significant lower serum triglycerides with the GG genotype compared to TT.

	TT (n=54)	**G (n=40)**	**GG (n=7)**
BMI	31.15 (27.77-35.78)	29.41 (27.46-32.43)	30.55 (29.27-32.09)
DM (%)	44	46	28.6
Glucose (mmol/L)	5.7 (4.8-8.33)	6.25 (5.2-8.83)	6.5 (5.4-7.1)
Total Cholesterol (mmol/L)	4.4 (3.9-5.13)	4 (3.5-4.9)	4.4(3.9-5.1)
LDL Cholesterol (mmol/L)	2.29 (1.71-3.19)	2.21 (1.61-2.93)	2.77(2.26-3.37)
Triglycerides	1.94 (1.49-2.74)	1.73 (1.23-2.27)	1.4 (1.15-1.54)

Table 3.15: Glucose and lipid profile of patients by genotype, median (IQR) or percentage of patients.

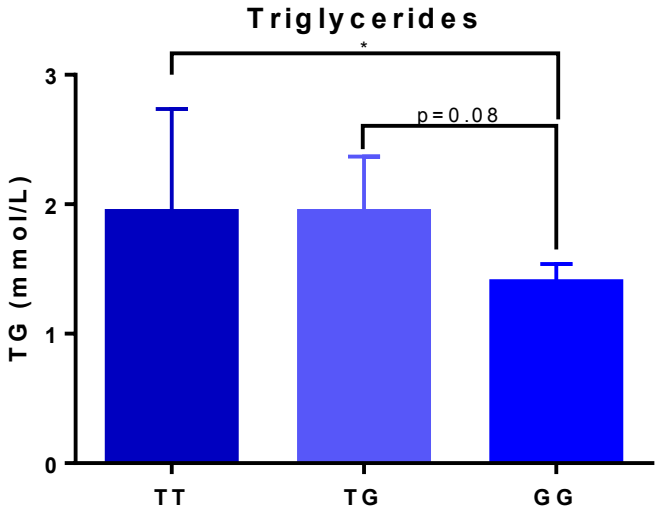


*Figure 3.22: Serum triglycerides by number of G alleles. *p<0.05*

3.3 Conclusions: Disease Associations of BAD

3.3.1 Disease associations with primary bile acid diarrhoea and low FGF19

I hypothesized that BAD, hypertriglyceridaemia, NAFLD and gallstones may share pathophysiologic mechanisms and commonly coexist. We have shown that BAD, as defined by a SeHCAT 7 day retention value <15%, does coexist with hepatic steatosis (OR 3.0), hypertriglyceridaemia (OR 3.3) and gallstones or cholecystectomy (OR 2). Unfortunately we cannot comment on the shared pathophysiological mechanism, as the number of patients with a FGF19 recorded and the tests required to investigate this (imaging, serum lipid and liver profile) was low at 19. Despite the low numbers of patients with FGF19 recorded there were non-significant trends for all 3 conditions to be more prevalent in patients with a FGF19<145pg/L. There was also a significant association of FGF19 <70pg/L and ALT>40IU/L (OR 5.1). These 4 conditions are connected, but the underlying shared pathogenesis may not involve FGF19. A study of 482 patients with cholelithiasis, found that it was an independent risk factor for NAFLD (OR 1.92), BMI>25 (OR 2.0) and hypertriglyceridaemia (OR 1.91).[184] This implies that these conditions do commonly co-exist, but the relationship with FGF19 is yet unknown.

There were a number of limitations placed on this study due to its retrospective design. In particular we cannot assure that the patients presented here as NAFLD do not have another cause of their deranged liver function. Viral hepatitis serology, alcohol consumption or liver biopsy results were not routinely collected, although patients were excluded if a liver disease other than NAFLD was mentioned on the documentation reviewed. Ideally diagnosis of NAFLD would be by hepatic biopsy, or at least a validated non-invasive scoring system. However, we were limited to using ALT and imaging findings as these are common tests that significant numbers of our patients had recorded. Ultrasound has sensitivity of 77-100% depending on the degree of steatosis, and ALT can be normal in up to 23% of patients with biopsy proven NAFLD.[185, 186] However ALT is widely accepted as a marker of severity for NAFLD and regardless of the aetiology of the liver disease, the incidence is remarkably higher in patients with BAD. It is also important to remember that our control patients

have chronic diarrhoea and are not healthy volunteers, there is likely to be an increased level of co-morbidity in this group compared to the general population.

The rationale for NAFLD being associated with BAD comes from the known association of both these diseases with hypertriglyceridemia and obesity. It is plausible that low FGF19 is a pathogenic factor in both diseases, since it is associated with obesity, hepatic insulin resistance and NAFLD in paediatric patients.[136] However the link between all these conditions is far from certain, we know that serum triglycerides are unaffected by FGF19.[187] Also we know that 30% of patients with a SeHCAT <15% have a high FGF19 and high triglycerides.[121] This probably accounts for the high prevalence of hypertriglyceridemia within our cohort. Therefore there is likely to be several different causes of pBAD, not just low FGF19 and the disease associations are likely to vary according to the underlying pathogenesis.

The finding that median SeHCAT values are lower in patients with gallstones without cholecystectomy is important. The mechanism behind post-cholecystectomy diarrhoea is not understood. It is associated with increased C4, indicating that increased bile acid production is a factor, but FGF19 does not change significantly.[188] Our data suggests that patients with bile acid diarrhoea are predisposed to gallstone disease, and probably subsequent cholecystectomy. A non-linear relationship between serum FGF19 and C4 is suggested by studies using bile acid sequestrants to suppress serum FGF19 which had much larger effect on serum C4 in healthy volunteers: an 87% reduction in serum FGF19 created an 18 fold increase in C4.[108] It is possible that in susceptible individuals with SeHCAT <15%, the small decrease in FGF19 caused by cholecystectomy, creates a much larger increase in bile acid production, causing BAD. Equally, it is possible that these patients had diarrhoea pre-dating their cholecystectomy.

In addition we also hypothesised that there would be an increased incidence of microscopic colitis and colorectal adenomas in patients with a SeHCAT <15%. Despite a large series of patients with colonoscopy (328) and colonic histology (200) the proportion of patients with either colonic adenoma/cancer or microscopic colitis was remarkably similar between patients with a SeHCAT<15% and those >15%. The proportion of patients with either colonic adenoma/cancer or microscopic colitis was 25% and 4% which is a little lower than a recent series of colonoscopies in 130,204 patients with chronic diarrhoea that reported rates of 29% and 8.6%.[189]

In conclusion, we have shown that BAD and particularly primary BAD is associated with increased incidence of NAFLD, hypertriglyceridemia and gallstones. The reason for these associations requires further investigation, and cannot be assumed to be due to low FGF19 alone.

3.3.2 Prevalence of primary bile acid diarrhoea in patients with non-alcoholic fatty liver disease

We describe here a 25% prevalence of subjective diarrhoea in NAFLD. Diarrhoea was significantly associated with female sex, white ethnicity, higher BMI, diabetes and metformin treatment. High BMI, diabetes and metformin are all factors known to be associated with chronic diarrhoea through a variety of mechanisms.

Using the cut off of FGF19 described by *Pattni et al.*[190] to detect BAD, 19 patients with diarrhoea met the criteria for BAD on FGF19 alone (<145pg/mL, sensitivity 74%, specificity 72%). Using the cut off for C4 described by *Brydon et al.*[100] 13 patients met the criteria for BAD (>74.88nmol/L, sensitivity 97%, specificity 74%). 11 patients met both these criteria, although the specificity of both markers together is not known, it can be assumed to be higher than 74%. Allowing for false positives, we calculate that 8 patients were identified with likely BAD out of the 96 that had FGF19/C4 measured. This incidence of 8.5% is higher than the 2.5% predicted from the retrospective database and the 1% reported in the general population.

Compliance with the stool diary completion was 75%. As per the study protocol, 3 patients were identified with >21 BMs per week, all these patients had a FGF19 <145pg/mL (Median 67.5), 2 had a C4 >74.88 nmol/L (Median 85.1). One off these patients had a Roux-en-Y gastric bypass, so almost certainly had BAD, therefore a SeHCAT scan was thought unnecessary, he was started on cholestyramine empirically with a good response. The other 2 had SeHCAT scans booked, but one declined further investigation, the other had proven pBAD with a SeHCAT of 12%. This patient had a cholecystectomy.

In patients with diabetes, which comprise 45% of our cohort, chronic diarrhoea has previously been reported to be as prevalent as 15.6%, conferring an OR of 1.69.[191] Our own OR was calculated as 2.6 (1.14-5.97). This effect is compounded by the high metformin usage in our diabetic cohort, making the diarrhoeal effect of diabetes and metformin inseparable. 54% of the patients with a BMI>30 were also diabetic. Higher BMI was associated with diabetes (median BMI 33.5 v 29.9) but this lost statistical significance when patients with DM were excluded, although the trend towards higher BMI with diarrhoea was still there (31.7 v 29.1). The prevalence of chronic diarrhoea in individuals of BMI>30 has been reported to be as high as 30%.[192] In non-diabetic patients prescribed metformin pre-operatively in a coronary bypass trial the prevalence of diarrhoea in the metformin arm was 21%, conferring an OR of 12.6.[193] Our own calculated OR of diarrhoea with metformin was 2.7. Clearly the links between obesity, diabetes and metformin use are strong, and differentiating the contributing effects of these to diarrhoea is impossible in our small cohort.

The mechanisms underlying diarrhoea in diabetes are multiple and include small bowel bacterial overgrowth, loss of α_2 adrenergic stimulation and medications (usually metformin).[194] However, BAD has never been considered to be associated. There is growing evidence that metformin interferes with bile acid metabolism and that this part of its diarrhoeal mechanism. In a study where patients stopped metformin for around 2 weeks then restarted, the total bile acid pool increased on metformin and faecal bile acids decreased (although not significantly so) on metformin

withdrawal.[195] Interestingly the authors conclude that part of metformin's insulin sensitising effect may be due to increased serum GLP-1 caused by increased TGR5 stimulation by colonic bile acids. Metformin's principal mechanism of action is through activation of AMP-activated protein kinase (AMPK). AMPK has recently been found to be regulator of FXR through phosphorylation.[196] Metformin reduced FGF15/19 in both human HepG2 cells and mice. This effect was seen in our cohort, with median FGF19 30% lower in patients on metformin, this is the first time this effect has been described in humans.

If there was a shared pathogenesis of NAFLD and BAD, we may expect the number of BMs/day to correlate with the severity of NAFLD. In our study this was measured by the NAFLD fibrosis score, Fibroscan and liver biopsy. The presence of diarrhoea was associated with a higher median NAFLD fibrosis score, but not higher Fibroscan scores, or fibrosis on biopsy. There were no significant correlations with these parameters and the number of BMs/day. Although the sample size of patients that returned stool diaries was small.

Serum FGF19 did not appear to change with NAFLD severity, but a C4 >70nmol/L was associated with a higher NALFD fibrosis score. To detect whether this effect was due to hepatic resistance to FGF19, as has been reported in patients with insulin resistance,[136] patients with C4>70nmol/L where divided in to those with FGF19</>145pg/mL and compared. No significant difference was found. High C4 was not associated with higher Fibroscan score or advance disease on biopsy.

53% of our cohort had liver biopsies. A weakness of our study is the variability in histopathology reporting. Most studies will use a standardised reporting score, we were limited to recording key words from the reports which were written by different pathologists using different criteria. This has resulted in a large number of patients with reported fibrosis, but no measure of the severity of fibrosis or steatosis.

A key finding of the retrospective database was the negative correlation of SeHCAT with ALT. In this prospective series we have seen the same negative correlation but with FGF19 and ALT. A similar correlation was also seen with AST, and a positive correlation with C4 and ALT and AST, but these narrowly missed statistical significance. A negative correlation of FGF19 and ALT has been reported before. *Wojcik et al* reported a correlation of FGF19 and ALT of r=-0.3 in obese adolescents.[136] This finding may explain the correlation seen with SeHCAT values, and why the association is stronger with pBAD, which is particularly linked with low FGF19. The fact that the correlation is strongest with ALT and AST but not other markers of fibrosis may suggest that low FGF19 is associated with active hepatic inflammation, rather than fibrosis that the NAFLD fibrosis score and Fibroscan are powered to detect.

SHP the principle end target of FXR in the hepatocyte is known to have antifibrosis effects in the liver and is thought to be mechanism by which OCA is effective in NAFLD.[197] However FGFR4 stimulation has been shown to inhibit the NFκB apoptosis pathway.[198] Together with stimulation of SHP, this may be the mechanism of the higher ALT in those with low FGF19. Possibly low FGF19 may be risk factor for worse hepatic inflammation in the presence of other inflammatory triggers and possibly faster progression to fibrosis as suggested by paediatric studies.[199] Assessment of this is complicated by the association of low FGF19 and obesity; the only way to discover the true relationship of this association will be with a longitudinal cohort study.

Emerging evidence suggests that in cholestatic diseases, intra-hepatic FGF19 transcription is up-regulated with a corresponding decrease in CYP7A1.[200] In this study of patients with PBC, serum FGF19 had no relationship severity of liver disease, but hepatic FGF19 expression was 69 times higher and CYP7A1 50% lower in patients with cirrhosis compared to healthy controls. This would suggest that in PBC with cirrhosis, serum FGF19 and low C4 should be expected to be normal. Hepatic FGF19 is thought to act in an autocrine or paracrine manner, rather than the endocrine action of portal FGF19. NAFLD is not a cholestatic disease, if anything our data suggests that the NAFLD fibrosis score

is associated with normal serum FGF19 and high C4, but this study is an example of how hepatic FGF19 response is different in disease. FGF19 is undetectable in the healthy liver, so hepatic FGF19 rather than serum FGF19 may be a better predictor of disease severity.

3.3.3 Prevalence of the rs12256835 DIET1 polymorphism in conditions associated with low FGF19

In the 1000 genomes project, the reported MAF of the DIET1 rs12256835 G allele was 0.31, in our cohort of NALFD it was 0.42. In a series of patients with chronic diarrhoea recruited from the same geographic area it was 0.44 indicating that our local population probably have a higher MAF than that reported in the 1000 genomes project.[unpublished data] There were no significant differences in the severity of NAFLD based on genotype, despite those carrying the G allele having a lower median FGF19. The only other significant difference with GG genotype was lower median serum triglycerides.

In a series of 78 patients with FGF19 and SeHCAT values my colleague, Jonathan Nolan showed that the GG genotype was associated with lower median SeHCAT and FGF19 compared to TT or TG (94.66pg/ml, 11% vs 174 pg/ml, 18%).[unpublished data] This 54% reduction in FGF19 between the TT and GG genotype was constant with our own 54% decrease, but we also found a significant effect between TT and the heterozygote TG genotype.

Thus we can conclude from these 2 studies that the effect of the GG Diet1 genotype is lower SeHCAT retention, FGF19 and triglycerides. These findings represent a distinct clinical group, since 30% of patients with a SeHCAT<15% have high FGF19 and triglycerides.[121] It also suggests that the GG genotype should be protective against NAFLD, as high triglycerides are a risk factor for NAFLD, the frequency of the GG genotype in the 1000 genomes project is 0.16, where in our cohort of NALFD it is 0.08 despite a higher heterozygote frequency, which would support this hypothesis. This may give a clue to Diet1 mechanism of action, since less FGF19 and less hepatic FXR stimulation would be

expected to increase triglycerides; the decrease in triglycerides must be through a FGF19 independent DIET1 action. It is unlikely that this effect is due to sample error, the same effect was seen in mice with a Diet1 mutation.[201] Diet1 is not expressed in hepatocytes, so this effect probably mediated in the ileum.[140]

4 Regulation of ileal FGF19 expression

4.1 Methods: Regulation of ileal FGF19 expression

4.1.1 Subjects

Patients were identified on attending for colonoscopy in the endoscopy department of Hammersmith Hospital. Any patient between the age of 18-80 was eligible for entry. The patient was asked to read the information sheet: *'Mechanisms of the regulation of intestinal bile salt absorption and diarrhoea'* after which they were consented for entry by a study doctor (information sheet and consent form are in appendix 2.1). This is an on-going study that was started in 2000, and the ethics committee approval was renewed in 2010 (Hammersmith and Queen Charlotte's & Chelsea REC, reference number: 2000/5795) and allows serum and DNA collection for investigation of bile acid signalling. It also allows up to 8 endoscopic biopsies to be taken from patients undergoing colonoscopy for clinical indications. At the time of consent a brief medical history was checked and the presence of IBD, BAD or other ileal disease recorded.

4.1.2 Endoscopic Biopsy Procedure

The colonoscopy was performed by a trained endoscopist and up to 8 biopsies taken from the terminal ileum using standard endoscopic radial jaw biopsy forceps (Boston Scientific, Malborough, MA, US). In addition 2 ileal biopsies were sent for routine histological examination. The histological and endoscopic finding were recorded for later comparison. If a stimulation experiment was planned, the 8 research biopsies were placed immediately in Dulbecco's Modified Eagles Medium (DMEM, Sigma-Aldrich Ltd, Gillingham, UK) and placed on ice for transfer (5-10 minutes) to the primary tissue culture lab. If only baseline expression was required, the biopsies were placed directly into RNAlater (Life Technologies, CA, US) and placed overnight in a 3-6^{o}C refrigerator overnight before freezing at -80^{o}C until RNA extraction.

4.1.3 Ileal Explant stimulation

The full explant protocol is available in appendix 1.4 and is an adapted version of a method set up by Sara Balesaria to investigate human duodenal transcription responses to vitamin D.[202] It has already been validated to measure FGF19 transcription in ileal explants,[21] but a brief description is given here.

1.9mls of complete media (CM, DMEM supplemented with 10% heat inactivated foetal calf serum (Life Technologies, CA, US), 100U/ml penicillin, 100ug/ml streptomycin (Sigma-Aldrich Ltd, Gillingham, UK), 50µg/ml leupeptin (Sigma-Aldrich Ltd, Gillingham, UK), and 50µg/ml soya bean trypsin inhibitor (Roche Life Science, Indianapolis, IN, US) were add to each well of a 6 well culture plate (Nunclon Δ Surface multidishes, ThermoScientific, Loughborough, UK).

Each well was designated as negative control (CM only), positive control (CDCA 50µM (Sigma-Aldrich Ltd, Gillingham, UK)) and the compound being investigated added to the other 4 wells in varying concentrations with or without bile acid, these compounds are detailed in table 4.1.

Bile Acid / Compound	Concentrations used	Supplier
Chenodeoxycholic Acid (CDCA)	50µM	VWR, Radnor, PN, US A1690
Obeticholic Acid (OCA)	1-5µM	Gift from Intercept Pharmaceuticals, San Diego, CA
Lithocholic Acid (LCA)	10-50µM	Sigma-Aldrich Ltd, L6250
Cafestol (CAF)	12.5-200µM	Cayman Chemicals, Ann Arbor, MI, USA
Resveratol (RSV)	12.5-200µM	Cayman Chemicals
Ursodeoxycholic Acid (UDCA)	12.5-100µM	Fisher Scientific, Loughborough, UK

Table 4.1: Bile Acids and compounds used during ileal stimulation experiments.

Once the compounds were mixed in the wells by pipetting, the biopsies were distributed between the 6 wells, in a manor that was visually equal. The lid was placed on the culture plate and the plate placed within a plastic box with a gas inlet and outlet (see figure 4.1). Once the lid was placed on the box, it was filled with 100% oxygen through the inlet valve for 15 seconds and placed within a tissue incubator at 37°C with 5% CO_2 for 6 hours. The box was removed every 2 hours and the oxygen renewed with 15 seconds of 100% O_2 through the inlet. After 6 hours the explants were removed from the wells a placed in 0.5ml microcentrifuge tubes with RNAlater. They were placed in a refrigerator and kept overnight at 3-6°C. The next morning the explants were frozen at -80°C until RNA extraction. The media supernatant in the well was mixed by pipetting and 1ml was stored at -80°C so that future studies could measure secretion.

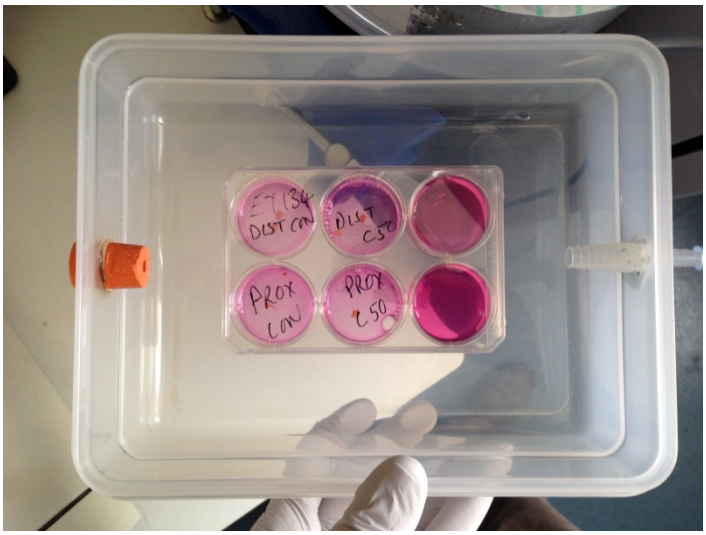


Figure 4.1: Endoscopic biopsies in a 6 well tissue culture plate within the oxygenation box, with the lid removed.

4.1.4 RNA extraction

RNA from thawed explants was extracted using a commercially available RNA extraction kit (RNAeasy Minikit, Qiagen, Hilden, Germany, 74106). The full protocol can be found in appendix 1.5, but a brief description follows.

4.1.4.1 Tissue lysis

Explants were removed from their storage microcentrifuge tube into another containing 600µl lysis buffer with 40mM dithiothreitol (DTT). Physical disruption of the tissue was performed with a handheld motorised pestle and mortar pellet mixer (VWR, Radnor, PN, US). This was applied until no visible tissue could be seen in the sample. The sample was then transferred into a QIAshredder spin column (Qiagen, Hilden, Germany). This spin column has a biopolymer shredding filter that aids disruption and increases RNA yield. The spin column was then discarded and the lysate kept for RNA purification.

4.1.4.2 RNA purification

This was performed using the RNAeasy silica membrane based spin columns. After one wash stage, DNA contamination was removed by adding 27 Units of RNAase-free DNAase (Qiagen, Hilden, Germany, 79254) directly to the silica membrane and leaving for 20 minutes. After 3 further wash steps, the RNA was eludated and diluted with 50µl of RNAase free H_2O. The diluted sample was passed through the spin column one last time to maximise the RNA concentration, before freezing at -80^{o}C or immediate cDNA synthesis.

4.1.4.3 Complimentary DNA synthesis

Complimentary DNA (cDNA) was synthesised from purified RNA using the SuperScript First-Strand Synthesis System Reverse Transcription kit (Invitrogen, Carlsbad, CA, US 11904-018). The full protocol is available in appendix 1.6. Briefly, 8µL of RNA per sample were added to 1 µL of a 10mM nucleotide mix and 50ng of random hexamers. The solution was incubated for 5 minutes at 65^{o}C to allow the RNA strands to anneal. Random hexamers were used as non-specific primers to allow the broadest analysis for multiple genes in qPCR later. 50U of superscript II reverse transcriptase was then added to each sample, with 4 µL of 25mM MgCl and RNase inhibitor (RNase OUT, 40U per sample). Each sample then underwent PCR at 42 ^{o}C for 50 minutes until it was terminated at 70 ^{o}C for 15 minutes and frozen at -20C until qPCR.

4.1.4.4 Quantitative PCR

Real time PCR was performed on thawed cDNA samples in either 96 or 384 well PCR plates. Depending on which plate was being used, the reaction amount varied, and are detailed in the protocol in appendix 1.7, but the proportions, thermal cycling or analysis was the same for either 96 or 384 well methods.

To target genes for amplification, specific primer pairs were used to start the DNA polymerase in specific gene locations. All the primers used have been validated in other studies (table 4.2), but were designed to have primer length of 18-22, similar melting temperatures (64-69^{o}C), similar G-C content and an amplicon size between 85-150 base pairs. All primer pairs had been previously tested in the genomic database BLAT (www.genome.ucsc.edu) to verify primer specificity and that amplicons span at least 2 exons to minimise genomic DNA amplification. All primers were supplied by Sigma-Aldrich Ltd, (Gillingham, UK) and were added to the primer master mix at a concentration of 10mM.

A primer master mix was prepared with Sybr Green JumpStart Taq ReadyMix (Sigma-Aldrich Ltd, Gillingham, UK, S4438) that contains DNA polymerase and a Jumpstart Taq antibody that prevents amplification at room temperature, but is inhibited at 70 ^{o}C. The mix also contains deoxynucleotides, $MgCl_2$ and Sybr Green I. Sybr Green is an intercalating fluorofore that emits fluorescence in the presence of annealed double stranded DNA. A rt-PCR machine (7900HT Fast Real-Time PCR System, Applied Biosystems, Paisley, UK) cycled the sample temperature between 40-95 ^{o}C 40 times. After each cycle the about of amplified DNA doubled and the machine measured the fluorescence emitted by annealed Sybr green. The cycle number when the amount of fluorescence crossed the threshold level (set at 1), was taken as the cycle threshold value (Ct) and used to calculate the quantity of target DNA (usually FGF19, but other genes were investigated, see table 4.2) relative to an endogenous housekeeping gene (always glyceralderhyde-3-phophate dehydrogenase: GAPDH). This was calculated using the method described in the next section.

Gene	Forward	Reverse	Reference
Fgf19	CGACCACTTTGTCAAAGCTCA	CCATCTGGGCGGATCTCC	[21]
Gapdh	CGACCACTTTGTCAAGCTCA	AGGGGAGATTCAGTGTGGTG	[21]
Fxr	AGGATTTCAGACTTTGGACCATGA	TGCCCAGACGGAAGTTTCTTATT	[21]
Shp	AGGGGACCATCCTCTTCAACC	TTCACACAGCACCCAGTGAG	[21]
Ostα	AGATTGCTTGTTCGCCTCC	ATTCGTGTCAGCACAGTCATTAG	[203]
Ostβ	GAGGAGCTGCTGGAAGAGAT	GACCATGCTTATAATGACCACCA	[21]
Ibabp	TCAGAGATCGTGGGTGACAA	TCACGCGCTCATAGGTCA	[21]
Asbt	GCCCCAAAAAGCAAAGATCA	GCTATGAGCACAATGAGGATGG	[21]
Sirt1	AAGGAAAACTACTTCGCAAC	GGAACCATGACACTGAATTATC	[204]
Srebp2	GAGAGGTCAATCATAAACTG	GGACATTCTGATTAAAGTCCTC	[205]

Table 4.2: Primer sequences used during rt-PCR

4.1.4.4.1 Statistical analysis

Cycle time (Ct) for each reaction was calculated for all samples using a plot of cycle number vs ΔRn. The Ct was recorded at ΔR=1.0. All samples were performed in triplicate. To minimise the effects of pipetting errors, Individual Cts that varied by more than 0.5 from the other 2 repeats were excluded from the analysis. The mean Ct of the remaining samples were used, if the standard error (SE) of the mean was $\geq$0.3 with one repeat excluded, the sample was excluded from the final analysis. Mean Cts $\geq$35 were also excluded.

Relative gene expression was calculated using RQ manager V1.2 software (Applied Biosystems, Paisley, UK). ΔCt was calculated from the mean Ct of all samples by using the equation:

$$\Delta Ct = Ct_{(GAPDH)} - Ct_{(target)}$$

Gene expression in unstimulated samples were expressed in arbitrary units (AU) using the widely accepted $2^{-\Delta Ct}$ method:[206]

Gene expression (AU) = $2^{-\Delta Ct}$

Gene expression in stimulated samples relative to control samples were expressed as fold change using the ΔΔCt which was calculated as:

$\Delta\Delta Ct = \Delta Ct_{(test\ sample)} - \Delta Ct_{(control)}$

Fold change was calculated using $2^{-\Delta\Delta Ct}$ method:

Fold change = $2^{-\Delta\Delta Ct}$

4.2 Results: Regulation of ileal FGF19 expression

4.2.1 Ileal Gene Expression in Primary Bile Acid Diarrhoea

4.2.1.1 Unstimulated ileal explants

Ileal biopsies were received from 10 patients with known SeHCAT values and histologically normal ileum. Correlations of the mean Ct for SIRT1 and SREBP2 were correlated against SeHCAT values and Cts of FGF19, FXR and BA transporters that had been performed previously by a colleague (J. Nolan).

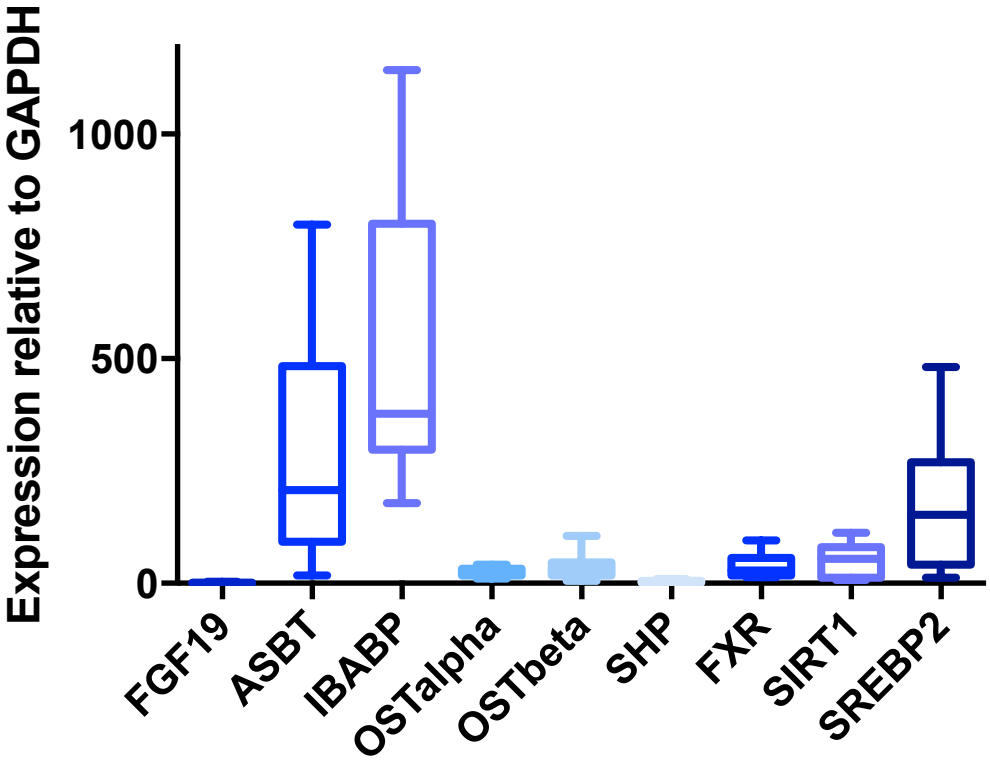


Figure 4.2: Box and whisker (median, IQR, 95% CI) plot of mRNA expression relative to GAPDH (arbitrary units) of FXR target genes.

No genes were found to correlate with SeHCAT value significantly, but there was a trend for increased FXR and FGF19 expression in higher SeHCAT values (rs= 0.56 p=0.10 and rs=0.49 p=0.15 respectively). Several correlations between genes were found to be statistically significant and are displayed in table 4.3 and figure 4.3.

	FGF19		ASBT		IBABP		OSTa		OSTb		SHP		FXR		SIRT1		SREBP2	
	rs	p	rs	p	rs	p	rs	p	rs	p	rs	p	rs	p	rs	p	rs	p
SeHCAT	0.49	0.15	0.47	0.18	0.18	0.63	0.19	0.61	-0.08	0.84	0.15	0.68	0.56	0.10	0.28	0.43	0.07	0.87
FGF19			0.89	0.001	0.36	0.31	0.25	0.49	0.02	0.97	0.62	0.06	0.84	0.004	0.45	0.19	0.44	0.20
ASBT					0.54	0.11	0.41	0.25	0.01	1.00	0.66	0.04	0.96	<0.0001	0.68	0.03	0.55	0.10
IBABP							0.92	0.001	-0.19	0.61	0.71	0.03	0.55	0.10	0.33	0.35	0.27	0.45
OSTa									-0.22	0.54	0.55	0.10	0.47	0.18	0.12	0.76	0.13	0.73
OSTb											-0.38	0.28	-0.04	0.92	0.26	0.47	0.14	0.71
SHP													0.68	0.03	0.14	0.71	0.05	0.89
FXR															0.60	0.07	0.43	0.22
SIRT1																	0.87	0.002

Table 4.3: Correlation matrix of Spearman's rank correlation and p values for FGF19, FXR, SIRT1, SREBP2 and BA transporter mRNA expression in unstimulated ileal biopsies. Statistically significant ($p<0.05$) results in purple. p values nearing significance in blue.

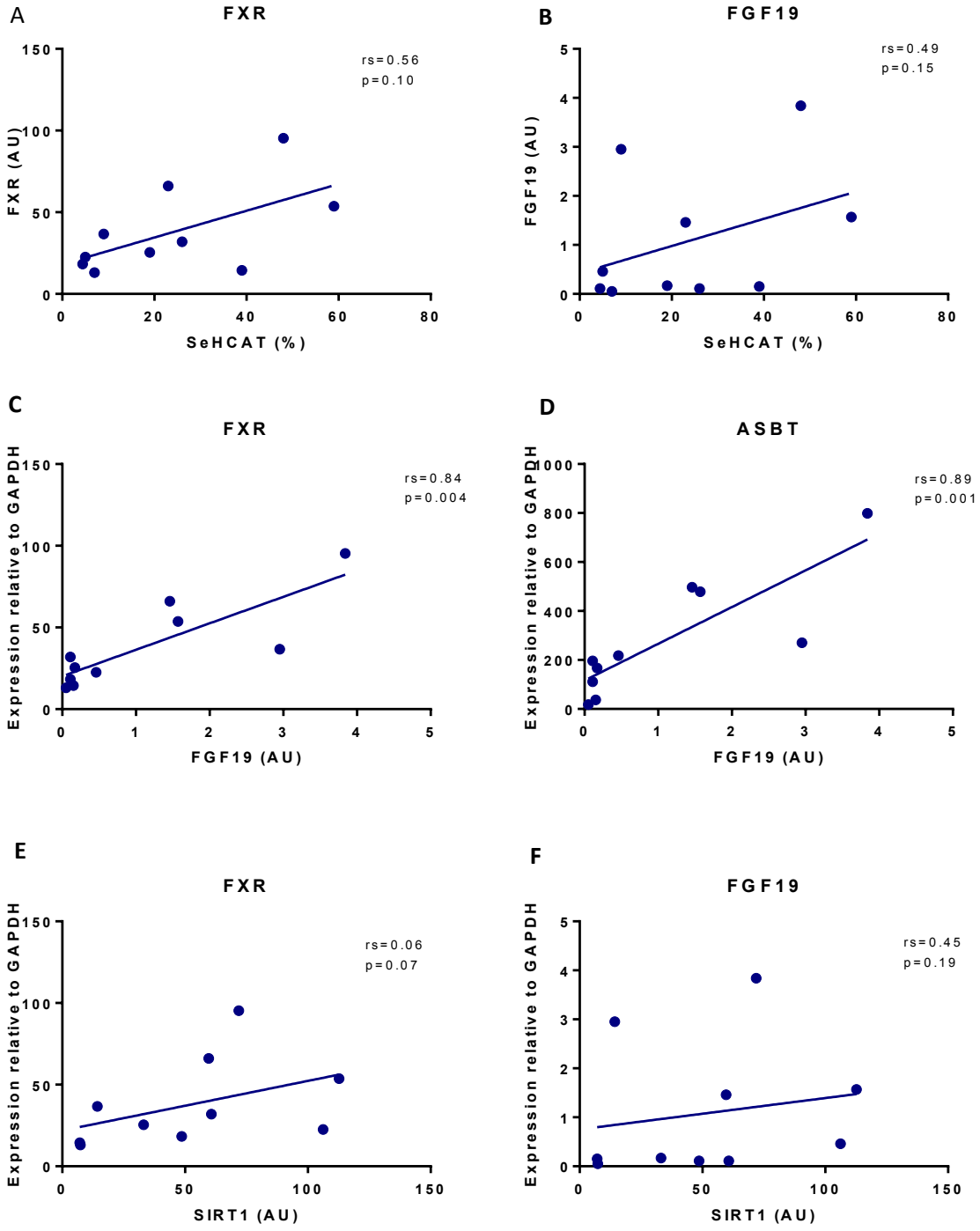


Figure 4.3: Correlation of SeHCAT value with FXR expression (A) and FGF19 (B). Correlation of FGF19 with FXR (C) and ASBT (D) expression. Correlation of SIRT1 with FXR (E) and FGF19 (F). mRNA expression (relative to GAPDH (arbitrary units (AU)) in unstimulated ileal explants. rs=Spearman's rank.

4.2.1.2 Stimulated ileal explants

Ileal explants were incubated from 16 patients with known SeHCAT values. 6 of these patients had a SeHCAT <15% and 5 <10%, all of these patients had primary BAD. There was a trend towards lower FGF19 and IBPAP expression in patients with lower SeHCAT (rs 0.47, p=0.09 and rs 0.48 p=0.06 respectively). Significant correlations were found between FGF19 and IBPAP (rs=0.60, p=0.03) and an inverse correlation between OSTα and SIRT1 (rs=-0.60, p=0.03). Other correlations are shown in table 4.4.

	IBPAP		FGF19		SIRT1		SREBP2		SHP		OSTα		ASBT	
	rs	p	rs	p	rs	p	rs	p	rs	p	rs	p	rs	p
SeHCAT	0.47	0.09	0.48	0.06	0.11	0.69	0.12	0.69	-0.16	0.59	-0.10	0.73	-0.30	0.29
IBPAP			0.60	0.03	0.30	0.30	0.09	0.75	0.01	0.97	-0.03	0.94	-0.07	0.82
FGF19					0.08	0.77	0.27	0.34	0.16	0.59	0.13	0.66	-0.004	0.98
SIRT1							0.44	0.12	0.05	0.86	-0.60	0.03	0.36	0.21
SREBP2									-0.13	0.70	-0.36	0.26	0.23	0.46
SHP											0.57	0.04	0.40	0.16
OST a													-0.19	0.51

Table 4.4: Spearman's rank correlations and p values for SeHCAT values, FXR target and effector genes mRNA expression in ileal explants after 6 hours incubation with 50uM CDCA. Statistically significant values are in purple, and near significant value in blue.

When categorised by SeHCAT result, a SeHCAT <15% (6 patients) did not reveal any statistically significant differences in median gene expression (figure 4.4). Using the 15% cut off there was a trend towards lower FGF19 expression in patients with SeHCAT <15% (p=0.14, Mann-Whitney U test). Using a SeHCAT value cut off of 10% (n=5), this trend reaches significance (p=0.019) and there is a non-significant trend towards lower IBPAP expression in patients with a SeHCAT <10% (p=0.18).

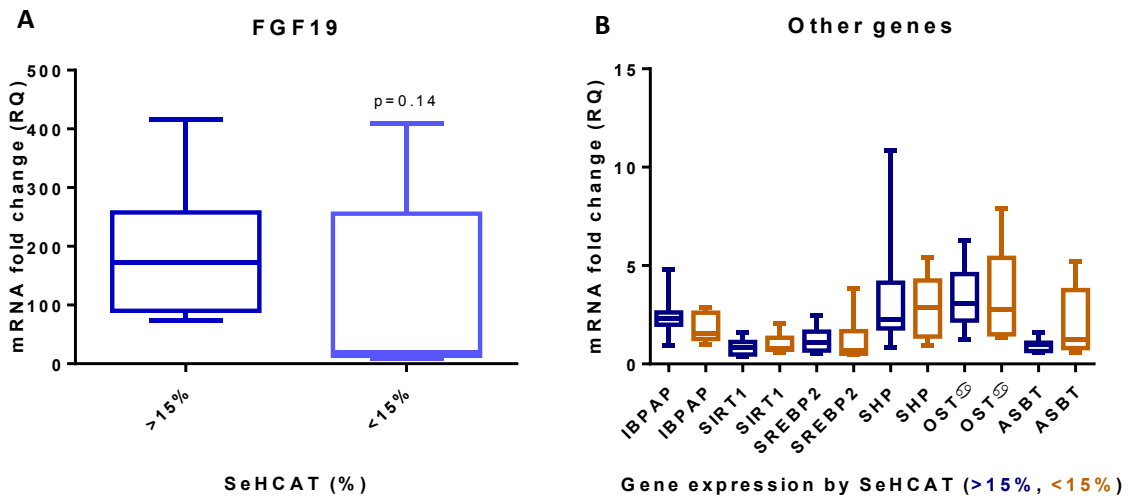


Figure 4.4: Box and whisker (median, IQR, range) plots of FXR target and effector gene mRNA expression after 6 hours incubation with 50uM CDCA. FGF19 displayed by SeHCAT above and below 15% (A) and other genes related to FXR signalling displayed by SeHCAT above and below 15% (B).

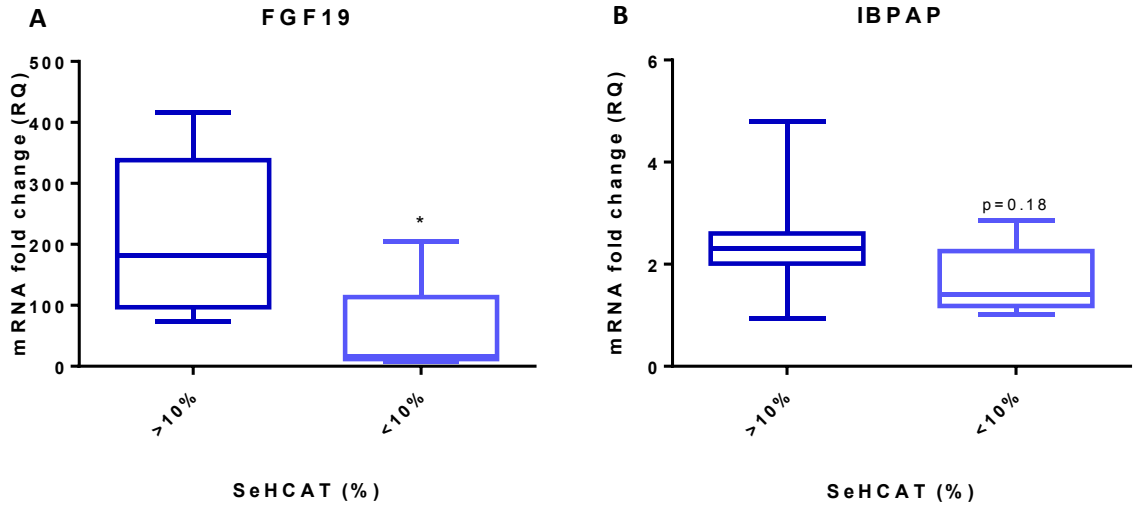


*Figure 4.5: FGF19 (A) and IBABP (B) mRNA expression after 6 hour in ileal explants after 6 hours incubation with 50uM CDCA divided by SeHCAT above and below 10%. *p<0.05*

4.2.2 Effects of Cafestol, Resveratrol and Ursodeoxycholic acid on ileal FGF19 expression

Over the 40 experiments performed with resveratrol, cafestol or ursodeoxycholic acid the median FGF19 RQ with CDCA 50µM was 78.2 (range 6.37-577.1). OCA 5µM had comparable efficacy with a median FGF19 RQ over 11 experiments of 71.95 (range 4.18-1392).

4.2.2.1 Resveratrol

Ileal explants were incubated for 6 hours with resveratrol (RSV) at concentrations of 12.5, 25, 50, 100µM, with a negative control (enriched media only) and a positive control (CDCA 50 µM).

4.2.2.1.1 FGF19

Despite a median 87.8 fold change in FGF19, with C50, there was no significant effect of RSV compared to controls (table 4.5)

	CON	C50	RSV12.5	RSV25	RSV50	RSV100
n	9	7	5	6	5	9
Median	1	87.8	1.359	0.946	1.219	1.123
Lower 95% CI	1	-15.97	0.311	0.6815	-0.4483	0.4716
Upper 95% CI	1	310.1	1.861	1.526	3.684	2.247

Table 4.5: median fold change (RQ) compared to negative control (CON) in FGF19 mRNA in ileal explant stimulated with CDCA 50 µM or RSV at varying concentrations (µM).

2 experiments were conducted with co-incubation C50 with RSV50 or RSV100µM. In one patient there was a 53% decrease with RSV50 and a 64% decrease in FGF19 expression with RSV100. In another patient there was no effect (Figure 4.6)

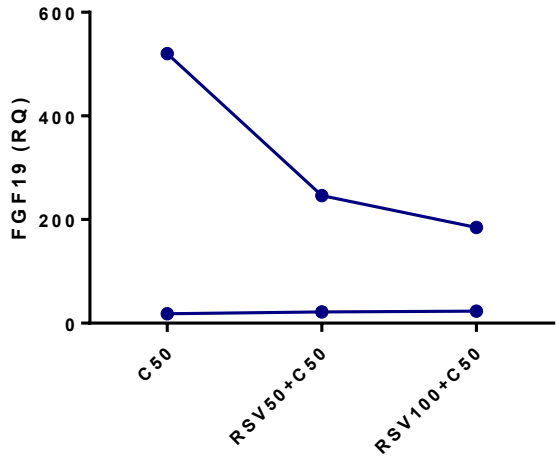


Figure 4.6: FGF19 mRNA expression relative to control in 2 patients ileal explants co-incubated with resveratrol and CDCA

4.2.2.2 SIRT1

SIRT1 was measured in 8 experiments. Median SIRT1 fold change after 6 hour incubation with RSV 12.5, 25, 50 and 100 µM was 1.9, 1.8, 1.5, 1.4 respectively. RSV increased SIRT1 expression by 89%, 75%, 45% and 40% for 12.5, 25, 50, 100µM concentrations respectively. This compares to 31% with C50. For the 2 lower concentrations of RSV, there was statistical significance compared to control.

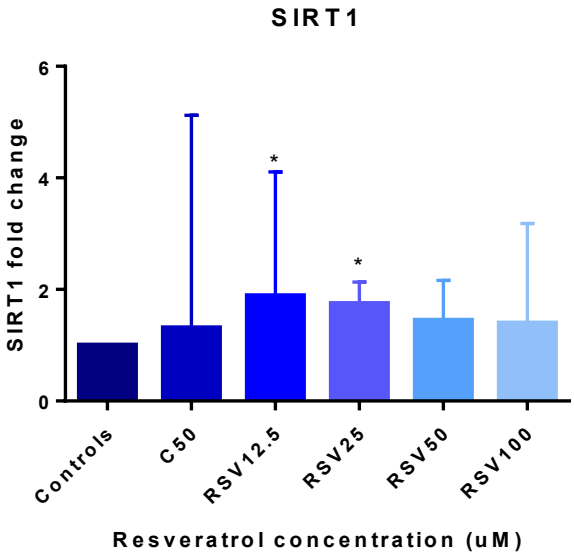


*Figure 4.7: Median (range) SIRT1 expression in ileal explants after 6 hours incubation with reveratrol (RSV). *p<0.05*

4.2.2.2.1 ASBT and FXR

As RSV may have actions on other parts of the bile acid signalling pathway, ASBT and FXR expression were also measured. 4 experiments measured ASBT and 6 measured FXR. There were no significant differences or discernable trends between RSV and negative controls.

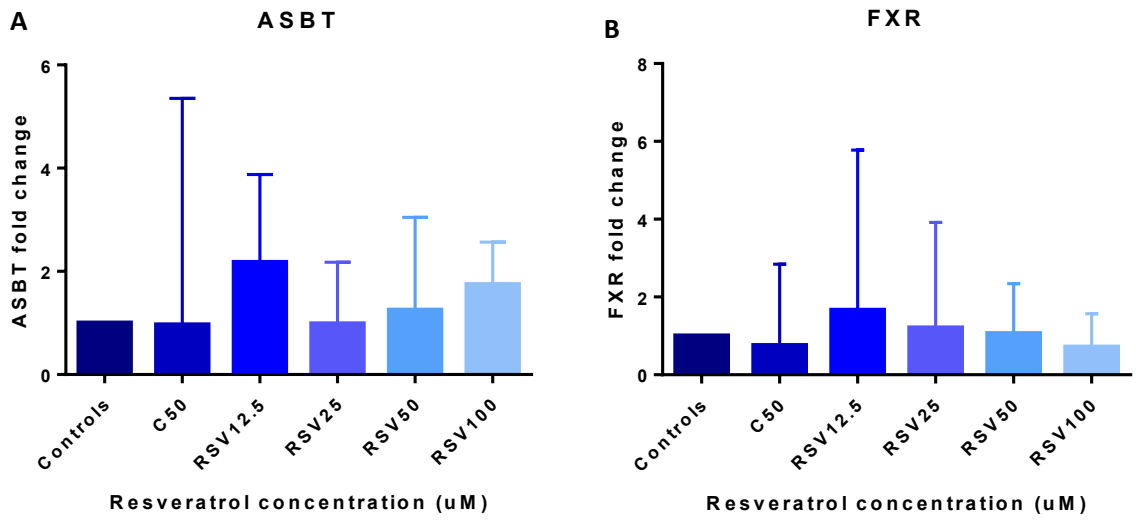


Figure 4.8: Median (range) ASBT (A) and FXR (B) expression in ileal explants after 6 hours incubation with resveratrol (RSV)

4.2.2.3 Cafestol

10 cafestol dose-ranging experiments were performed. Median fold change compared to controls in FGF19 with CDCA 50 µM was 106. CAF at concentrations of 12.5, 25, 50 and 100µM produced a median fold change in FGF19 of 1, 1.7, 2.4 and 2 respectively. The median increase in FGF19 was 0%, 74%, 240%, 104% for CAF concentrations of 12.5, 25, 50 and 100µM respectively. The maximum median was seen with CAF50 and this was statistically significant compared to controls. The increases seen with CAF25 and CAF100 were also statistically significant. The range for the lowest dose, CAF12.5 was very large (0.4-27.7) and is not shown in figure (4.9(B)).

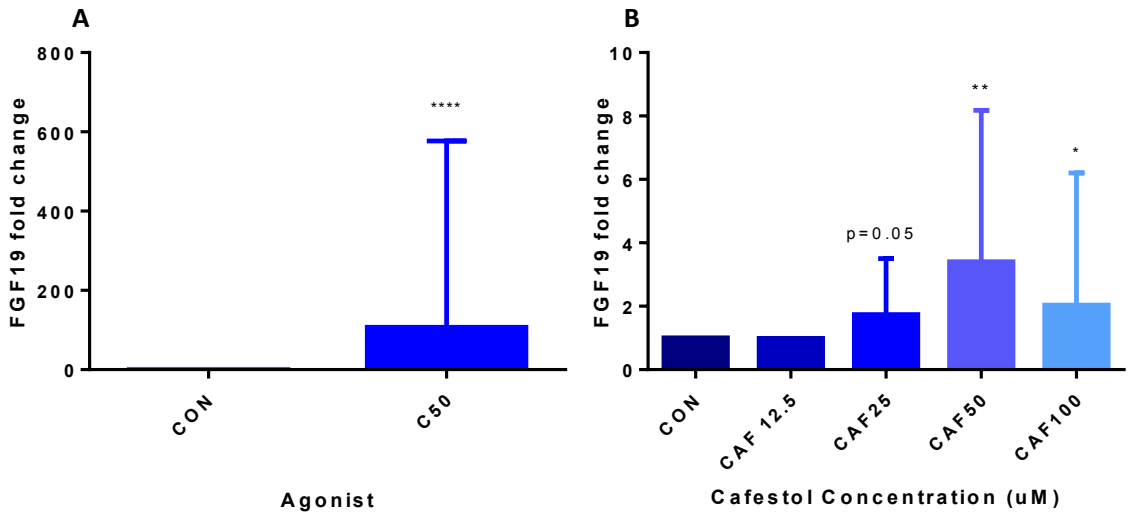


*Figure 4.9: Median (range) FGF19 mRNA expression in ileal explants after 6 hours incubation with varying concentrations (in uM) of Cafestol (CAF) (A) and CDCA 50 uM (C50) with Cafestol (B). *p<0.05, **p<0.01, ****p<0.0001*

Co-incubation with 50µM CDCA was performed in 7 experiments. FGF19 expression was decreased by 95% with CAF50 and 35% with CAF100 (p=0.6 and 0.12 respectively compared to C50 alone).

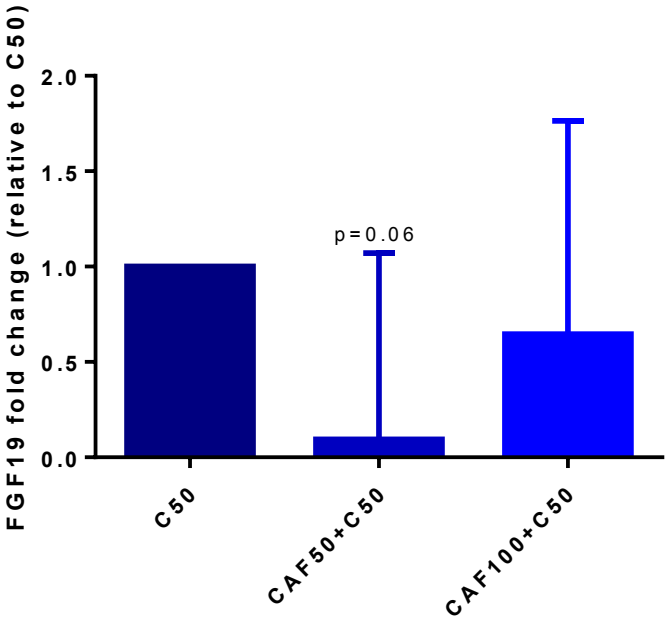


Figure 4.10: Median (range) fold change compared to CDCA 50µM (C50) in FGF19 mRNA expression with addition of Cafestol (CAF) 50 µM or 100uM.

4.2.2.4 *Lithocholic acid*

To investigate if other weak FXR agonists acted as competitive inhibitors in the presence of CDCA, 1 experiment was performed with LCA and CDCA. LCA at a concentration of 50 µM produced a 1.15 fold increase in FGF19 compared to the negative control, 50 µM CDCA in the same patient produced a 41 fold increase. Addition of 50 µM LCA to 50 µM CDCA decreased FGF19 expression from 48 fold increase for CDCA alone to an 18 fold increase. When compared to C50, addition of LCA at 10 and 50 µM reduced FGF19 expression by 69 and 55% respectively (figure 4.11 (B)).

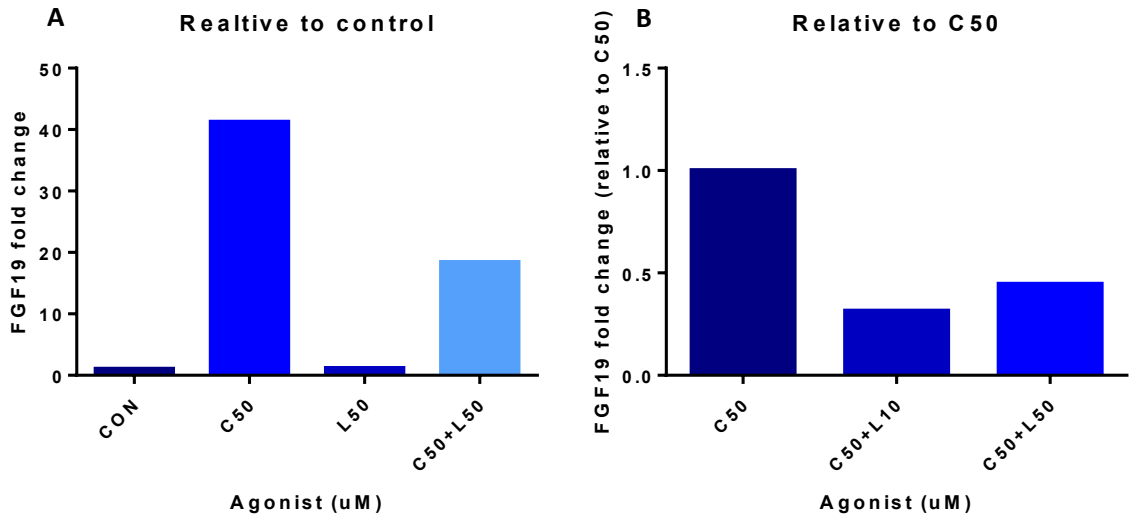


Figure 4.11: Median fold change in FGF19 relative to negative control (CON)(A) and positive control CDCA 50µM (C50)(B). L=LCA

4.2.2.5 *Ursodeoxycholic Acid*

20 ileal explant experiments were conducted in total, 5 were dose ranging experiments of UDCA 12.5-100 µM, 6 were combined incubations of OCA with UDCA, 3 were combined incubations with CDCA and 6 were both. One OCA experiment was excluded because of a complete lack of stimulation, thought to be experimental error, leaving 19 for analysis.

CDCA 50 µM and OCA 5 µM had similar potencies with median FGF19 stimulation of 67 and 75 fold change relative to control. Although the maximum range for OCA 5µM was much larger than that of CDCA 50µM (1392 vs. 621 respectively) (figure 4.12 (A)).

Median FGF19 fold increase with UDCA alone compared to controls were 2.85, 2.19, 3.78 and 9.01 for UDCA 12.5, 25, 50 and 100 µM respectively. Stimulation with UDCA 100 µM produced a statistically significant increase on Wilcoxon testing (p=0.008) and 50 µM neared significance (p=0.078) (figure 4.12 (B)).

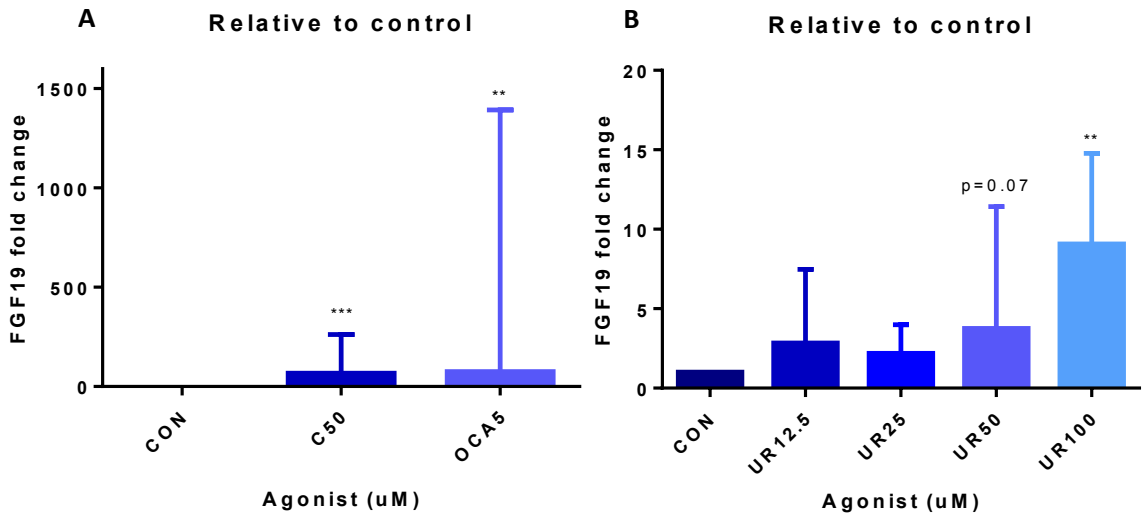


*Figure 4.12: Median (range) fold change in FGF19 mRNA expression relative to controls (CON) with CDCA and OCA (A) and varying concentrations of UCDA(B). **p<0.01, ***p<0.001.*

4.2.2.5.1 Co-incubation of CDCA with UDCA

When co-incubated with 50 µM CDCA, 100µM UDCA increased FGF19 stimulation compared to CDCA alone, with a median increase of 129% (fold change 2.29 relative to CDCA 50µM). There was no increase in FGF19 expression with CDCA co-incubated with UDCA 50µM (fold change 0.89 relative to CDCA. These findings were not statistically significant due to low numbers (n=3) (figure 4.13).

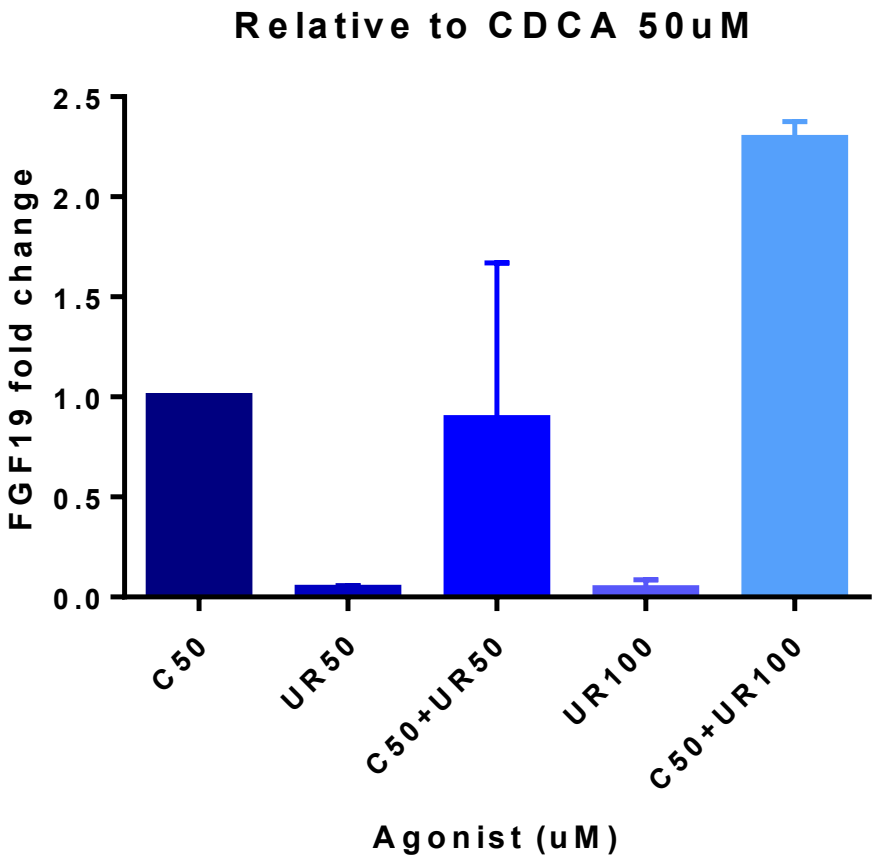


Figure 4.13: Median (range) fold change of FGF19 mRNA expression with co-incubation with UDCA (UR) and CDCA (C50) relative to CDCA 50uM alone.

4.2.2.5.2 Co-incubation of OCA with UDCA

As OCA is likely to be used clinically with UDCA in patients with PBC, we examined the effect of co-incubation with UDCA in ileal explants. Addition of UCDA 50 or 100 µM to OCA 5 µM increased FGF19 fold change relative to OCA 5 µM alone by 70% and 145% respectively (p=0.12 and 0.04 on Wilcoxon testing).

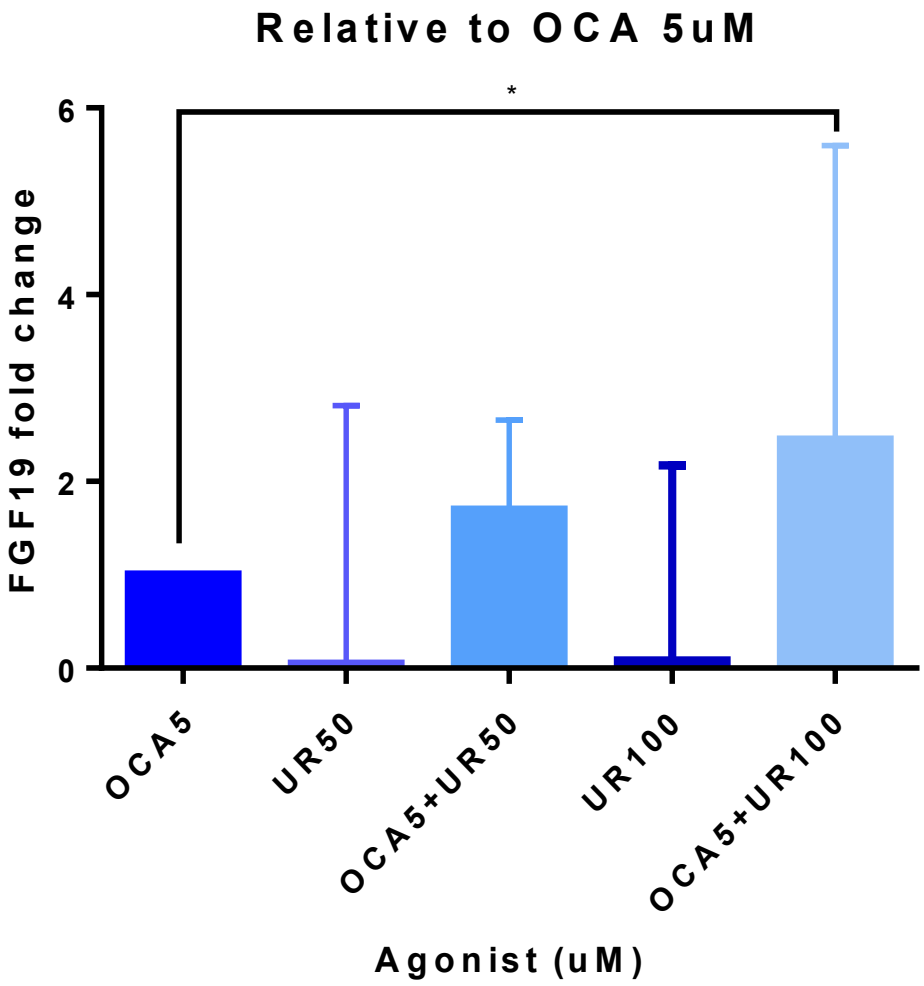


*Figure 4.14: Median (range) fold change in FGF19 expression relative to OCA 5 uM with co-incubation with UDCA (UR). *p<0.05*

4.3 Conclusions: Regulation of ileal FGF19 expression

4.3.1 Ileal FGF19 expression in pBAD

We have shown that ileal FGF19 transcription is reduced in patients with primary BAD. There is also trend suggesting that lower IBABP transcription is also associated. As IBABP is also a FXR transcription target it is possible that this represents a global deficiency in FXR controlled transcription. We do not see the same decrease with other FXR target genes, but the fold increase in these in response to FXR stimulation is much smaller of those of FGF19 and IBPAP (2 fold compared to 100 fold and 8 fold respectively), so a difference may be difficult to detect. There is also evidence that IBABP is required for BA binding to FXR, so reduced IBABP could result in reduced FGF19 transcription.[207] The search for associated polymorphisms in FXR or IBABP has not revealed any significant abnormalities of these genes,[208] but the evidence presented here suggests that the low serum FGF19 found in pBAD is a result of altered transcription within the ileum.

These findings may explain the clinical findings of *Borup et al.[209]* who compared fasting and postprandial FGF19 in 26 patients undergoing SeHCAT testing. Patients who had a SeHCAT <10% did not exhibit an increase in postprandial FGF19, whereas the diarrhoea controls exhibited a 33% increase. A primary defect in ileal FXR mediated FGF19 transcription would explain this lack of response. Work done my colleague, Ian Johnston, suggests that this low-low FGF19 phenotype is only present in 47% of patients with a SeHCAT <15%, although these patients tended to have a lower SeHCAT value than the other phenotypes (low-high, high-high).[121] Which in turn may explain the variance of the FGF19 response to stimulation that we observed in our SeHCAT series, the low-high and high-high patients were probably selected out by reducing the cut off of SeHCAT to 10% rather that 15%, which is when the difference in FGF19 stimulation reached significance.

There is a growing appreciation that control of FXR-mediated transcription is more complex than previously thought. FXR-mediated transcription can be modified in substrate specific manner and an ever increasing list of co-factors are emerging in the literature. At least 12 transcriptional co-factors

and even more post-translational modifiers of FXR are now known.[210] These include AMPK that seems to have an inhibitory effect on FXR signalling in response to feeding,[196] and SIRT1 that has the opposite effect on fasting.[147] SIRT1 is of particular interest since low activity can be induced by obesity in mouse models, but SIRT1 knock-down mice also develop obesity.[145] Since obesity can influence FGF19 levels,[211] this leads to interesting hypothesis that obesity can cause low FGF19 and diarrhoea, rather than low FGF19 causing both.

Unfortunately we have not shown any difference in SIRT1 expression in our simulated explant experiments, although levels of SIRT1 mRNA did have a significant negative correlation with OSTα. This is of interest as OSTα is a FXR transcriptional target who's ileal expression has been shown to be reduced in patients with chronic diarrhoea compared to FXR.[208] A significant reduction in OSTα expression was also seen in intestinal SIRT-1 KO mice, which only produced a modest decrease in FGF15 that did not reach statistical significance.[147] It is possible that SIRT1 is the co-factor responsible for this and it is possible that these patients represent another subset BAD, possibly with high BMIs (although we do not have BMI data for those in this cohort).

4.3.2 Effects of Resveratrol, Cafestol and Ursodeoxycholic acid on ileal FGF19 expression

4.3.2.1 Resveratrol

We have shown that resveratrol increases SIRT1 expression but without effecting FGF19. A recent publication has suggested that SIRT1 does not directly deacetylate FXR, but probably acts through PGC-1α which has been shown to have direct interactions with both SIRT1 and FXR.[196] Possibly the 6 hour incubation time was not long enough to see this effect.

Resveratrol acts through many mechanisms and one study suggested that it promotes the degradation and reduces mRNA expression of ASBT.[176] Another study of mice with a hepatic SIRT1

deletion showed significantly decreased FXR expression.[212] However, ASBT and FXR mRNA expression were unaffected by resveratrol in our experiments.

Significant SIRT1 stimulation was only seen at the lower doses of resveratrol (12.5-25µM). Studies that have demonstrated SIRT1 stimulation in human cells, have done so at the concentrations of 1-10µM.[213] There are several explanations why stimulation was not seen at higher doses. Resveratrol may exhibit tachyphylaxis at higher doses. Drug trials in NAFLD using higher doses have not achieved the serum concentrations reported by trials using lower doses.[214] Alternatively, toxicity has been shown with HepG2 cells with concentrations above 50µM.[215] And the multiple mechanisms of resveratrol may mean that there is an inhibitory effect through an alternative pathway.

The resveratrol series were some of the first experiments performed by myself and an attached undergraduate using the ileal explant system and as a consequence many samples had to be excluded from the final analysis as the final PCR result did not meet the required standard of a ΔCt SE<0.3. This meant that although 9 samples were analysed for 100µM resveratrol, only 5 were available for 50µM. However, this is a common problem when there are low amounts of the PCR product and may reflect the lack of action of RSV on our target genes. The 95%CI of the FGF19 results with resveratrol are comparable to those of ileal explant experiments with other compounds and poor ΔCt SEs are probably due to poor resolution of the high Ct seen with some impact of sub-optimal laboratory technique.

Since resveratrol does not act directly on FXR, we did not expect there to be any inhibition with co-stimulation, but from the 2 experiments performed no conclusions can be made on this and further experiments are warranted.

4.3.2.2 Cafestol

We have shown a dose dependant simulation of FXR with cafestol. The maximum stimulation of FGF19 was 3.4 fold with 50µM cafestol, which fell to 2 fold at 100µM. This is comparable to the ~4 fold change in FGF15 seen in mice fed 400mg/kg cafestol.[154] The reasons for the reduction in stimulation at 100µM compared to 50µM are unknown, the EC50 for cafestol is not known, as is the concentration that affects cell viability. Either of these reasons could be explanations and should be addressed in future work. This modest but significant effect may be an explanation to why coffee is protective in chronic liver disease.[216]

The 3.4 fold FGF19 stimulation with cafestol led us to question whether in the presence of a more potent FXR agonist, cafestol acted as a competitive inhibitor. We had already proved the principle in a pilot experiment with LCA which produced a 1.15 fold increase in FGF19 at 50µM, but when added to CDCA 50µM, reduced FGF19 by 55% compared to CDCA 50µM alone. This was only one experiment, so these findings were not statistically significant. We performed 7 experiments where CDCA 50µM and cafestol 50µM or 100µM were added together. These produced a decrease in FGF19 expression of 35% and 95% respectively. The change of 95% with 50µM neared statistical significance compared to CDCA alone ($p=0.06$). Why the effect seen is more pronounced with the lower concentration of cafestol is hard to explain, but it is interesting that the same inverse relationship was seen in the LCA experiment (reduction of 69% and 55% with 10 and 50µM respectively). Although the effect of the sample variance would be limited by further experiments and, the effect of the higher dose of partial agonist would possibly be equal or greater the lower dose. Further experiments are necessary to confirm these preliminary findings.

4.3.2.3 Ursodeoxycholic acid

In our ileal explant system UDCA did stimulate FXR, as evidenced by 9 fold change for UDCA 100µM compared to controls, which was statistically significant. At the concentration of 50µM there was a

3.78 fold stimulation that nears significance with a p value of 0.07, this compares to a median fold change of 67 for CDCA 50μM/ml in the same patients. Thus we can conclude that UCDA is a weak FXR agonist but is 17.7 times less potent than CDCA at the same concentration. This supports the findings of *Campana et al*. which is the only published study comparing CDCA and UCDA on FXR activation in luminal cells (albeit in colon adenoma derived cells).[159] This study looked only at IBABP transcription, which we have shown to transcribed at one hundredth of the quantity of FGF19 in response to CDCA 50μM. However, they did show that FXR stimulation with UDCA starts at 100μM, up to 500μM, which would be in keeping with our results.

The addition of 100μM UCDA to either 50μM CDCA or 5μM OCA increased FGF19 transcription by 129% and 145% respectively. This combined effect is much higher that the relative potencies of these agonists added together (9 fold, 67 fold and 75 fold for UCDA 100μM, CDCA 50μM and OCA 5μM respectively) so suggests a multiplier effect of UCDA. This finding was unexpected, we expected UCDA to reduce FGF19 expression in the presence of a more potent FXR agonist, in this case CDCA, like the other partial agonists tested before (cafestol and LCA). Indeed, *Campana et al* found that addition of 100μM UCDA reduced CDCA 50μM stimulated transcription of IBABP by 50%.[159]

A possible explanation for this paradoxical effect is provided by *Fang et al*.[207] In their study in Caco-2 cells they reported that UCDA at a concentration of 125μM increased FXR activity by 2-5 fold compared to CDCA alone. In addition they discovered that this effect was dependant on the presence of IBABP and that IBABP was essential for FXR activation by any BA. The suggested mechanism for this is that UCDA binds to a single site on IBABP, whereas CDCA can bind to either the same site or another. Binding of UCDA to IBABP increases the affinity of IBABP at the second binding site for CDCA, and since this site can't bind UCDA, there is no competitive inhibition.

These findings are likely to be relevant clinically. UCDA is found in concentrations of 50μM in faecal water of patients taking UCDA, meaning that the concentrations required to affect FGF19 transcription are achievable physiologically.[217] Since OCA is likely to taken together with UCDA in

patients with PBC, the additive effect of UDCA is likely to influence the actions of OCA. OCA is likely to be first used in UDCA non-responsive patients, in whom UDCA will have been stopped. One potential problem with long term OCA use may be an increase in the pruritus experienced by patients with PBC.[218] The findings that the action of OCA can be potentiated by UCDA may provide a rationale for restarting UDCA in UCDA non-responders to improve response or to allow dose reduction of OCA in patients with problematic pruritus.

4.3.3 Technical limitations of the ileal explant model

As expected CDCA 50µM was a potent simulator of FGF19 transcription, but this effect displays large inter-individual variability with a median of 78.2, but a range of 6.37-577.1 with CDCA 50mM/ml and median of 71.95 (range 4.18-1392) with OCA 5mM/ml. This compares to a median FGF19 RQ of 136.1 (range 8.59-477.6) with CDCA 50µM/ml in the 16 patients with SeHCAT values (median 172 in the 10 patients with SeHCAT>15%) and 350 in the 24 patients reported by *Zhang et al.*[21]

There are several potential explanations for these differences in stimulation. Firstly, the patients used for these experiments did not have SeHCAT tests (otherwise they would have been in the previous SeHCAT series). Therefore there is potential for patients with undiagnosed BAD to be within our series, these patients would be expected to have constitutionally lower FGF19 stimulation. Anecdotally, we have never been able the repeat the high median values reported by *Zhang et al,* despite using the same lab and protocols, many individual samples have recorded FGF19 RQs of 350 or above, but as an median value for 24 individuals, this level of stimulation is probably not a true representation of the population. In our experience since those early experiments were published, individual levels of FGF19 stimulation vary widely. For this reason, comparative tests are paired to account for this variability.

There are other reasons for the differences in stimulation levels seen between series. The protocol used has changed slightly over time, this mostly reflects practical considerations and changing locations, but also familiarisation and standardisation of techniques and concentrations used. One such example of this is that the original Zhang paper quantified the RNA extracted using nanodrop spectrophotometry and diluted the RNA to a standard 125µg/mL before cDNA synthesis. Over the years, this step was removed as the dilutions of the RNA samples varied very little and were always within the range required for the PCR reaction. When we moved labs, we no longer had access to a nanodrop spectrophotometer and this step was removed completely.

In a small series comparing proximal and distal TI biopsies I showed that the FGF19 stimulation varied significantly depending on the site, but not by whether it was from proximal or distal TI. When we started performing co-incubation experiments, the number of biopsies reduced from 3 to 2 or sometimes 1 per well as more needed to be done with the same amount of tissue, this may affected the final concentration of RNA, and by using less biopsies per well, the variability of the biopsy site would have a greater effect. In order to correct for these potential confounders, the ΔCt against GAPDH was calculated to standardise for the quantity and metabolic activity of the tissue. To correct of pipetting error during PCR, any samples with ΔCt SE between the 3 wells (or 2 with one outlier excluded) >0.3 were also excluded from the analysis.

The ΔCt SE were affected by Ct's >35, were the PCR assay reached the limit of its detection. Any Ct>35 was excluded from the analysis, but to check that the quantity of tissue did not effect the ΔCt SE at lower Ct values, I calculated the correlation between the Ct for GAPDH and the ΔCt SE of the target gene for the series that had the most excluded samples (resveratrol). ΔCt SE did not correlate with the Ct(GAPDH) ($n=42$, $r_s=0.09$, $p=0.55$) indicating that tissue quantity was not causing the problem. For the resveratrol series, I suspect cell viability with concretions >50μM was probably the cause of the variable results.

Working with RNA and PCR techniques has a steep learning curve. At various times during the experiment series new undergraduates were working with me, learning these techniques. Each time a new student started, a predictable fall in the quality of the extracted RNA and PCR results would occur. These effects on the final results are minimised by excluding or repeating PCRs on samples with a ΔCt SE>0.3. In most cases when the PCR was repeated, the ΔCt SE would still be >0.3 indicating that there was problem with the RNA purity that could not be corrected and that sample would be excluded.

Finally the concentration of BA in the wells may not have been exactly as described. Unconjugated BAs are not very soluble requiring their dilution in DMSO or ethanol. During the course of the

experiments we used different solutions of CDCA aliquoted by different people within the previous 2 years. An early experiment compared the stimulation by 3 different aliquots made by 3 different people, the 3 CDCA aliquots, all used at a concentration of 50µM produced FGF19 RQs of 32, 44 and 140 in biopsies from the same patient. After this we only used one batch of aliquots, but caution is advised when comparing RQs between patients, but interpreting differences within individuals and relative differences between individuals minimises the potential effect of these.

One key limitation of these experiments is that they study the ileum in isolation. BA, FXR and FGF19 signalling in the liver is likely to be just as important in disease processes. In bile acid diarrhoea, hepatic resistance to FGF19 would explain the 53% of patients with high FGF19 levels reported in Ian Johnston's study.[121] In cholestatic liver disease, FXR signalling and FGFR4 are upregulated, but the effects in other liver diseases are not known. This means that the potential benefit of OCA in NAFLD can't be attributed to local effects in the liver or increased FGF19 from the ileum. This distinction will have to be made before OCA becomes widely available and second-generation FXR agonists developed.

5 Effects of conventional and colonic release cholestyramine

5.1 Methods: Effects of conventional and colonic release cholestyramine

5.1.1 Conventional and colonic release cholestyramine in healthy volunteers

Data were received from the colonic release cholestyramine (CRC or A3384) arm of a phase I study of the ASBT inhibitor A4250. The full title of the study was *'A phase I, double-blind single and multiple ascending dose study to assess safety and pharmacokinetics of A4250 as monotherapy, and in combination with colonic release cholestyramine (A3384) or commercially available cholestyramine (Questran™) in healthy subjects'.* It was performed by Quotient Clinical Translational Pharmaceutics (Nottingham, UK) on behalf of the sponsor Albireo AB (Gothenburg, Sweden). EudraCT Number: 2013-001175-21.

The study was designed to assess the feasibility of co-administration of CRC (A3384) or cholestyramine powder (Questran) and an ASBT inhibitor: A4250. There were 7 cohorts of varying doses and combinations of the investigational medicinal products (IMPs). 6 patients within Cohort 4 were given CRC (1g BD) without A4250, and 2 were given a placebo form of CRC only. 2 other cohorts were given CRC placebo with A4250, 2 patients within each cohort were administered A4250 placebo, so these patients were added to the CRC placebo cohort for analysis. One cohort were administered Questran (4g BD) with A4250, the 2 patients that were administered Questran with A4250 placebo were used for analysis for the Questran arm.

A summary of the trial protocol is available in appendix 2.2. Briefly, healthy volunteers were recruited through a newspaper advert and screened for suitability and other medical conditions prior to admission to the clinical trials unit on day -2. Between day -2 and -1 a 24 hour stool collection was performed for faecal BAs. On day 1 at 07.30 blood tests were performed for baseline FGF19, C4 and total BA and the first dose of CRC or placebo given. On day 1 further blood test were taken at 4 hours

(11.30) and the following morning (24 hours or day 2). The evening dose was administered at 19.30. As Questran was being administered with an A4250 placebo, the dosing of the Questran was 4 hours after the placebo (07.30 and 19.30), to prevent IMP binding in the GI tract, but the timing of blood tests was the same as the CRC group. All patients continued CRC, Questran or placebo BD for 7 days. On the last day of IMP, day 7, a 24 hour stool collection was performed for faecal BA and blood tests performed in a day series at 0, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12 and 24 hours after 07.30. The last dose of IMP was administered at 19.30 on day 7 (11.30 for Questran group) and the patient was discharged 24 hours later. They returned 5-7 days later for follow up blood tests off the IMP.

The data received from the trial was restricted patients taking CRC alone (n=6), CRC placebo and CRC placebo with A4250 placebo (n=2+4) and Questran with A4250 placebo (n=2). Individual serum FGF19, C4, CDCA, TCDCA and GCDCA were received for these groups as well as stool CDCA, TCDCA and GCDCA. For serum and faecal total BAs only the mean, median, SD and numbers were received for each group.

Analysis between groups and time points was performed using one-way ANOVA and Dunns multiple comparisons test with a correction for a false discovery rate of 1%. Comparison of pairs of groups were performed using Wilcoxon tests of paired data and Mann-Whitney U tests for non-paired.

5.1.2 A double blind trial of colonic release cholestyramine (A3384) in primary bile Acid diarrhoea

A double-blind, randomized, placebo-controlled, study to demonstrate the efficacy and safety of 250mg or 1 g A3384 administered orally twice daily for two weeks to patients with bile acid malabsorption (BAM) / bile acid diarrhoea (BAD) was an industry sponsored multicentre phase II drug trial. A summary of the protocol is available in appendix 2.3.

5.1.2.1 Subjects

Men and women aged 18-80 with a BMI between 18.5-35 were eligible for inclusion in the trial. Recruitment was split equally between 3 centres, one in the UK, 2 in Sweden.

BAD was defined as:

- SeHCAT 7 day retention of <10% within the last 5 years with no change in symptoms since.
- 3 stools of Bristol stool form scale (BSFS) ≥5 per day on average, calculated from 7 days off therapy.
- ≥21 bowel motions (BMs) in the last 7 days before randomisation, of which ≥50% are BSFS≥5.
- Macroscopically normal colonoscopy with no histological evidence of microscopic colitis.
- No previous biliary surgery, excluding cholecystectomy.
- No evidence of other diarrhoeal or structural GI abnormality.

In addition, patients were excluded if they had used antibiotics within 2 weeks of trial entry, or loperamide or codeine after trial entry. A full list of the inclusion and exclusion criteria are available within the study protocol in appendix 2.3.

The study was carried out in accordance with the International Conference on Harmonisation of Good Clinical Practice and the principles of the Declaration of Helsinki. The study protocol was approval by the East Midlands Regional Ethics Committee (13/EM/0374), the Medicines Regulatory Health Authority (36216/0003/001-0001) and the local NHS Research and Development Office

(Imperial Healthcare Joint Research Compliance Office (13HH0889)). All patients provided informed consent before inclusion in the trial.

5.1.2.2 Trial design

This was a multicentre, double-blind, placebo controlled phase II trial. It was carried out across 3 sites, one in the UK (Hammersmith Hospital) and 2 in Sweden (Sahlgrenska Universitetssjukhuset in Gothenberg and Kärnsjukhuset in Skövde). Eudract number: 2013-002924-17. Recruitment started on the 03/04/2014 and closed on the 18/12/2014.

The trial design is shown in figure 5.1. After an initial screening visit there was a one week run in period during which the patients were asked to take their usual medications, including bile acid sequestrants (BAS), but excluding loperamide or codeine. A daily stool diary was started on visit one. On visit 2 the compliance with the stool diary was checked and the patient was asked to stop any BAS medication until after the last visit (visit 5). After a 2 week (weeks 2 and 3) baseline washout period, the patients returned for visit 3. On visit 3 the stool diary was reviewed and if the criteria for randomisation ($\geq$21 stools with a $\geq$50% BSFS$\geq$5 during the last 7 days of the baseline period), was met, the patient was randomised to either 1g CRC (A3384) BD, 250mg CRC BD or placebo BD in an 1:1:1 blinded fashion. After a 2 week treatment period (weeks 4 and 5), the patient returned for assessment at visit 4, where any remaining trial medication was collected and compliance checked with a pill count. A one week wash-out period (week 6) followed before the last follow-up visit (visit 6) where the patient's regular BAS was restarted. At all visits any adverse events (AEs) were recorded. All concurrent medications were kept at the same dose 4 weeks before and during the trial and this was checked at visits. The use of any anti-diarrhoeal medication was not permitted between weeks 2 and 6.

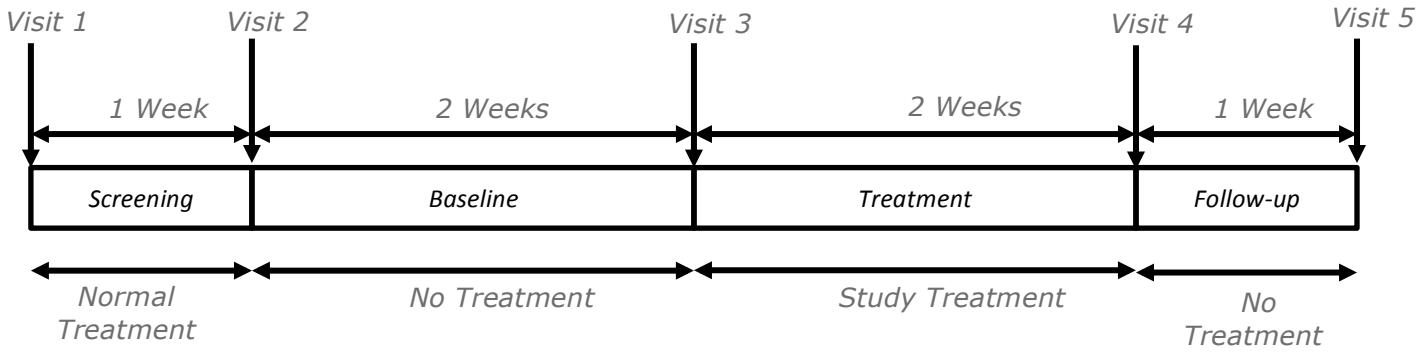


Figure 5:1: Schematic of the design of A3384 in BAM/BAM

5.1.2.3 Assessment

At all visits the patient's general health were assessed with clinical blood tests (full blood count, urea and electrolytes, liver function tests) and urine dip-stick, with a urinary pregnancy test for females. Details of the activities on each visit are shown in table 5.1.

5.1.2.3.1 Symptom recording

A daily stool diary was started from visit one and continued throughout the trial until visit 4 this recorded the time and BSFS of each bowel movement (BM). In addition each day the patient was asked 3 questions asking the severity of diarrhoea, abdominal discomfort and bloating on a 0-10 Likert scale. During the first week, the patients were also asked if they had taken their regular BAS that day.

On the last day of each week the patient was asked to rate their diarrhoea, abdominal discomfort and bloating during the last 7 days on a 1-5 Likert scale. They also recorded their global symptoms over the last 7 days on a 0-10 Likert scale.

During the 2 treatment weeks the patient was also asked to compare their symptoms of diarrhoea, abdominal discomfort, bloating and global symptoms to those on their regular medication over the last 7 days on a 1-7 Likert scale. The patients were also asked to rate the treatment effectiveness over the last 7 days on a 1-7 Likert scale.

The stool diaries were collected at each visit and a new one issued for the forthcoming period.

	Screening - Baseline Period for symptom registrations		**Treatment Period**		**Follow-up Period**
	Visit before Baseline 1	**Visit before Baseline 2**	**START**	**END**	
Study Days	–21	–14	1	15	22
Allowed days for the Visit	–21 to –24	–14 to –17		15 to 18	22 to 25
Visits	Visit 1	Visit 2	Visit 3	Visit 4	Visit 5
Informed consent	•				
Randomization			•		
Inclusion/exclusion criteria	•	•	•		
Demographics	•				
Medical and surgical history	•				
Physical examination	•			•	•
Vital Signs	•	•	•	•	•
GI symptom evaluation (paper diary)	•	•	•	•	
Clinical laboratory tests and blood for pharmacodynamic markers	•	•	•	•	•
Pregnancy test	•	•	•	•	•
Study medication dispensed			•		
Study medication compliance evaluated				•	
Concomitant medication documented	•	•	•	•	•
AEs documented	•	•	•	•	•

Table 5.1: Breakdown of study activities by visit. AE: adverse event.

5.1.2.3.2 Pharmacodynamic markers

At each visit the patient attended fasted for 6 hours previously and venous blood was collected for FGF19, C4 and serum BAs. The serum was separated by centrifuge and frozen at -80^{o}C until analysis by Quotient Bio Analytical Sciences, Cambridgeshire, UK.

5.1.2.3.3 Endpoints

The primary efficacy endpoint was a reduction in mean daily number of BMs in the last 7 days of treatment (week 5).

The secondary efficacy endpoints were based upon the patients stool diary recording in the last 7 days of treatment and included:

- Reduction in stool consistency as measured by the BSFS
- Reduction in abdominal discomfort using rating scales
- Reduction in bloating using rating scales
- Reduction in the severity of diarrhoea using the rating scales
- Reduction in the degree of global symptoms using the rating scales
- Comparison of the treatment efficacy compared to regular treatment using the rating scales

The exploratory efficacy endpoints assessed serum FGF19, C4 and BAs on and off treatment.

The primary safety endpoint was the incidence of treatment-emergent serious adverse events (SAEs) based upon patient reports and the visit blood tests and clinical assessments.

The secondary safety endpoints were the occurrence of treatment-emergent adverse effects (AEs) based upon patient reports and the visit blood tests and clinical assessments.

5.1.2.3.4 Safety reporting

The occurrence of all AEs were recorded within the electronic clinical research file (eCRF) from first visit to the follow up visit. All AEs occurring after first dosing were considered treatment-emergent.

Worsening of diarrhoea was not considered to be an AE. Serious AEs were recorded up to the follow-up visit and were defined as a life-threatening or permanently disabling medical event. These were reported to the sponsor immediately.

5.1.2.3.5 Statistical methods

The sample size was determined to detect a 40% reduction in mean number of BM during the last week of treatment in the group dosed with 1g BD CRC compared to placebo with a power of 90% and an alpha of 0.05. This assumed a mean number of BMs/day of 4 in the placebo group as reported by *Jacobsen et al*.[148] Based on these calculations 12 patients per treatment arm were required.

Both the safety and efficacy end points were calculated including all patients who had undergone randomisation and had taken at least 1 dose of the trial mediation. This was the intention-to-treat (ITT) population.

A per-protocol (PP) population consisted of those patients who were at least 80% compliant with study medication and 60% compliant with patient reports and was used for some of the exploratory endpoints.

Data on baseline demographics and AEs were presented descriptively. The primary and secondary endpoints were calculated for the ITT population, as the sum of the number of BMs, BSFS or rating scales for the last 7 days on treatment. These were compared against the placebo population using Mann-Whitney U tests and against the last 7 days of the baseline period using paired Wilcoxon tests.

5.2 Results: Effects of conventional and colonic release cholestyramine

5.2.1 Effects of conventional and colonic release cholestyramine in healthy volunteers

10 patients completed the trial, 6 received CRC 1g BD, 6 CRC placebo and 2 Questran 4g BD. All completed the trial as per protocol apart from one patient in the CRC arm that did not return for the follow-up visit and one in the Questran arm that did not perform a faeces collection on day 7. Blood tests were performed on day 1 of starting the medication and on the last day (Day 7). 24 hour faeces collection was performed on day -2 (baseline) and day 7 (treatment). Due to the necessity for Questran administration to be separated from other medications, the dosing schedule was 4 hours behind that of CRC and placebo. Therefore direct comparison was only available at 5 time points when the times were corrected for the dose timing. Therefore the following figures are shown in uncorrected first dose (day 1), day 7 day series, a corrected day 7 day series and (uncorrected for does timing) morning samples throughout the trial period (figures 5.2 and 5.3).

5.2.1.1 FGF19

The diurnal effect of FGF19 rising in afternoon was observed in all 3 groups, despite the difference in dosing timings (Fig 5.2). There were no significant differences between the treatment groups as analysed by Mann-Whitney U tests. There were significant differences within the CRC group, with increased FGF19 at 8 and 12 hours dose day on day 7 compared to 3 hours post dose (mean differences 171 and 202pg/mL respectively, both $p<0.05$ on Dunns multiple comparison test).

When corrected for the dose timings, there were no significant differences in FGF19 on day 7 of treatment between the groups. Despite figure 5.3(A) showing rise in a rise in median FGF19 at 2 hours, this is due to one outlier and not statistically significant on grouped analysis.

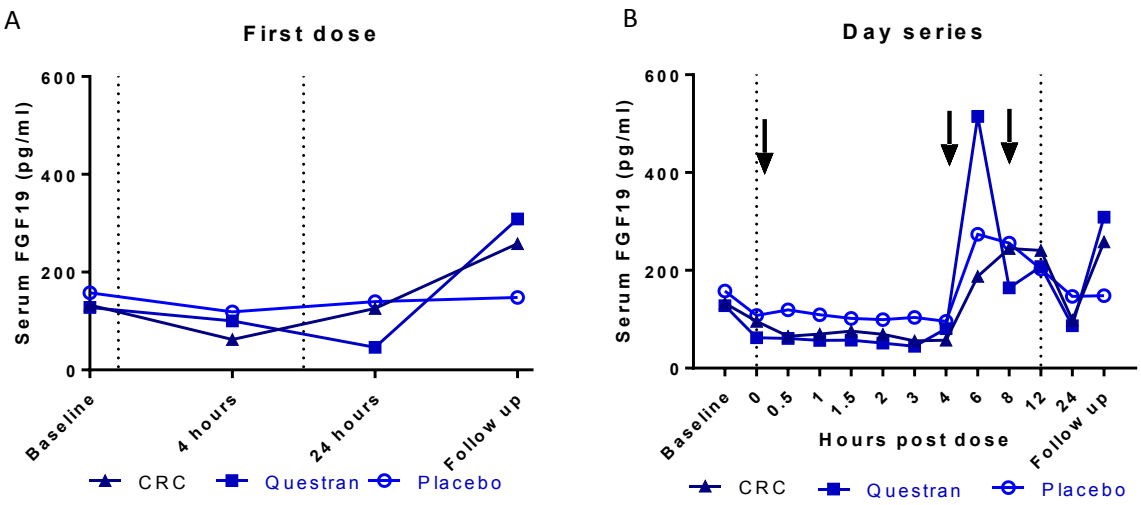


Figure 5.2: Serum FGF19 after first dose of CRC, questran or placebo (A) and after taking CRC, questran or placebo for 7 days (B). Timings are not corrected for dosing, dotted lines indicate time doses given, arrows indicate meal times.

There was a trend towards lower FGF19 in the Questran group in the mornings (the most reliable time to measure FGF19) compared to placebo and CRC. The reduction 58% from baseline after 7 days of treatment nears significance ($p=0.08$ on Dunns multiple comparison test), despite a 30% reduction in the placebo and CRC groups. Despite a similar baseline FGF19 in all groups, the Questran and CRC

groups had higher FGF19 on follow up compared to placebo, possibly indicating over-compensation in the treatment groups, although this trend was non-significant (p=0.4, figure 5.2(B)).

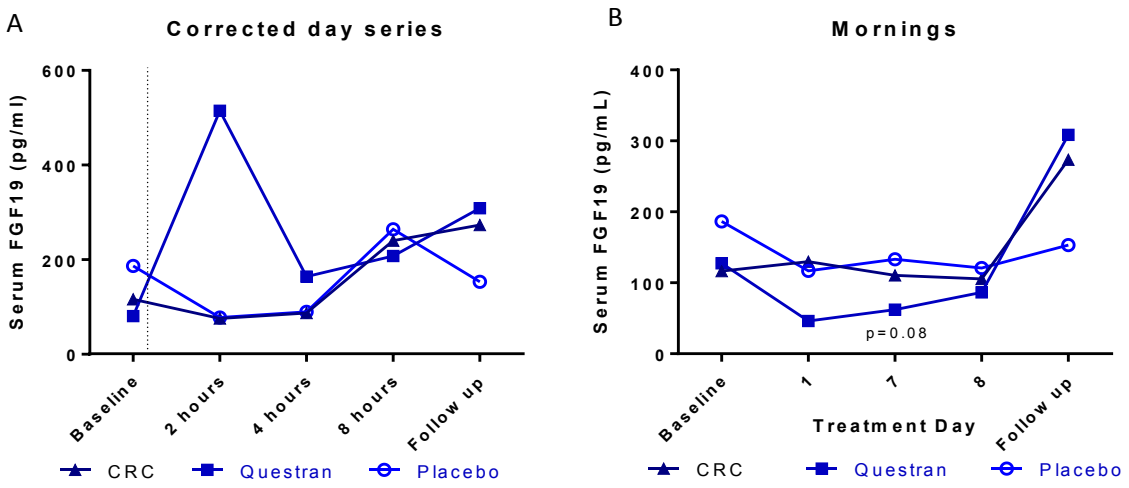


Figure 5.3: Day 7: Day series of serum FGF19 after receiving CRC 1g, Questran 4g or placebo, corrected for dose timing (A). FGF19 pre-dose morning measurements, uncorrected for dose timing (B). Dotted line denote time of dose.

	Baseline Day 1	**Treatment Day 2**	**Treatment Day 7**	**Treatment Day 8**	**Follow Up Day 14**
Placebo (n=4)	114 (53-264)	44 (28-190)	82 (35-131)	76(34-199)	139 (27-208)
Colestyramine (n=2)	167 (134-199)	72 (33-112)	71 (50-91)	116 (88-143)	389 (329-449)
CRC (n=6)	133 (48-172)	126 (44-198)	96 (50-243)	98 (43-158)	258 (98-456)

Table 5.2: Morning FGF19 in healthy volunteers on bile acid sequestrants.

5.2.1.2 C4

Serum C4 was measured at the same time-points as FGF19 in all groups. There was a definite trend towards higher C4 in the Questran group compared to CRC and placebo (figure 5.4). There was a 5 fold increase in serum C4 on Questran treatment from baseline, maximum change for both A3384 and placebo was a 2-fold change. The change in C4 was statistically significant for Questran when compared to baseline values for all days on treatment and serum C4 returned to baseline values when Questran was stopped.

There were almost identical baseline and follow up C4 between all groups. 24 hours after the first dose there is a significant rise in C4 in the Questran group compared to the placebo and CRC arms (figure 5.4(A)). This trend continues with 7 days of treatment with higher C4 in the Questran group at all time points and nearing significance at 3 times on day 7 (0.5 and 8 hours, p=0.07). The difference in C4 reached statistical significance on the mornings of day 7 and 8.

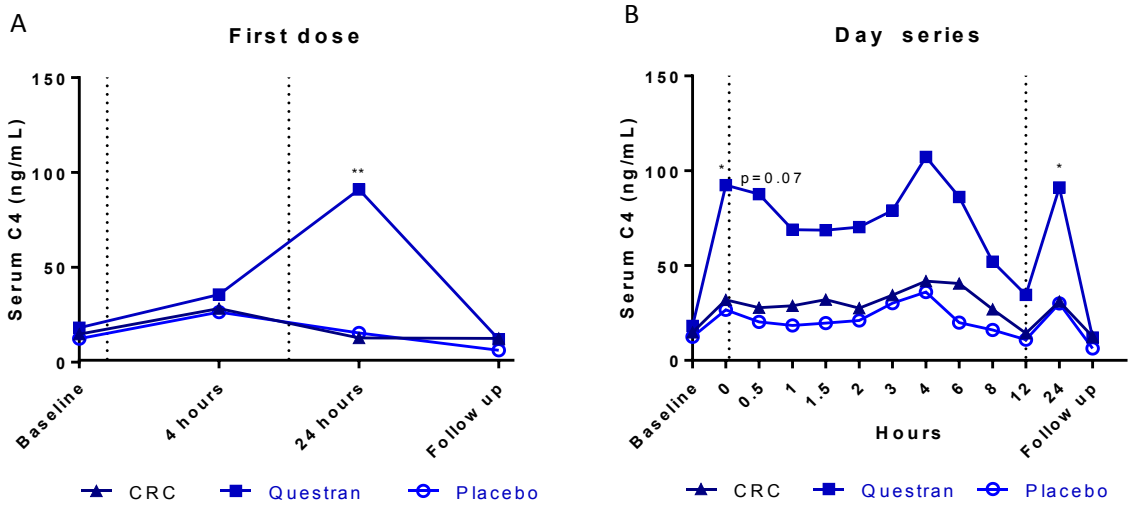


*Figure 5.4 Serum C4 after first dose of CRC, Questran or placebo (A) and after taking CRC, Questran or placebo for 7 days (B). Timings are not corrected for dosing, dotted lines indicate time dose given.*p<0.05 **p<0.01*

When corrected for dose timing the trend for higher C4 in Questran arm continues, but does not reach statistical significance. C4 on the morning, pre dose samples, showed an approximately 5 fold difference in C4 in the Questran group compared to the placebo and CRC arms on all mornings after treatment (days 1, 7, 8). These changes were statistically significant, and C4 normalised to comparable values to the CRC and placebo groups 7 days after treatment cessation (figure 5.5(B)).

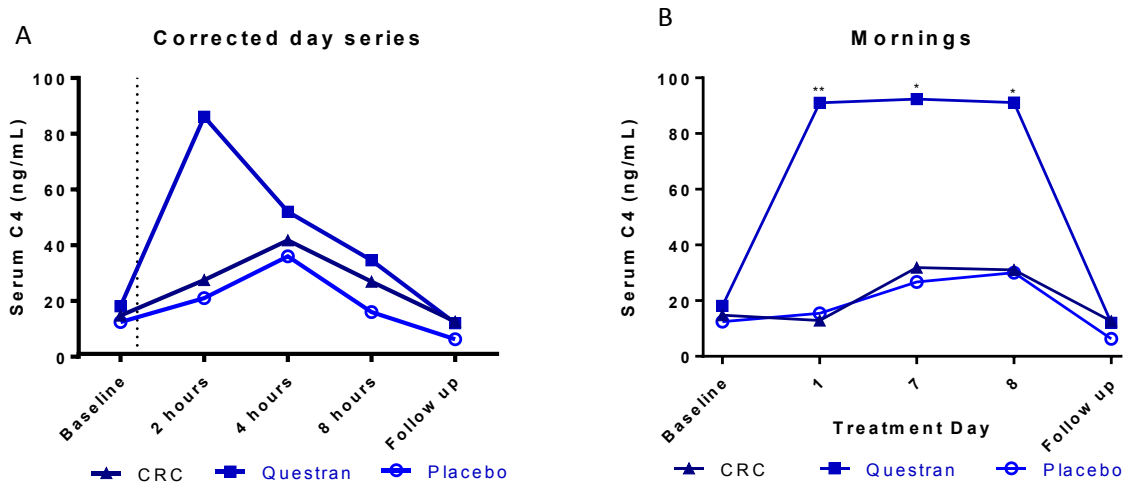


*Figure 5.5 Day 7: Day series of serum C4 after receiving CRC 1g, Questran 4g or placebo, corrected for dose timing (A). FGF19 pre-dose morning measurements, uncorrected for dose timing (B). Dotted lines denote time of dose. *p<0.05, **p<0.01*

5.2.1.3 Serum total bile acids

Serum total BAs were measured at the same time points as FGF19 and C4. Overall there was a trend towards higher serum total BAs in the placebo group compared to the Questran group, with the CRC group in between. However, the placebo group also exhibited higher serum BAs at baseline and follow up. There were no significant changes in serum BAs after first dose (Figure 5.6). In the day 7 day series there was a significant difference between the placebo and Questran groups 8 hours after dosing, but no difference in the CRC group when compared to placebo or Questran groups.

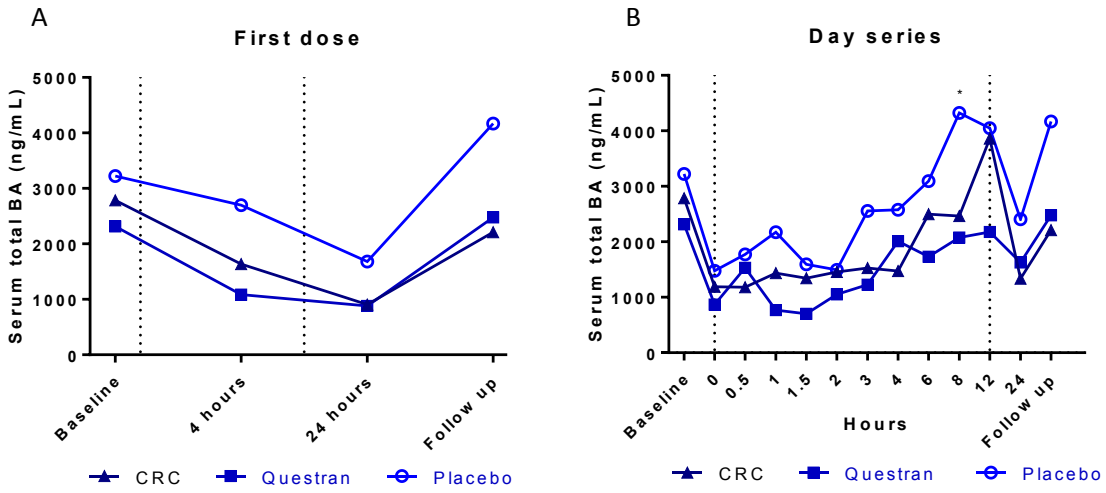


*Figure 5.6 Day 1: Serum total BAs after first dose of CRC, Questran or placebo (A) and after taking CRC, Questran or placebo for 7 days (B). Timings are not corrected for dosing, dotted lines indicate time dose given. *p<0.05*

The lower serum BAs at 8 hours in the Questran group, maintained statistical significance when corrected for dose timing (figure 5.7(A)). There were no significant changes in serum BAs in the morning, pre-dose samples.

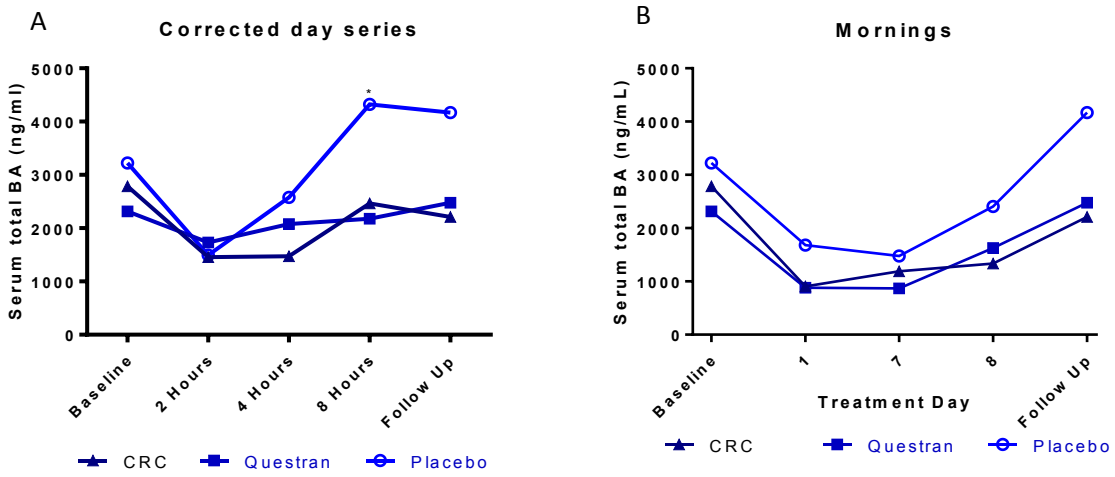


*Figure 5.7: Day series of serum total BAs after receiving CRC 1g, Questran 4g or placebo, corrected for dose timing (A). serum total BAs pre-dose morning measurements, uncorrected for dose timing (B). *p<0.05.*

5.2.1.4 *Faecal bile acids*

Faecal BAs were measured the day before starting treatment (Baseline) and on day 7 (treatment). Due to the need to produce a stool sample on day 7, only 1 of the patients on the Questran arm provided a sample and 3 from the placebo arm. All 6 patients with the CRC arm produced samples for analysis. There was a trend for higher faecal BAs in the Questran group compared to CRC and placebo, and in the CRC group compared to placebo, but these trends did not reach statistical significance (figure 5.8)

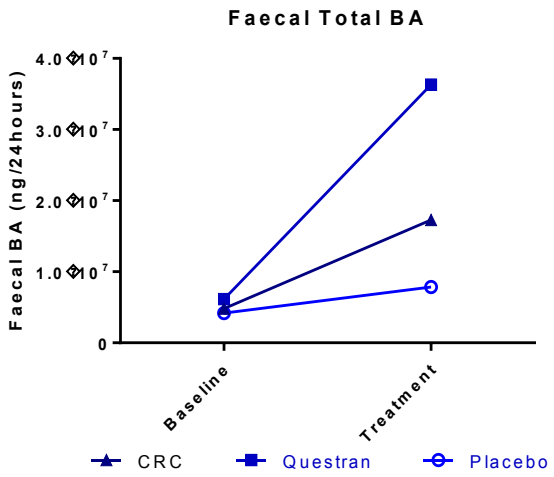


Figure 5.8 Faecal total BA over 24 hours on and off treatment.

5.2.1.5 CDCA Conjugates in serum and faeces

Serum CDCA and its conjugates with taurine (TCDCA) and glycine (GCDCA) were measured in serum on the morning before starting treatment and on the morning of day 7 on treatment. 24 hour faeces collections were also performed on these days. There were no significant trends seen between the treatment groups.

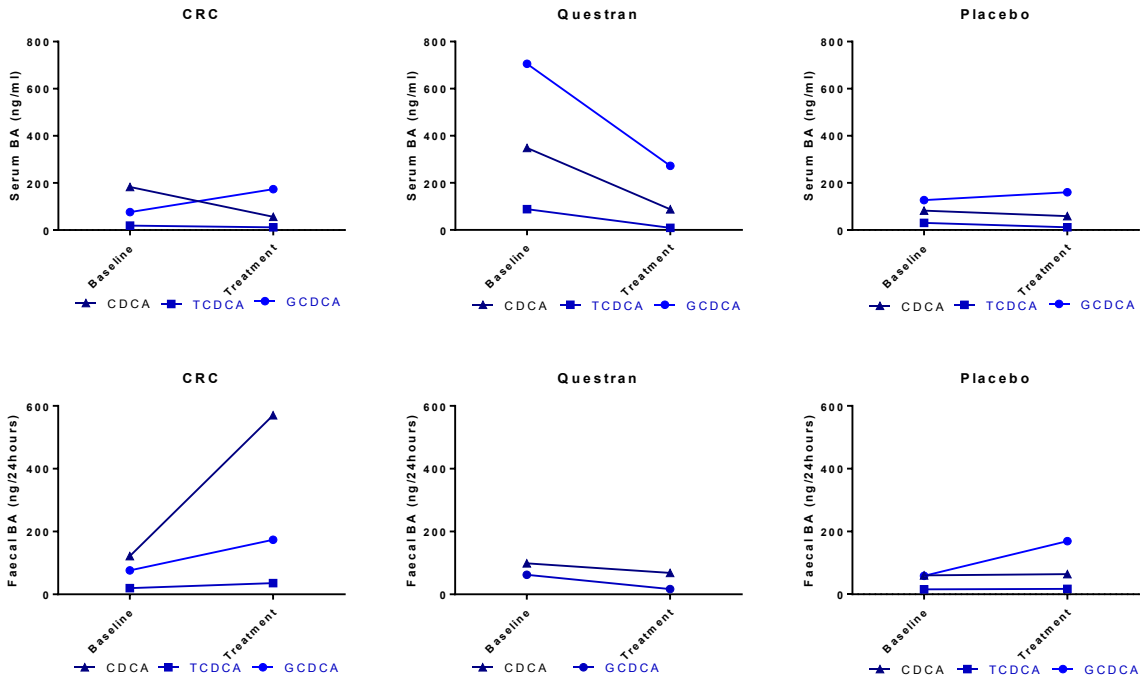


Figure 5.9: Change in serum (top row) and faecal (bottom row) CDCA and its conjugated forms during treatment with either CRC, Questran or placebo.

5.2.2 Effects of bile acid sequestrants and colonic release Cholestyramine in primary bile acid diarrhoea

The A3384 in BAD study was stopped early by the sponsor due to the high rate of GI adverse advents and lack of effect on FGF19 in the A4250 healthy volunteer study (reported in section 5.2.1). The study had planned to screen 48 patients, of whom 36 would reach randomisation. By the time of trial closure, 34 patients had been screened, 19 had entered the trial, forming the intention-to-treat cohort (ITT) of whom 15 completed the trial as per protocol (PP), these were patients that showed at least 80% compliance with the IMP and 60% compliance with reporting.

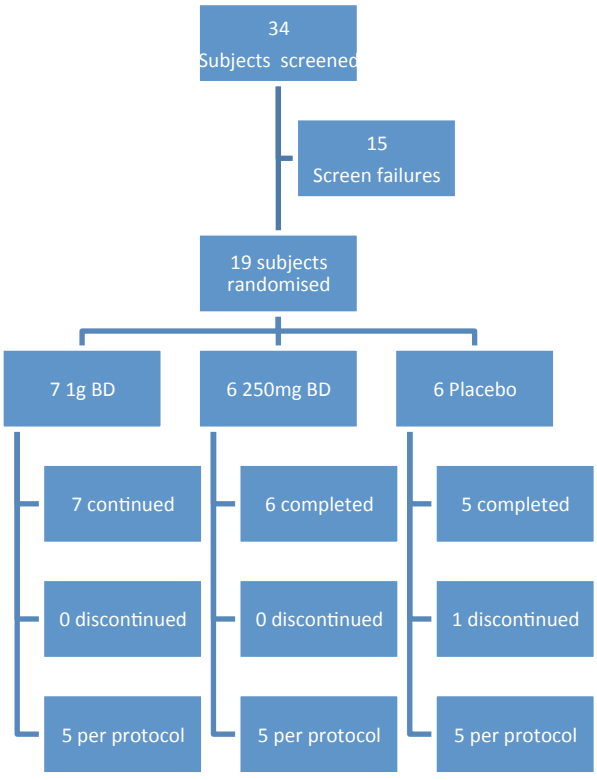


Figure 5.10: Flow diagram of patient recruitment during the A3384 in BAD trial.

5.2.2.1 Demographics

Of the 19 patients in the ITT population, 11 were recruited at the Hammersmith Hospital, 6 in Gothenburg and 2 in Sovde. Demographics are shown in table 5.3.

	1g CRC	250mg CRC	Placebo	All
Female n (%)	3 (42.9)	2(33.3)	3(50)	8(42.1)
Mean age (SD)	46.4(20.4)	50(21.28)	38.7(17.57)	45.1(19.31)
Caucasian n(%)	5 (71.4)	5 (83.3)	4 (66.7)	14 (73.7)
Mean BMI (SD)	28.7(5.1)	28.4(5.17)	26.6(4.44)	27.9(4.79)

Table 5.3: Demographics of the ITT population.

5.2.2.2 Safety

1 SAE was reported during the study. This patient was diagnosed with metastatic cancer 3 months after receiving 1g BD CRC, since the first symptom of this cancer was mild shoulder pain that was recorded as an AE during the baseline period of the study, it was decided that this should be reported as SAE, but was deemed not related to the IMP.

There were 14 AEs reported in 9 patients. 9 of these were treatment-emergent AEs, 3 of these were judged to be possibly related to the IMP, these were all in the same patient who was taking 1g CRC and reported cardiac palpitations, upper abdominal pains and hyperhidrosis. All were graded as mild and the patient completed the trial. One patient discontinued the IMP after a hospital admission with non-cardiac chest pain on day 11 of treatment, which was graded as moderate. This patient was in the placebo arm. All other AEs were graded as mild apart from the incidence of cancer metastasis that was graded as severe. The details of all treatment-emergent AEs are in table 5.4.

	Ig CRC	250mg CRC	Placebo	Total
Total n in ITT	7	6	6	19
n of any AE (%)	2 (28.6)	3 (50)	4 (66.7)	9
Abdominal pain			1	1
Upper abdo pain	1*			1
Serum calcium increased		1		1
Headache			1	1
Hyperhidrosis	1*			1
Lower respiratory tract infection		1		1
Metastasis	1			1
Nasopharyngitis			2	2
Neuralgia	1			1
Non-cardiac chest pain			1	1
Palpitations	1*			1
Urinary tract infection		1		1
Vomiting			1	1

*Table 5.4: Treatment emergent adverse events by diagnosis *denotes possibly related to IMP.*

5.2.2.3 *Primary efficacy endpoint*

The primary efficacy endpoint was a reduction in the mean daily number of BMs over the last 7 days of treatment for CRC compared to placebo. 50% patients receiving placebo reported a decrease in BMs from week 3 (baseline, off treatment) to week 5 (second week of treatment), compared to 67% for the 250mg A3384 and 71% for 1g A3384 and 69% for both dose of A3384 combined. These changes were not statistically significant, and are shown in table 5.4 and figure 5.11(A).

	Placebo	**250mg CRC**	**1g CRC**	**250mg+1g CRC**
No reduction	3	2	2	4
Any reduction	3	4	5	9
%	50	67	71	69
p (Fisher's)		1.0	0.59	0.62

Table 5.5: Number and percentages of patients achieving any reduction of weekly BMs between W3 (baseline) and W5 (treatment).

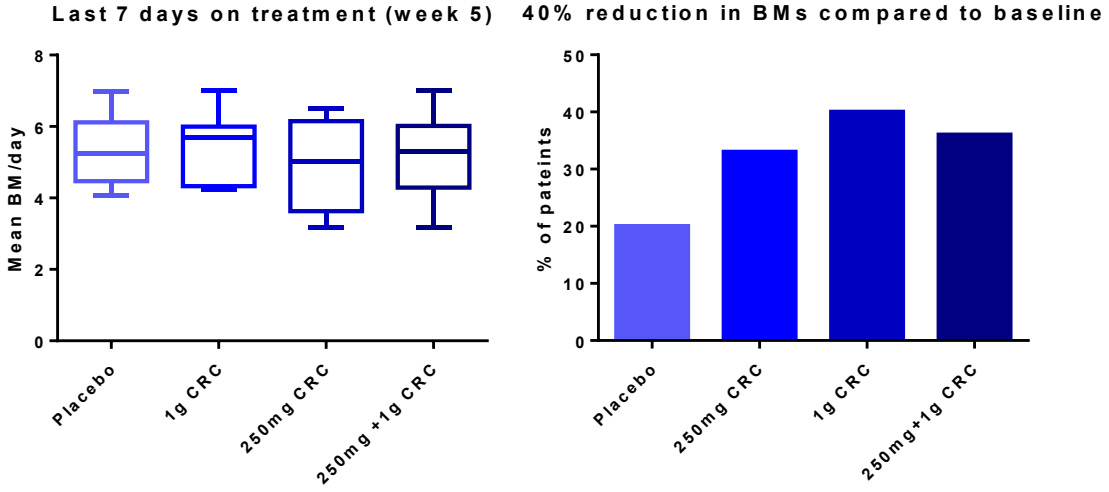


Figure 5.11: Primary efficacy endpoint: Box and whisker plot (Median, IQR and 95% CI) of Mean BMs/day over the last 7 days on treatment by treatment group (A). Percentages of patients achieving a 40% reduction of weekly BMs between W3 (baseline) and W5 (treatment) (B).

Another definition was a 40% reduction in number of BMs in second treatment week (W5) from the second baseline week (W3). The percentage of patients reaching the primary endpoint in the placebo group was 20% (1 patient). This compared to 33% in the 250mg BD group (2 patients) and 28% in the 1g BD group (2 patients) in combined treatment group the response rate was 31%. These trends were non-significant on fisher's exact tests (table 5.6 and figure 5.11(B).

	Placebo	250mg CRC	1g CRC	250mg+1g CRC
<40%	5	4	5	9
>40%	1	2	2	4
%	20	33	28	31
p (Fisher's)	1	1	1	1

Table 5.6: Number and percentages of patients achieving a 40% reduction of weekly BMs between W3 (baseline) and W5 (treatment).

5.2.2.4 Secondary efficacy end points

Each subject was asked to record their subjective symptoms on a Likert scale in 2 different questions:

- Daily they were asked '*How would you rate your diarrhoea / abdominal discomfort / bloating during the last 24 hours? Please use the following scale from 0 to 10 where: 0= no diarrhoea / abdominal discomfort / bloating and 10 = very severe diarrhoea / abdominal discomfort / bloating'* For analysis, the mean of the score for the last 7 days on treatment (W5) and the last 7 days of the baseline period (W3) were used and the difference calculated.
- Weekly they were asked '*On average, how would you rate you diarrhoea / abdominal discomfort / bloating during the past 7 days? 1=none, 2=mild, 3=moderate, 4=severe, 5= very severe.'* The change from W3 to W5 was calculated.

5.2.2.4.1 Change from Baseline in average severity of diarrhoea during the second treatment week or the last 7 days of reporting

Answers to the daily diarrhoea questions showed a significant trend towards less severe diarrhoea with 250mg CRC (median change -2.24, p<0.01), both treatment groups combined (250mg and 1g CRC, median change -2.14, p<0.01) when compared to placebo (median change +0.71). 1g CRC showed a non-significant trend towards less severe diarrhoea (median -1.41 p=0.10).

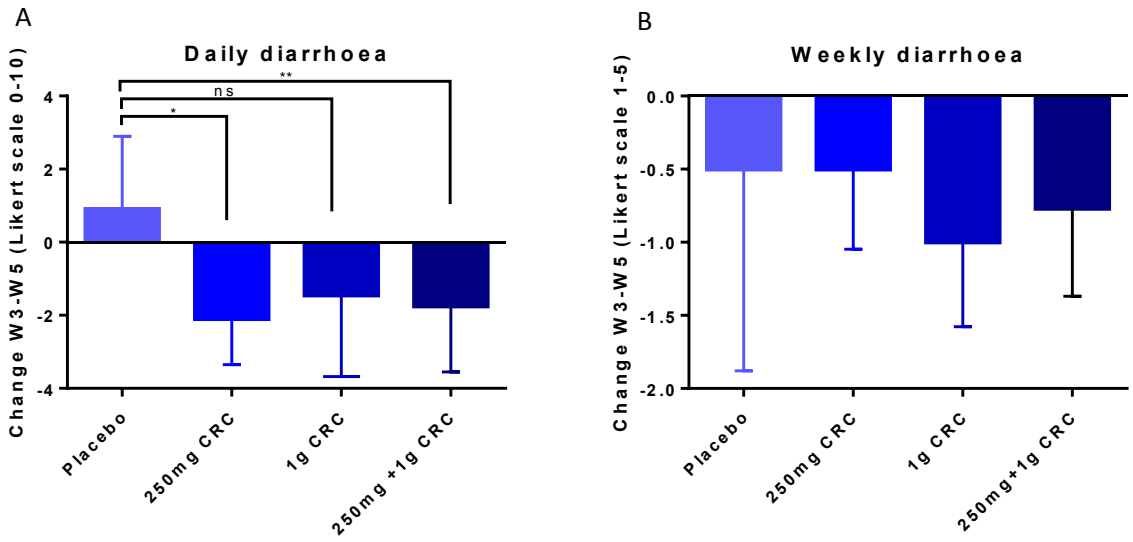


*Figure 5.12: Change in reported severity of daily diarrhoea (A) and weekly diarrhoea from week 3 to week 5 (B). *p<0.05 **p<0.01 ns=non-significant.*

In addition, the change in the average daily number of liquid stools (types 6 and 7) were calculated between week 5 and week 3 as an objective measure of severity.

There were no significant differences in the reported diarrhoea in the weekly questionnaire, or in the number of type 6 or 7 stools /day between the treatment arms. Although all groups showed an improvement in diarrhoea while on treatment (figures 5.13(B) and 5.14).

As an objective measure of change in diarrhoea, the frequency of liquid stools (BSFS 6/7) in week 5 was compared to week 3. There were trends for all groups for a reduction in BSFS 6/7 but these were non-significant when compared to placebo (median change (95%CI); placebo -0.9 (–3-0.8), 250mg CRC -1.6 (–5.6-1.3), 1g CRC -2 (–3.1-0.1), combined 250mg and 1g (–0.3-–0.3).

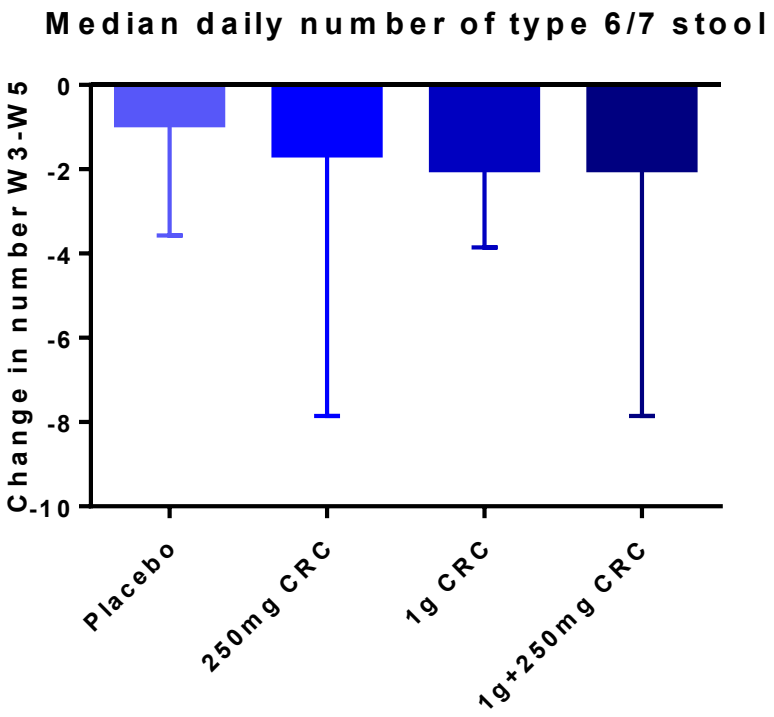


Figure 5.13: Median (with range) change in number of stools of BSFS type 6 or 7 from week 3 (baseline) to week 5 (treatment).

5.2.2.4.2 Change from Baseline in average severity of abdominal discomfort during the second treatment week or the last 7 days of reporting

Abdominal pain was recorded on a Likert scale of 0-10 for daily symptoms and 1-5 for average symptoms over the last week. For daily symptoms the median Likert scale over the last 7 days of treatment was compared to the median score for week 3 of the baseline period. There was a non-significant trend towards less daily abdominal discomfort on treatment; median values are displayed in table 5.7.

	Placebo	**250mg CRC**	**1g CRC**	**250+1g CRC**
Daily discomfort	+0.3 (-3.6-2.6)	-0.9 (-3.2-0.4)	-2.6 (-5.2-2)	-1.3 (-3.3-0.3)
Weekly discomfort	-1 (-2.5-0.9)	0 (-0.9-1.2)	0 (-2-0.3)	0 (-1.1-0.3)

Table 5.7: Median (95%CI) change in self-reported abdominal pain from week 3 (baseline) to week 5 (treatment).

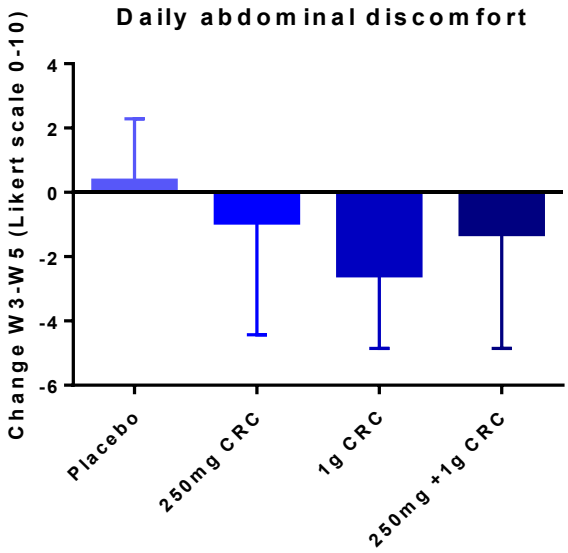


Figure 5.14: Median change in daily abdominal discomfort as recorded by Likert scale. Error bars represent the range.

5.2.2.4.3 Change from Baseline in average severity of abdominal bloating during the second treatment week or the last 7 days of reporting

Bloating was recorded on a Likert scale of 0-10 for daily symptoms and 1-5 for average symptoms over the last week. For daily symptoms the median Likert scale over the last 7 days of treatment was compared to the median score for week 3 of the baseline period. There was a non-significant trend towards less daily bloating on treatment, the median values are displayed in table 5.8 and figure 5.15. There were no significant changes in the weekly score.

	Placebo	**250mg CRC**	**1g CRC**	**250+1g CRC**
Daily bloating	0.3 (-3-2.6)	-1.1 (-4.8-1.2)	-0.4 (-3.7-3)	-1.4 (-3-0.9)
Weekly bloating	-0 (-1.6-1.2)	-1 (-0.9-2.5)	0 (-0.2-1.3)	0 (0-1.4)

Table 5.8: Median (95%CI) change in self-reported bloating from week 3 (baseline) to week 5 (treatment).

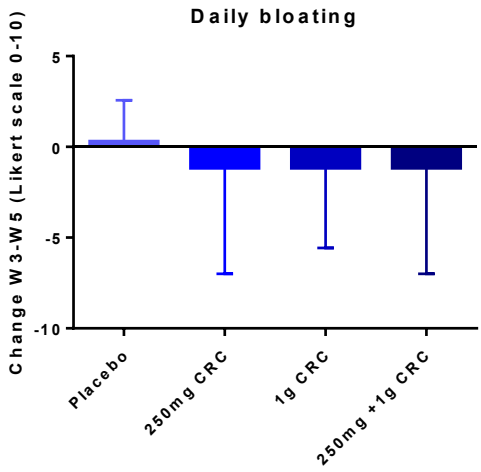


Figure 5.15 Median change in daily abdominal bloating as recorded by Likert scale. Error bars represent the range.

5.2.2.4.4 Change from Baseline in average BSFS during the second treatment week or the last 7 days of reporting

The median stool type as measured by BSFS reduced in all groups from week 3 to week 5 (Figure 3(A)). Median change in the placebo arm was -0.1 (0.8-0.5), 250mg A3384 -1.4 (-4.1-0.4), 1g A3384 -0.4 (-1.4 - 0) and in both A3384 doses combined -1.2 (-2.2- -0.2). This narrowly missed statistical significance for the 250mg and combined A3384 arms (p=0.058 and 0.065 respectively).

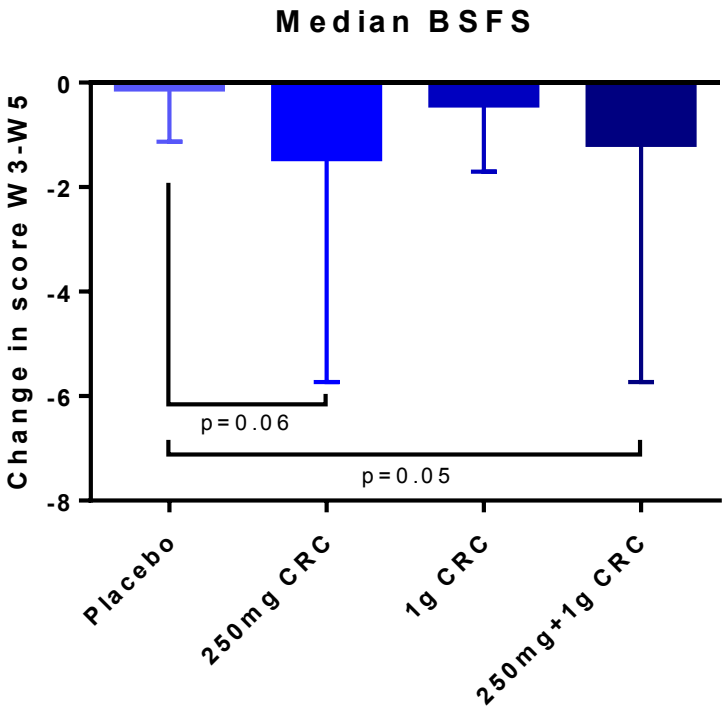


Figure 5.16: Median (range) change in BSFS from week 3 to week 5 for all treatment groups

5.2.2.4.5 Change in symptom reporting from week 1 on BAS to week 5 on treatment.

5.2.2.4.5.1 Diarrhoea

There were no discernable trends on daily or weekly diarrhoea scoring during week 5 (on treatment) compared to week 1 (no treatment, or on BAS). Most patients reported that their diarrhoea was unchanged or somewhat relieved when compared to their regular medication, regardless of what group they were in.

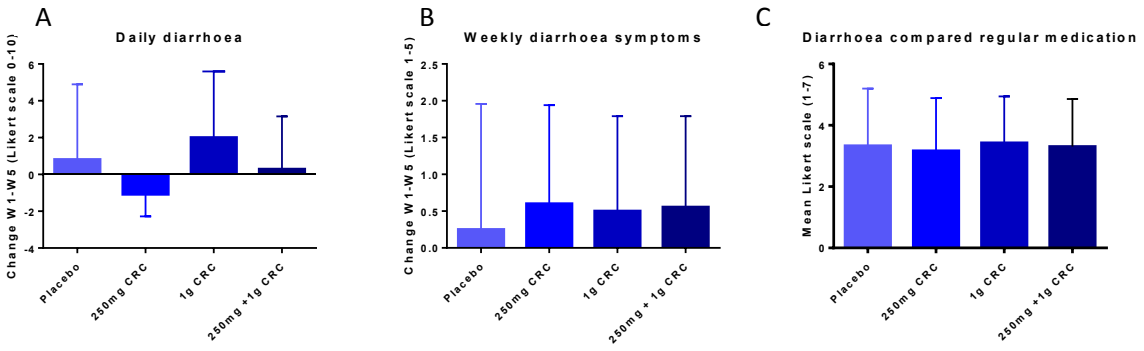


Figure 5.17: Median (range) change in self-reported daily diarrhoea scores (A), weekly diarrhoea (B) during week 5 (on treatment) compared to week 1 (not on treatment or on BAS) and diarrhoea compared to regular medication (C).

5.2.2.4.5.2 Abdominal discomfort

On weekly scoring there was a trend towards more discomfort on treatment rather than placebo. This reaches statistical significance for 250mg CRC compared to placebo (median change placebo; -1.5, 250mg CRC; 0, p=0.03). This effect is not seen on the daily discomfort scores, which show a non-significant trend towards less discomfort with 250mg CRC compared to placebo (median change placebo 0.1, 250mg CRC -0.4, p=0.6). Nor is it reported when compared to regular treatment; where there was no change in reported discomfort for any of the groups. On further analysis the increased reported abdominal pain in the treatment groups compared to placebo can be attributed to higher discomfort scores for the placebo group in week 1 while on conventional BAS, (median (95%CI) placebo 4 (3.1-4.9), 250mg CRC 2.5 (1.4-3.9)) than higher scores while on treatment during week 5 (median (95%) placebo 3 (1.4-5), 250mg CRC 4 (2.6-4.8).

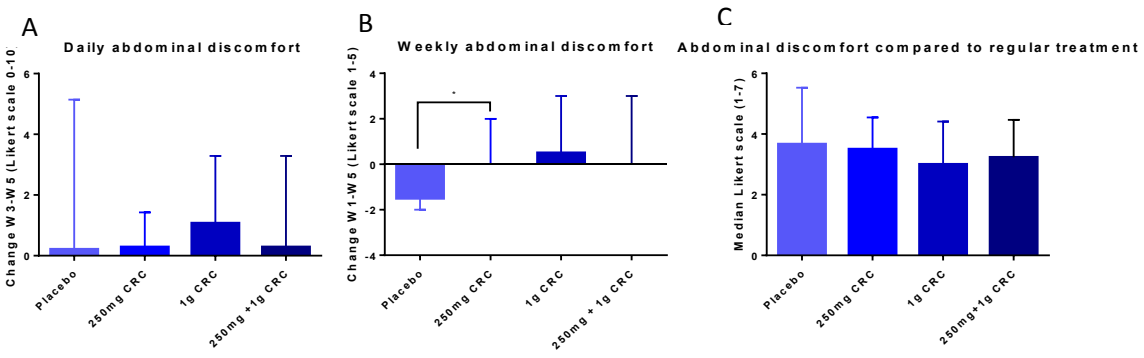


*Figure 5.18: Median (range) change in self-reported daily abdominal discomfort scores (A), weekly abdominal discomfort (B) during week 5 (on treatment) compared to week 1 (not on treatment or on BAS) and abdominal discomfort compared to regular medication (C). *p<0.05.*

5.2.2.4.5.3 Bloating

There were no discernable trends for abdominal bloating in week 5 compared to week 1 or regular treatment.

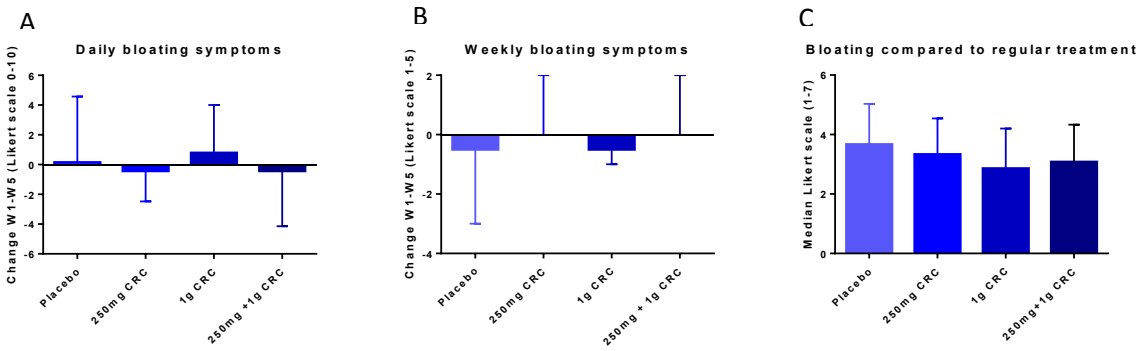


Figure 5.19: Median (range) change in self-reported daily abdominal bloating scores (A), weekly abdominal bloating (B) during week 5 (on treatment) compared to week 1 (not on treatment or on BAS) and abdominal bloating compared to regular medication (C).

5.2.2.4.5.4 Global symptoms

There was a non-significant tread for global symptoms to be worse on treatment compared to placebo (median (95%CI); placebo -3 (-8.8-4.8); 250mg CRC 0 (-4.5-6.5), 1g CRC 1.5 (-1.8-4.8), combined 250mg+1g CRC 1 (-1.4-3.8)). There were no differences in global symptoms when compared to regular treatment.

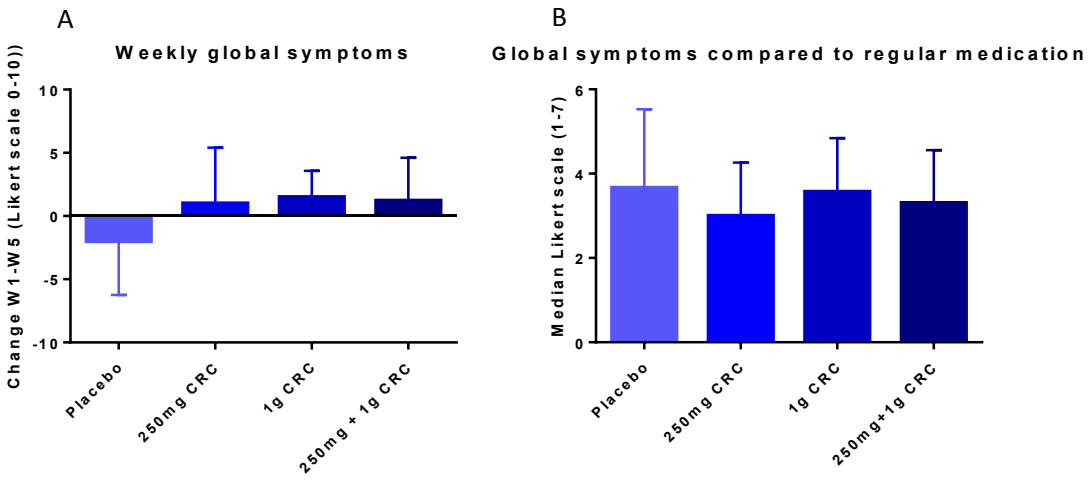


Figure 5.20: Median (with range) change in self-reported global symptoms between week 1 (on no treatment or regular BAS treatment) and week 5 (on trial treatment) (A) and global symptoms on trial medication when compared to regular medication (B).

5.2.2.5 Exploratory end points

5.2.2.5.1 Effect of BAS and CRC withdrawal on FGF19 and C4

Thirteen patients across all treatment groups were taking conventional sequestrants in week one (7 cholestyramine, 4 colesevelam, 2 colestipol). On withdrawal of conventional sequestrants, FGF19 increased by 28% (median FGF19 visit 2; 58.6pg/ml (21.9-95.3), visit 3; 74.8pg/ml (33.7-184), p=0.01, (Fig.5.22A). This was supported by a 58% decrease in serum C4 (median C4 visit 2; 109ng/ml (65.8-168.7), visit 3; 45.7ng/ml (33.9-99.6), p=0.0005 (Fig.5.22B).

In addition there was a significant difference between median FGF19 on visit 3 and visit 1 (28pg/mL), where administration of BAS could be assumed, but was not explicitly recorded, but there was no significant difference between visit 1 and 2. A similar increase was seen in median C4 in the same patients (Visit 1 90 ng/ml and V3 45.7 ng/mL), which was statistically significant.

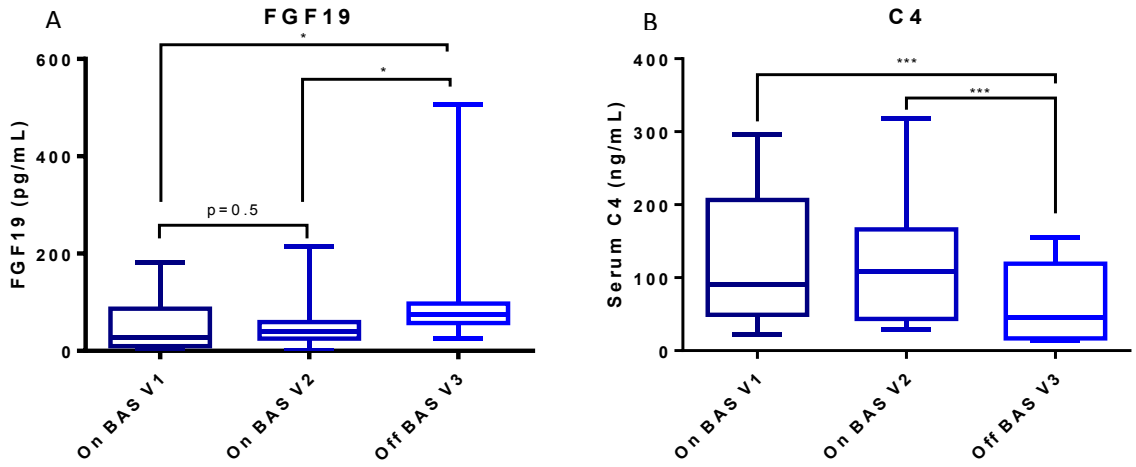


*Figure 5.21: Box and whisker plots (median, IQR, 95% CI) of FGF19 (A) and C4 (B) on and off BAS treatment on visits 1,2 or 3 (V1,2,3). *p<0.05 ***p<0.005.*

A comparison of the effects of BAS and CRC withdrawal was made by calculating the difference between the FGF19 and C4 on visit 5 and visit 4 for the placebo and CRC groups and visit 3 and visit 2

for the patients taking BAS. Overall there was a trend towards higher FGF19 and lower C4 on stopping BAS and the reverse on stopping either dose of CRC. There was a 145pg/ng mean decrease in FGF19 on withdrawal of 250mg CRC that was not statistically significant compared to placebo (mean change 0.36.5pg/mL, p=0.7), but there was a significant increase in FGF19 on stopping BAS (50.24pg/mL) compared to placebo and 250mg CRC. There were no significant differences between the 2 CRC arms and placebo in either FGF19 or C4 (figure 5.23(A)).

Serum C4 decreased on withdrawal of BAS, but increased on withdrawal of CRC (mean change, placebo 34.8, 250mg CRC 31, 1g CRC 20.3, BAS -50.5) These changes in C4 were statistically significant for all treatment arms when compared to BAS (figure 5.23(B)).

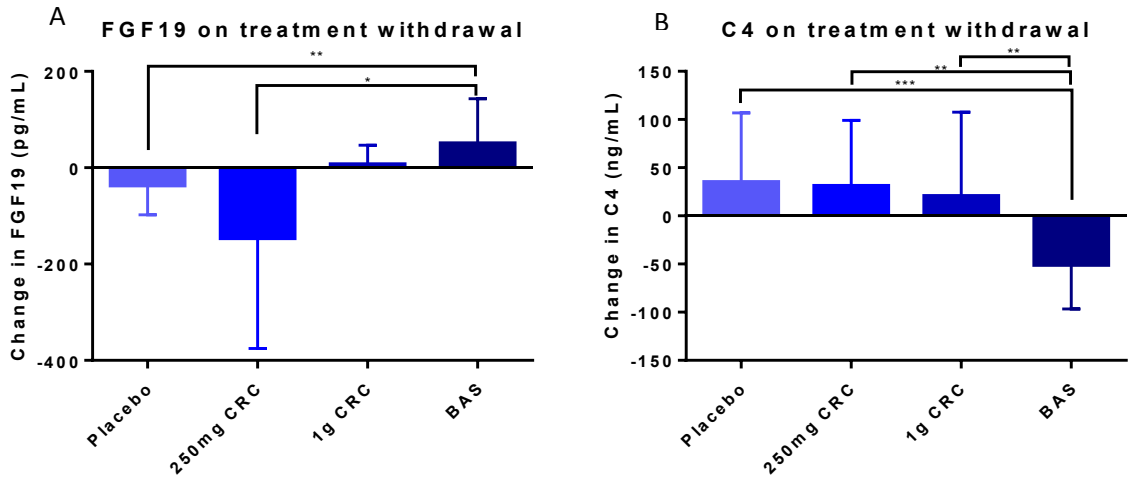


*Figure 5.22: Mean change in FGF19 (A) and C4 (B) on withdrawal of treatment. *p<0.05 **p<0.01 ***p<0.005*

There were no significant changes in FGF19 or C4 on starting either dose of CRC compared to baseline on visit 3. There were significant differences in both FGF19 and C4 on treatment when both doses of CRC were combined compared to visit 1 and visit 2, with a trend towards higher FGF19 and lower C4 on CRC treatment (figure 5.24).

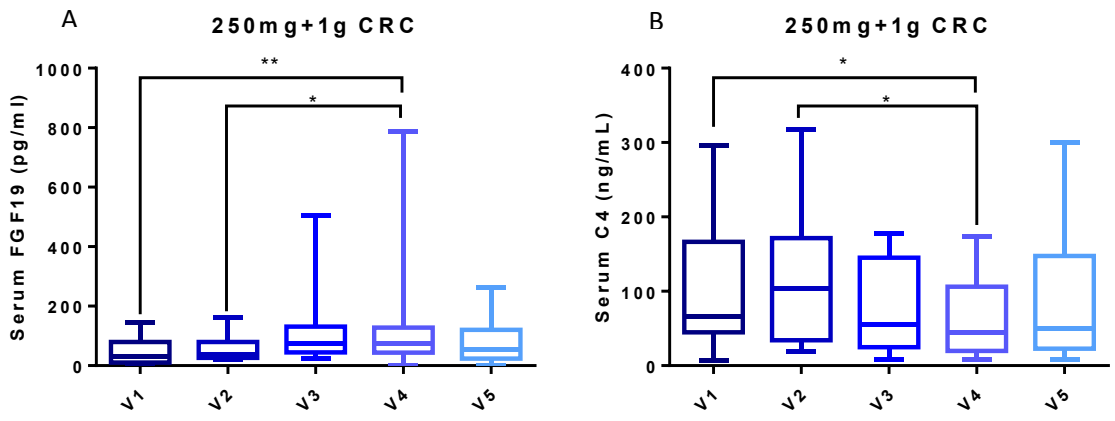


*Figure 5.23: Box and whisker plot (median, IQR, 95%CI) of serum FGF19 (A) and C4 (B) in patients taking either 250mg or 1g CRC by visit. *p<0.05 **p<0.01.*

5.2.2.5.2 Effect of BAS and CRC withdrawal on BAs

5.2.2.5.2.1 Total BAs

Total BAs were calculated by adding together the separate serum BAs and their glycine and taurine conjugates. There was a significant increase in total BAs on BAS withdrawal from visit 1 (median 562 ng/L 95%CI 142-1720) to visit 3 (median 791, 95%CI 465-2086) with a paired Wilcoxon test of p=0.001), however the change at week 3 was not statistically significant when compared to visit 2, when BAS use was being recorded (median 594, 95%CI 595-1974) p=0.14. There were no other significant changes in the treatment groups (figure 5.25).

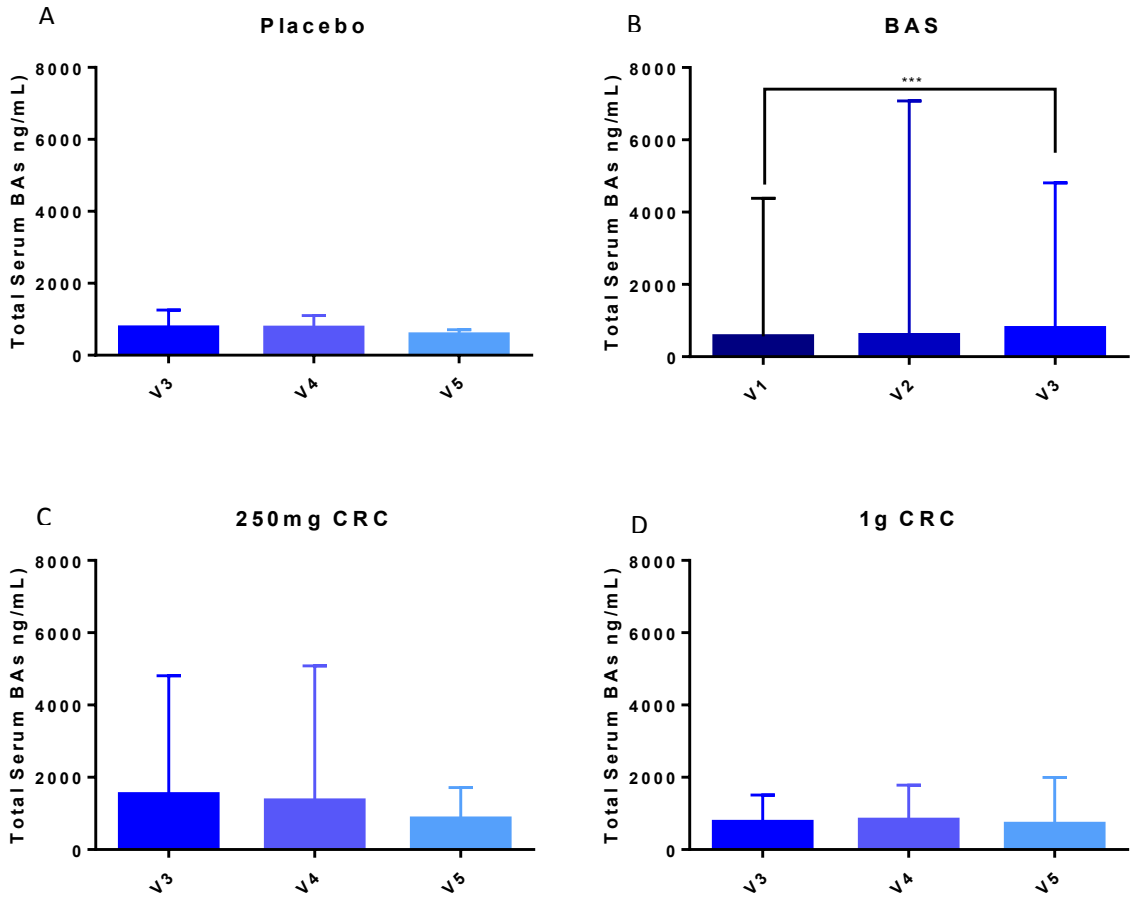


Figure 5.24: Median (+range) serum total BAs by visit for placebo (A), BAS (B), 250mg CRC (C) and 1g CRC (D).

5.2.2.5.2.2 Primary and Secondary BAs

There was a significant increase in total primary BAs (unconjugated) in the BAS treatment group from visit 1 and visit 3, but similar changes were not seen in other groups. There were no significant differences in serum unconjugated secondary BAS (figure 5.26 and 5.27).

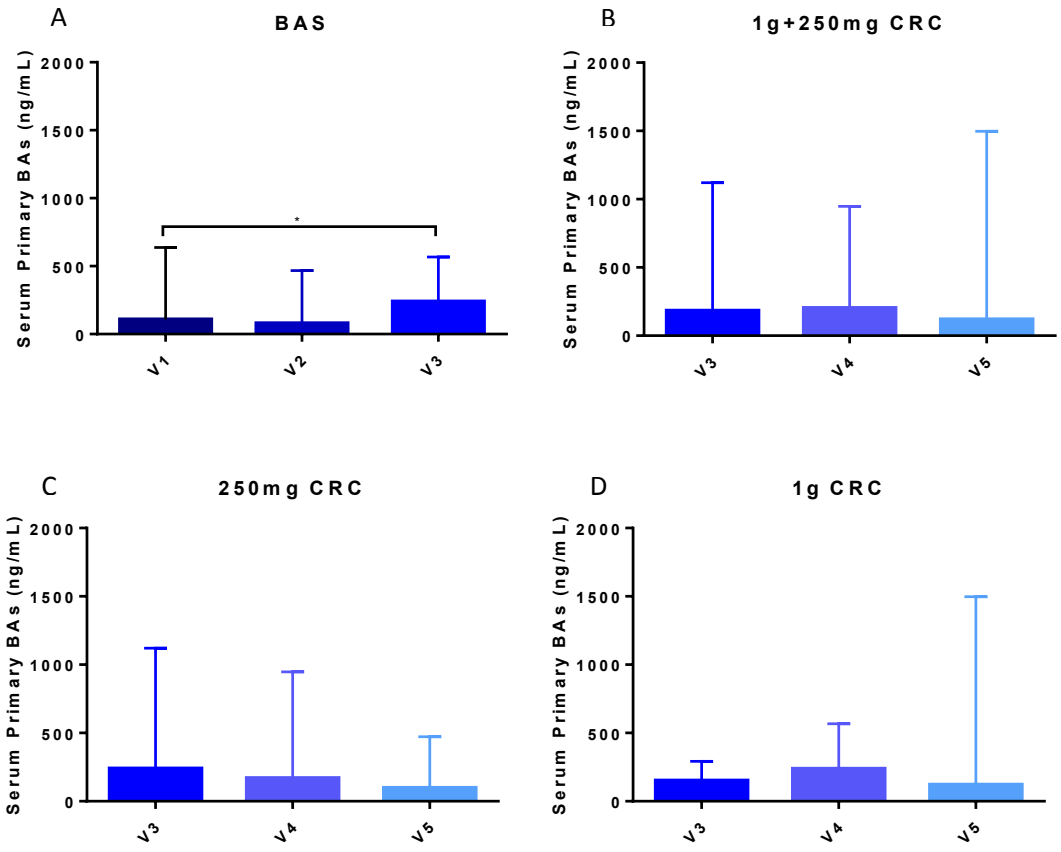


*Figure 5.25: Median (+range) serum primary BAs by visit for BAS (A), 1g+250mg CRC (B), 250mg CRC (C) and 1g CRC (D). *p<0.05.*

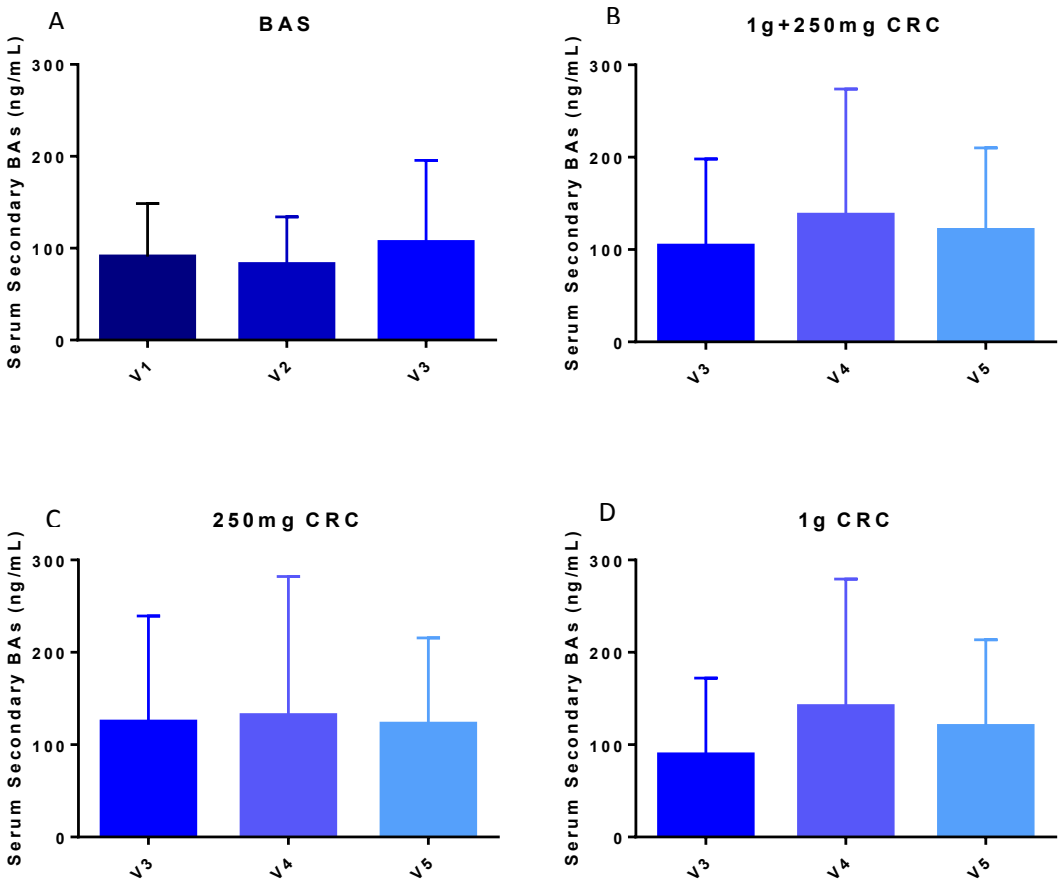


Figure 5.26: Median (range) serum secondary BAs by visit for BAS (A), 250mg +1g CRC (B), 250mg CRC (C) and 1g CRC (D).

5.2.2.5.2.3 Conjugated and unconjugated BAs

Within the CRC treatment groups there was a trend towards lower conjugated BAs on withdrawal that was the reverse of the trend observed on BAS withdrawal. Only with the CRC treatment groups combined was there near significant change from V4 to V5 (Median 513, [95%CI 168-1716] vs 448 [265-732], p=0.052).

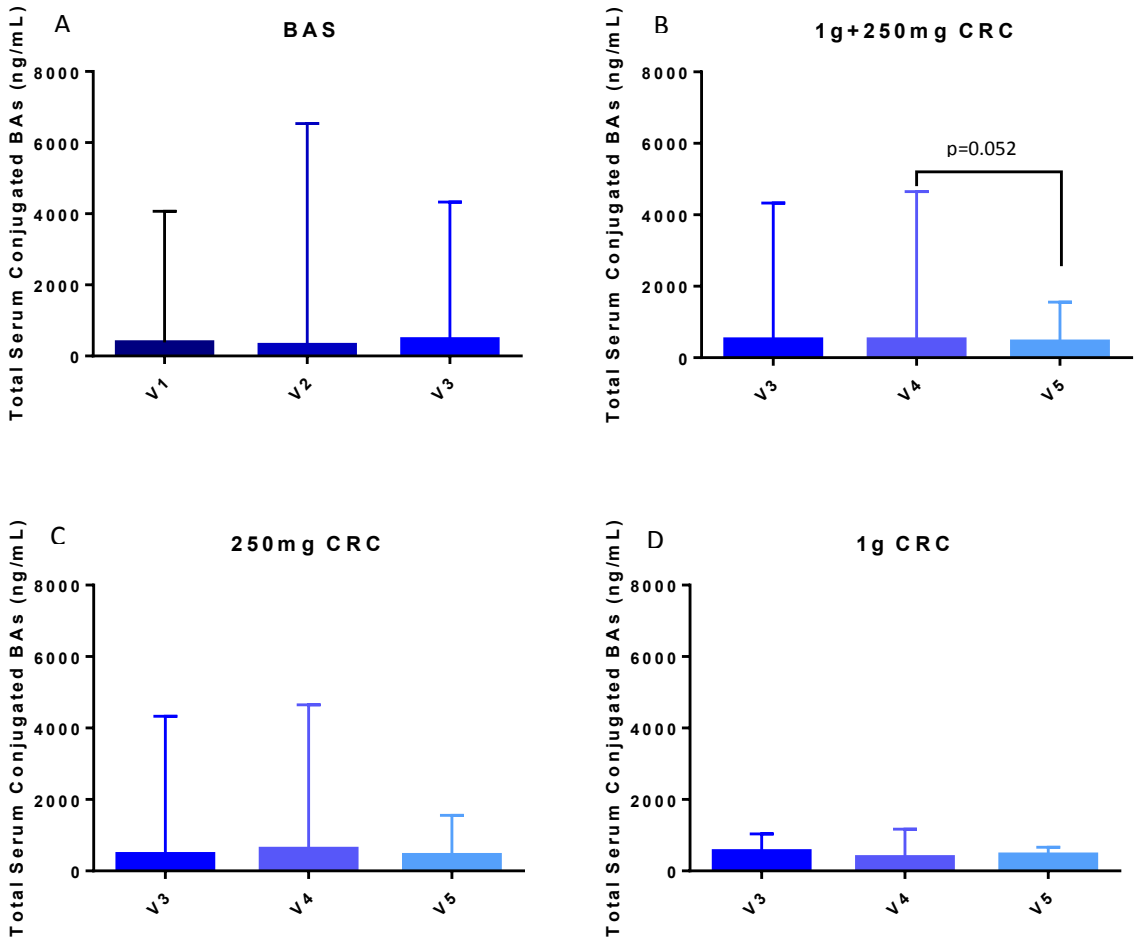


Figure 5.27: Median (+range) serum conjugated BAs by visit for BAS (A), 1g +250mg CRC (B), 250mg CRC (C) and 1g CRC (D).

Overall there were trends towards higher unconjugated BAs on treatment with BAS or CRC than off treatment. Only in the 1g CRC group was this significant (median (95%CI) visit 3; 270ng/mL (105-608), visit 4; 389 (201-712), p=0.039). This increase was partially reversed on treatment withdrawal, but not to a statistically significant extent. (visit 5 median, 306, 95%CI 113-855).

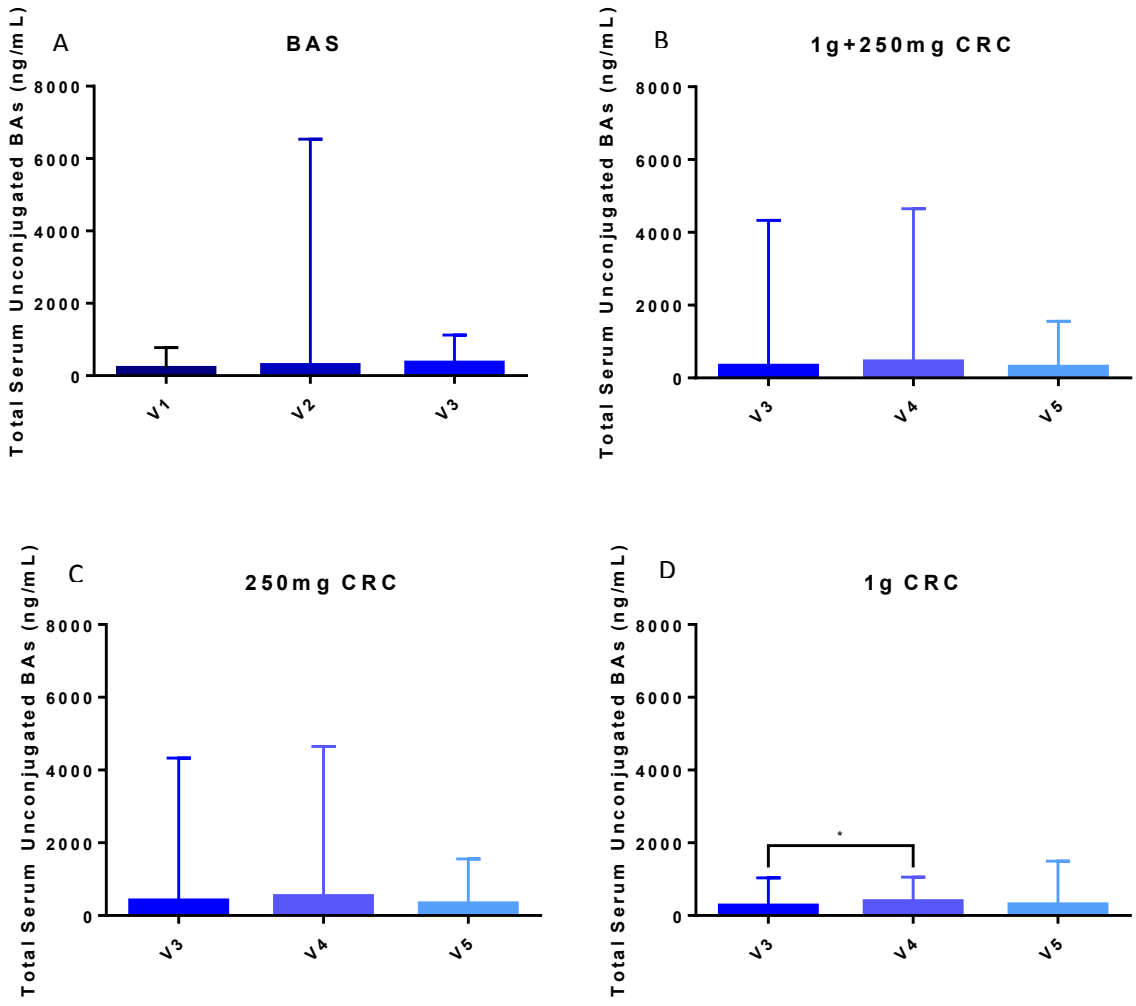


*Figure 5.28: Median (range) serum unconjugated BAs by visit for BAS (A), 1g +250mg CRC (B), 250mg CRC (C), 1g CRC (D). *p<0.05.*

5.3 Conclusions: Effects of conventional and colonic release cholestyramine

We have shown for the first time that administration of conventional BAS reduces FGF19 by 28% in patients with bile acid diarrhoea. This decrease is not seen when administering BAS in a colonic release capsule in BAD patients. We suspect that the reported 28% reduction in FGF19 is an underestimate, since we also report a 58% decrease in FGF19 in healthy volunteers taking conventional cholestyramine, although this possibly reflects a difference in baseline FGF19 between HV and BAD patients. More convincingly, the functional marker of FGF19, C4, increased in healthy volunteers taking BAS (410%) and in patients (58%). This the 18 fold increase in C4 reported by *Lundasen et al.[108]* in healthy volunteers and 2.9 fold increase reported by *Camilleri et al.[219]* in patients with IBS-D.

Serum C4 exhibits the same diurnal variation as serum FGF19 and values may vary within the same patient by up to 4 fold from baseline depending on timing.[120] This may explain some of the changes seen within the placebo groups, and the large ranges reported in the other arms. Despite the natural variances, and small number of patients, significant differences in C4 have been found between the treatment groups. Intact delivery of the A3384 capsule to the colon was not assessed in this study, but the lack of decrease in FGF19 in the A3384 arms, suggests that capsule behaved as designed. There is a potential for type II error in this comparison since the sample sizes for the A3384 and placebo arms are smaller than those of the BAS group (n=6-7 vs. n=13 respectively).

Serum total bile acids increased when conventional BAs were stopped. This is probably due to increased faecal losses while on BAS causing depletion of the BA pool. In this context the decrease in FGF19 and increase in C4 are appropriate physiological responses to restore BA homeostasis. The added benefit of increased cholesterol excretion in the form of BAs is this used therapeutically. But the effects of long-term FGF19 suppression are not known. The predicted effects outside BA metabolism are not desirable such as insulin resistance and white adipose tissue deposition.

Increasingly, FGF19 is understood to act on the central nervous system, which in-turn alter glucose metabolism and maintain lean mass though adrenergic pathways.[220]

The A3384 in BAD study was stopped early and was underpowered to detect the predicted 40% reduction in daily BMs. However, there was a trend towards a reduction in daily BMs on both doses of A3384, with 71% of patients on 1g BD A3384 reporting a reduction in daily BMs, compared to 50% on placebo. This placebo response rate is higher than that typically reported in IBS trials (20-40%).[221] We can only speculate for the reason for the high placebo response rate and possibly if the trial had been fully recruited, the response rate would have fallen. BM reporting during the baseline period may have been artificially high as patients were aware that that there was a minimum criteria to reach randomisation, although they were not told what this criteria was. Some placebo compounds are also active bile acid binders, such as hydroxypropyl cellulose, however the placebo we have used, is not thought to have this effect.[222]

The trend towards lower number of daily BMs with A3384 is further supported by a near-significant reduction in BSFS on treatment compared to placebo (-1.1 point reduction in BSFS, p=0.06) and significant decreases in daily diarrhoeal symptoms recorded by Likert scale. This discrepancy between objective BMs frequency recording and subjective diarrhoea severity recorded may be accounted for by a decrease in urgency, which was not specifically recorded in this trial, but is frequently quoted by patients as a significant contributor to chronic diarrhoea's impact on quality of life.[223]

Conventional BAS are poorly tolerated in around 40% of patients.[224] This is partly because cholestyramine is administered as a gritty powder, but also because all BAS can cause upper abdominal symptoms. A colonic release formulation of BAS will circumvent both these problems. Bile acids exert effects on small bowel motility through the TGR5 receptor, binding of BA in the upper GI tract may cause slowing of small bowel motility and colesevelam has been shown to slow gastric empting times.[225, 226] Colonic release BAS should avoid these effects, so it is of interest that there

was a small, non-significant decrease in reported daily pain and bloating, where we may have expected an increase with conventional BAS.

We have shown that conventional bile acid sequestrants may have detrimental effects on bile acid metabolism, and that these effects are not seen with a colonic release preparation of cholestyramine (A3384). A3384 was well tolerated and was efficacious in reducing diarrhoea. Further development of A3384 and other colonic release formulations of cholestyramine is warranted with the aim to find a novel treatment for bile acid diarrhoea restoring the physiological bile acid regulation.

6 Discussion

6.1 Answer to the hypothesis

The hypothesis presented at the beginning of this thesis was:

'Low FGF19 and, by association, primary bile acid diarrhoea may be contributory to several metabolic diseases such as hypertriglyceridaemia, reduced insulin sensitivity, non-alcoholic fatty liver disease, gallstones and overweight. These conditions together with primary bile acid diarrhoea will comprise a 'metabolic syndrome of low FGF19'. Low serum FGF19 and the rs12256835 DIET1 polymorphism will be more prevalent in these disease populations.'

'FGF19 expression can be modulated in ileal explants using novel compounds that have already been proven to be safe in humans. Colonic release cholestyramine will have beneficial effects patients with diarrhoea and cause less reduction in serum FGF19 than currently available formulations.'

In order to answer this I will discuss each sentence of the hypothesis in turn.

6.1.1 Low FGF19 and, by association, primary bile acid diarrhoea may be contributory to several metabolic diseases such as hypertriglyceridaemia, reduced insulin sensitivity, non-alcoholic fatty liver disease, gallstones and overweight. These conditions together with primary bile acid diarrhoea will comprise a 'metabolic syndrome of low FGF19'.

Not proven. Chapter 3 has provided significant proof that bile acid diarrhoea and in particular the primary form of BAD is associated with hypertriglyceridaemia, NAFLD and gallstones. The association with reduced insulin sensitivity was not tested and the link with obesity was already proven. Despite the proven disease associations with BAD as defined by SeHCAT value <15%, the link with low FGF19 could not be proven, despite strong correlation between SeHCAT retention and serum FGF19. There are suggestions that FGF19 is of importance in NAFLD, such as the significant negative correlation of FGF19 with ALT, but in order to detect hard endpoints such as fibrosis, our sample size was too small.

One explanation for the associations with BAD but not with FGF19 is the heterogeneity of FGF19 responses in patients with SeHCAT <15%. Since only 47% of these patients can be expected to have a low ileal FGF19 response, only 47% of these patients can be expected to have the proposed metabolic syndrome of low FGF19. The only way to prove this hypothesis is a prospective population study with serum FGF19, fasting glucose, lipid profile, liver function tests, liver ultrasound and medical history. It is interesting that high C4 was associated with higher NAFLD fibrosis score and this may indicate that the C4 is more reliable indicator of BA dysmetabolism, which warrants further investigation. There is now convincing data that low FGF19 is associated with lower serum triglycerides, but low SeHCAT retention is associated with hypertriglyceridaemia.[121] This is a clear example of how the phenotypes of the low FGF19 and low SeHCAT cohort differ, and disproves the hypertriglyceridaemia part of the hypothesis.

An alternative wording of the hypothesis that could be proven and presented as statement using this data is; '*There is a syndrome of altered bile acid metabolism comprising of primary bile acid diarrhoea, hypertriglyceridaemia, NAFLD and gallstones.*'

6.1.2 Low serum FGF19 and the rs12256835 DIET1 polymorphism will be more prevalent in these disease populations.

Not proven. We have proven that the rs12256835 Diet1 polymorphism is associated with low FGF19 in humans, but not that it is associated with conditions hypothesised to be associated with FGF19. This may be because the hypothesis that these conditions are associated with low FGF19 is flawed, since it has not been proven in the previous section.

We can only report on the prevalence of this polymorphism in NAFLD and hypertriglyceridaemia as the other conditions were not investigated. An increased prevalence of the G allele has already been described in patients with SeHCAT retention <15%.[Nolan J, unpublished data] We detected a MAF of 0.43 in NALFD, which is higher than that reported in the 100 genomes project (0.31), so an increased prevalence is possible, but without a direct comparison to a local control population without NAFLD we can't say the increased prevalence is proven.

The association between the Diet1 polymorphism and lower serum triglycerides is proven by this data, and together with the association with low FGF19 matches with Ian Johnston's findings. This may represent as distinct phenotype of patients with BAD with low FGF19 (possibly with low postprandial FGF19) and low triglycerides.

It is tempting to postulate that patients with reduced ileal FGF19 transcription in response to CDCA stimulation (section 4.2.1.2) are the same low-low serum FGF19 phenotype that Ian Johnston has reported and these patients will carry the minor G allele, but that requires further investigation.

A rewording of the hypothesis that can be proven by this data and that of the studies before this by Jonathan Nolan is: *The rs12256835 Diet1 polymorphism is associated with low SeHCAT, lower serum FGF19 and triglycerides.'*

6.1.3 FGF19 expression can be modulated in ileal explants using novel compounds that have already been proven to be safe in humans.

Proven. Although resveratrol did not affect ileal FGF19 transcription, Cafestol and Ursodeoxycholic acid did have significant increases of 2.4 and 3.8 fold respectively. Since these 2 compounds are widely available for human consumption, their therapeutic use could be tested relatively easily in a phase II trial, however, their actions in vivo and in the presence of other BAs requires further investigation. It is unlikely that the small fold increases in FGF19 seen with these 2 compounds will produce the level of suppression of CYP7A1 required to affect bile acid production and diarrhoea; when it is considered that the fold change in ileal FGF19 with OCA at equivalent doses is >1000, that produces a doubling in serum FGF19.[21, 227]

The finding that cafestol in the presence of CDCA reduces ileal FGF19 reinforces this view. Although this trend was technically non-significant ($p=0.06$), I believe this effect of competitive inhibition to be true and puts the findings that UCDA increases FGF19 in the presence of CDCA into contrast. It remains to be seen if the additive effect of UCDA can be exploited clinically, but these findings clarify previously conflicting studies (summarised in section 4.3.2.3).

This previously under-recognised property of UCDA calls into question the currently accepted theory that UCDA works in PBC by replacing the BA pool with a less fibrogenic BAs. If this was true then UCDA, which does not cause diarrhoea could possibly be used effectively in BAD. An alternate hypothesis to the mechanism of UDCA in PBC is by up-regulation of the FXR-FGF19 axis. Further work on this and role of IBABP should be a priority.

6.1.4 Colonic release cholestyramine will have beneficial effects patients with diarrhoea and cause less reduction in serum FGF19 than currently available formulations.

Proven. The evidence for this, due to the early closure of the trial is questionable, but I think on balance the hypothesis can be proven with the evidence we have. There were non-significant trends towards less bowel motions per day on CRC and although the primary endpoint was not reached, there were significant benefits in subjective diarrhoea, and near-significant improvement in BSFS (p=0.05) on treatment. Had the trial reached its recruitment target I think there was a reasonable chance that the primary endpoint would have been met.

Although we can't conclude that CRC does not affect serum FGF19 compared to placebo, we can say confidently that CRC affects FGF19 less than conventional bile acid sequestrants, which are the currently available formulations.

A phase III trial of CRC against conventional BAS is warranted from these findings.

6.2 Future work

6.2.1 Characterisation of pBAD

The heterogeneity of patients with diarrhoea and a SeHCAT<15% is only starting to be appreciated. Work in this thesis proves that there are multiple disease associations, but possibly that they don't occur in the same patients. Together with Ian Johnston's work on postprandial FGF19 response and serum triglycerides the possibility arises that different phenotypes of bile acid diarrhoea and associated conditions can be described using these blood tests as discriminators.

In the table below (table 6.1) I have attempted to organise how these phenotypes may be characterised using the known markers that may form part of a hypothesis for further study. I have also included the known genetic polymorphisms in BAD or IBS-D, the likely disease associations based upon the likely pathophysiology and a possible tailored treatment. This table is by no means exhaustive, but is meant to give an appreciation of how BAD may be characterised in the future. In particular, it is likely that multiple genetic polymorphisms will result in the same phenotype. Some of the proposed phenotypes will be quite rare, since around half will have the low-low FGF19 response, characterising the low-high and high-high responders will require a large number of patients, the FXR rs61755050 variant for example has a MAF of 0.0016.[1000 genomes project]

There are still a lot of unknowns in the this field, some of which will be discussed later in this chapter, but the contribution of transcription factors to FXR signalling is likely play a role, as is the largely ignored BA receptor TGR5 which a multitude of functions outside the gastrointestinal tract, such as monocyte differentiation.

The reader may notice that conventional bile acid sequestrants aren't featured in the table. As it is a prediction of the future, I predict that these will be largely superseded by colonic release cholestyramine due to the benefits described in the thesis.

FGF19 Postprandial response	Serum Triglycerides	Pathophysiology	SNP	Disease Associations	Treatment
Low-low	Low	Decreased ileal FGF19 transcription	Diet 1 rs12256835	NALFD Obesity IR	OCA / M70
	High	Increased hepatic receptor affinity	FGFR4 rs376618	NALFD Obesity Gallstones	CRC
Low-high	Low	Decreased ileal FGF19 transcription	FXR rs61755050	NAFLD Obesity	OCA
	High	Dysbiosis	None	Gallstones	CRC / ABX
High-high	Low	Hepatic receptor resistance	Klothoβ rs1015450	NAFLD Obesity IR	OCA / M70
	High	Increased colonic sensitivity to BA	TGR5 rs11554825	?monocyte dysfunction	CRC

Table 6.1: Hypothetical BAD phenotypes characterised by FGF19 postprandial response and serum triglycerides. IR= insulin resistance, OCA=obeticholic acid, CRC=colonic release cholestyramine, ABX=antibiotics, M70=synthetic FGF19.

6.2.2 Variation in FXR signalling (co-factors)

Studies within this thesis have shown for the first time the relationship between low FGF19 and the Diet1 polymorphism in humans. How Diet1 causes low FGF19 remains unknown. Other studies looking for the genetic polymorphisms in primary BAD have only found plausible polymorphisms in small proportions of patients in genes critical to the FXR-FGF19 axis implying that up or down stream modulators may have a role in the pathogenesis. IBABP is one such example. Before the discovery of FGF19, IBPAP was considered to be the most important transcription target of FXR. Now, its importance in BA stimulation of FXR and subsequent FGF19 transcription is only just being understood, highlighted by our findings of the additive effect of UCDA on FGF19. This raises the question of whether the reduced IBABP transcription we found in patients with low SeHCAT is the product of a global reduction in FXR mediated transcription or the cause of it? On current evidence it is impossible to say and it requires further work to delineate this.

Other co-factors for both FXR and FGFR4 are known, and all require further investigation to discover their effects of BA metabolism and to discover potential polymorphisms that may have importance in pBAD. Although within this thesis we have failed to prove a link between SIRT1 and FXR activation, the possibility of a link remains of interest. Obesity is a known risk factor of BAD and, so far, has been assumed to be metabolic consequence of low FGF19. But endoplasmic reticulum stress causes inhibition of FXR, probably through activation of activating transcription factor 4 (ATF4) and SIRT1.[228] The most common cause of ER stress is obesity, so this raises the intriguing question of whether low FGF19 and BAD can be secondary to obesity in at least some patients.

6.2.3 Differences in FXR signalling in hepatocytes and ileum in health and disease

As the evidence for FXR agonists in liver disease increases, one critical question remains unanswered: Are the benefits derived from ileal FGF19 release or from direct effects on FXR in the liver? FXR agonism with CDCA has been shown to prevent apoptosis in autoimmune hepatitis in mice but as in many other hepatology studies, end product cytokines have been measured, but the levels of FGF15/19 either intrahepatically or peripherally have not. This means that the relative contribution of ileal or hepatic FXR agonism to hepatoprotection are unknown. Equally, how FXR exerts its protective effects in the liver is unknown and can't be assumed to be due to FGF19 transcription alone.[229]

It quite possible that both ileal and hepatic FXR pathways are important, and contribute differing amounts to the therapeutic effects depending on the disease. For example, in PBC where hepatic FXR and FGFR4 are upregulated, there is likely to be greater effect on the hepatocyte when a FXR agonist is administered, but in NALFLD, the benefits of the same drug may be due to peripheral FGF19 release and improvement of metabolic risk factors.

Additionally there is confusion about whether FXR agonism or antagonism is of benefit in NAFLD. Intestine specific FXR knockout mice were protected from hepatic triglyceride accumulation, through a novel mechanism of lower ceramide levels suppressing SREBP-1c mediated de novo fatty acid synthesis in the liver.[230] In the same study they showed beneficial effects of increased Tauro-β-muricholic acid in wild-type mice, which was achieved through microbiome manipulation with antibiotics. Tauro-β-muricholic acid is thought to act a partial FXR agonist, much like cafestol in our experiments. An accompanying editorial rightly pointed out that that significant differences in mice and human exist, particularly that murichoic acid is not found in humans.[231] Clearly these effects need be to be better understood before OCA can be truly considered a treatment for NAFLD.

The upregulation of FXR and the hypothesised paracrine action of FGF19 in PBC hepatocytes is just one example of how the hepatic actions of FXR are different from the ileum and in disease. An

explant study of hepatic biopsies incubated with FXR agonists, along the lines of our ileal explant series may reveal some of these differences in signalling pathways.

6.2.4 Partial agonists.

The findings of *Jiang et al.* with tauro-β-muricholic acid imply that partial agonists maybe protective against NAFLD.[230] The search for bile acids with equivalent actions in humans would be logical next step. Within this thesis, 2 partial agonists, one endogenous; LCA and one xenobiotic (cafestol) have been shown to reduce FGF19 stimulation in the presence of CDCA. It is likely that other endogenous BAs have similar effects and requires investigation. The therapeutic use of these would be preferable to LCA which is cytotoxic. Understanding the effects of the microbiome in changing the proportion of BAs that are FXR agonists and partial agonists may lead to targeted microbiome therapies for NAFLD and some patients with BAD. The administration of cafestol may benefit those patients in whom there is not a dysbiosis, but the selection of those patients who will benefit from either of these treatments or a FXR agonist needs to be studied.

6.2.5 Tumourgenicity of FXR agonists

FGF19 is a growth factor and induces the expression of the pro-tumorigenic connective tissue growth factor (CTGF) in hepatoma cells.[232] Small molecule inhibitors of FGFR4 are in development for use in HCC.[233] There is no evidence for increased tumour genesis with OCA in humans in either preclinical studies or the clinical trials in PBC, but it remains a concern and the risks of FXR agonists will have to be fully understood with long-term human data before they can be widely used in a benign disease such as BAD.

6.2.6 TGR5

This thesis has necessarily concentrated on the role of FXR, but the contribution of the other BA receptor, TGR5 in BAD and its associated diseases is yet to be studied. Increased BA delivery to the colon resulting in GLP-1 release through TGR5 has been postulated to be the mechanism behind the improvement in diabetes seen after Roux-en-Y gastric bypass. TGR5 agonists have been shown to increase GLP-1 and PYY release, but their effects on bowel function has not been studied.[234] TGR5 polymorphisms are associated with faster small bowel transit, but not with increased faecal BAs.[93] It is possible that TGR5 is important in the pathogenesis of a subset of IBS, possibly with increased BA sensitivity.

Since TGR5 is a G-protein coupled receptor, its functions can't measured using the molecular biology techniques used within this thesis, although pilot studies measuring GLP-1 in our explant system are currently underway. As it is predominately situated basolaterally, apical BA transport and IBPAP binding are likely to have effects on TGR5, just as they do on FXR. To confuse the matter further, simulation of FXR lowers GLP-1 and administration of colesevelam to mice increased GLP-1, suggesting that the effect of FXR on GLP-1 predominates.[235]

6.2.7 The microbiome FXR / TGR5 switch

The primary BAs have high affinity for FXR, the secondary BAs for TGR5. Since the luminal microbiome is responsible converting primary BAs to secondary BAs, it is plausible that this is one mechanism by which the microbiome affects bowel function. The proposition that prebiotics, probiotics or faecal transplant can create a switch to predominately FXR simulation to TGR5 is complicated by the range of other bacterial modifications that can occur such as deconjugation and desulphation. However both a prebiotic (inulin) and a probiotic have been shown to alter the faecal BA profile in mice.[236] So far no-one has tried to target these effects towards secondary BA

creation, or visa-versa. It is possible that the microbial enzymes involved in BA metabolism could be targeted directly by oral small molecule inhibitors.

6.2.8 OCA dose reduction using UCDA

In a phase 3 trial of OCA in PBC, 80% of patients reported pruritus as an adverse event. In 13% this was so severe that treatment was discontinued.[218] At the lower dose of 10mg, 47% of patients reported pruritus, which was no different from placebo. Although the median serum FGF19 of the 3 treatment groups (10,25,50mg) are not quoted in the paper, from the box and whisker plot of median FG19 values, the increase in FGF19 from 10mg to 25mg does not look larger than the 70-145% increase we achieved with UCDA supplementation in our ileal explants. Therefore administration of UCDA to the 13% of patients with intractable pruritus on OCA may allow reduction to 10mg with preservation of the FGF19 response. The increased proportion of UCDA within the BA pool should reduce pruritus through competitive inhibition of peripheral TGR5, the proposed mechanism of its symptomatic benefit in cholestatic pruritus, creating a dual action. Since the PBC study was performed in UCDA unresponsive patients at high risk of liver transplant, the ability to be able to restart OCA in the 13% would be of great prognostic benefit in this cohort of patients.

6.2.9 OCA in BAD

A daily, well tolerated treatment remains the holy-grail in the treatment of BAD. Within this thesis I have proven that several metabolic conditions are associated with BAD and BAS treatment may exacerbate FGF19 deficiency. Therefore a direct FGF19 agonist remains attractive. The encouraging results from the pilot trail of OCA in BAD have not yet led to a phase II trial, although studies are planned. Since the cost of OCA is likely to be prohibitive for chronic, benign conditions for the foreseeable future, the short term use of it as diagnostic test for BAD may be more acceptable. This would be of considerable use in countries where SeHCAT is not available.

6.2.10 Colonic release BAS

Cholestyramine has been used for over 30 years without any safety concerns. Had the trial of A3384 reached completion it could possibly have been licenced for the treatment of BAD. The appeal of this low-cost and proven treatment in BAD is undeniable, and the multiple benefits of colonic release coatings in tolerability and preservation of ileal FGF19 production should mean that CRC will become the treatment of choice for BAD in the future. However we need a placebo-controlled trial that clearly states symptomatic benefit, unfortunately this will require a similar trial to one performed to reach its treatment targets.

7 References

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Appendix 1. Laboratory Protocols

A1.1. Collection of FGF19/C4 serum samples

Procedure

1. Ensure the patient has been fasted for at least 6 hours
2. Each sample will be collected into a 6 mL K2 EDTA tube or yellow tube serum tube.
3. If time to centrifuge will be >20 minutes, place on ice
4. Samples will be inverted 4 times and then centrifuged at 2000 g for 10 min at 4^{o}C
5. Transfer plasma into 4 labelled (study code, patient id., date of collection, visit number) polypropylene tubes. All four tubes should contain at least 0.25 mL plasma.
6. Samples to be processed and frozen within 120 min of collection.
7. Samples to be stored at -80 ˚C

A1.2. DNA Purification from Blood

Important points before starting

- All centrifugation steps are carried out at room temperature (15–25°C).
- 200 µl of whole blood yields 3–12 µg of DNA.

Things to do before starting

- Equilibrate samples to room temperature (15–25°C).
- Heat a water bath or heating block to 56°C for use in step 4.
- Equilibrate Buffer AE or distilled water to room temperature for elution in step 11.
- Ensure that Buffer AW1, Buffer AW2, and QIAGEN Protease have been prepared according to the instructions:
    - **QIAGEN Protease stock solution (store at 2–8°C or –20°C)**
        - QIAamp DNA Blood Mini Kit (50), pipet 1.2 ml protease solvent* into the vial containing lyophilized QIAGEN Protease, as indicated on the label.
        - QIAamp DNA Blood Mini Kit (250), pipet 5.5 ml protease solvent into the vial containing lyophilized QIAGEN Protease, as indicated on the label.
        - Dissolved QIAGEN Protease is stable for up to 2 months when stored at 2–8°C. Storage at –20°C is recommended to prolong the life of QIAGEN Protease, but repeated freezing and thawing should be avoided. For this reason, storage of aliquots recommended.
    - **Buffer AL† (store at room temperature, 15–25°C)** Mix Buffer AL thoroughly by shaking before use. Buffer AL is stable for 1 year when stored at room temperature. If a precipitate has formed in Buffer AL, dissolve by incubating at 56°C.
    - **Buffer AW1† (store at room temperature, 15–25°C)** Buffer AW1 is supplied as a concentrate. Before using for the first time, add the appropriate amount of ethanol (96–100%) as

indicated on the bottle. Buffer AW1 is stable for 1 year when stored closed at room temperature.

- **Buffer AW2* (store at room temperature, 15–25°C)** Buffer AW2 is supplied as a concentrate. Before using for the first time, add the appropriate amount of ethanol (96–100%) to Buffer AW2 concentrate as indicated on the bottle.

Procedure

1. Pipet 20 µl QIAGEN Protease (or proteinase K) into the bottom of a 1.5 ml microcentrifuge tube.
2. Add 200 µl sample to the microcentrifuge tube. Use up to 200 µl whole blood, plasma, serum, buffy coat, or body fluids, or up to 5 x 106 lymphocytes in 200 µl PBS.
3. Add 200 µl Buffer AL to the sample.
4. Mix by pulse-vortexing for 15 s.
5. Incubate at 56°C for 10 min.
6. Briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from the inside of the lid.
7. Add 200 µl ethanol (96–100%) to the sample
8. mix again by pulse-vortexing for 15 s.
9. Briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from the inside of the lid.
7. Carefully apply the mixture to the QIAamp Mini spin column (in a 2 ml collection tube) without wetting the rim. Close the cap
8. Centrifuge at 6000 x *g* (8000 rpm) for 1 min.
9. Place the QIAamp Mini spin column in a clean 2 ml collection tube (provided), and discard the tube containing the filtrate. If the lysate has not completely passed through the column after centrifugation, centrifuge again at higher speed until the QIAamp Mini spin column is empty.
10. Carefully open the QIAamp Mini spin column and add 500 µl Buffer AW1 without

wetting the rim. Close the cap

11. Centrifuge at 6000 x *g* (8000 rpm) for 1 min.

12. Place the QIAamp Mini spin column in a clean 2 ml collection tube (provided), and discard the collection tube containing the filtrate.*

13. Carefully open the QIAamp Mini spin column and add 500 µl Buffer AW2 without wetting the rim. Close the cap

14. Centrifuge at full speed (20,000 x *g*; 14,000 rpm) for 3 min. Label new 1.5ml microcentrifuge tubes while waiting

15. Optional: Place the QIAamp Mini spin column in a new 2 ml collection tube (not provided) and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min. This step helps to eliminate the chance of possible Buffer AW2 carryover.

16. Place the QIAamp Mini spin column in a clean 1.5 ml microcentrifuge tube (not provided), and discard the collection tube containing the filtrate.

17. Carefully open the QIAamp Mini spin column and add 200 µl Buffer AE or distilled water. Incubate at room temperature (15–25°C) for 1 min, and then centrifuge at 6000 x *g* (8000 rpm) for 1 min.

18. Use DNA immediately for PCR or freeze at -20ºC

A1.3. SNP Genotyping PCR

Before you start

1. You will need:
    a. TAQMAN genotyping mastermix (Life Technologies, Lutterworth, UK, Cat no: 4371355)
    b. Molecular grade H_2O
    c. 40x SNP TAQMAN Primers (Life Technologies, Lutterworth, UK)
    d. Accompanying SNP primer CD
2. Step up experiment on the Stepone machine,
    a. Select design new genotyping experiment
    b. Go through step as guided on the machine
    c. Insert the primer CD and save the details as a primer profile on the machine
    d. Change reaction amount to 15µl
    e. Save and send to machine

Protocol

1. Defrost and vortex DNA samples
2. Make primer MM (amounts per well):
    a. 7.5µl Mastermix
    b. 0.375µl 40x SNP primer
    c. 6.125µl molecular grade water
3. Add 14 µl of MM to each well
4. Add 1µl of sample to each well, samples do not need to be run in triplicate
5. Add I µl of control samples to wells
6. Cover with plate sealer

7. Briefly centrifuge
8. Insert in to StepOne machine (pull drawer out)
9. Press start experiment on the machine
10. Collect results with a memory stick.

A1.4. Ileal explant incubation

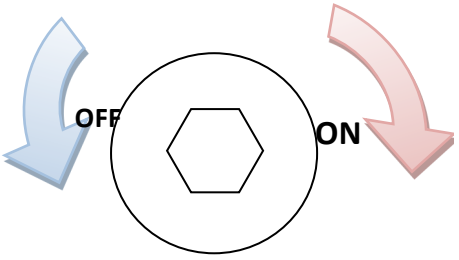
(protocol written by J. Zhang, updated by R. Appleby)

Before Bx collection

1. Get a foam box, put some ice in (from room next to the 'other' lab, opp lifts).
2. From the 6th floor lab -20 freezer, get one each per experiment of:
    a. 1 soybean trypsin inhibitor eppendorf
        i. Aliquot: 665mL of 1mg/mL
    b. 1 leupeptin eppendorf
        i. Aliquot: 665mL of 1mg/mL
    c. 1 Bile Acid /Agonist eppendorf
        i. Aliquot: Variable, usually 100µL of 10mM
        ii. (Optional): other compounds if needed, variable, usually 100µL of 10mM
    d. 1 falcon tube with 12 ml Complete media (CM) (DMEM +10% FCS)
        i. To make up 1 batch: 500ml DMEM + 2x25ml aliquots FCS + 1x10ml Aliquot PCS (all from freezer in opp. lab)). Aliquot in 12ml into 15ml falcon tubes
    e. Put all in the foam box with ice
3. Check that there are adequate pipette tips in the lab – if not, get some from the basement lab and bring up.
4. From cold room 6th floor CW, put DMEM into 25ml universal (found in 2nd drawer in cabinet in cell culture room)
5. If possibility of collecting colon samples for organoid study, put Leibovitz media into 25ml universal.
6. Cap and put media tubes on ice with the other eppendorfs.
7. Deliver DMEM falcon tubes and ice box to endoscopy.
8. Ensure enough consent forms (in green folder).

After Bx collection:

1. Label each DMEM+explant tube lid with experiment number matching consent form. Place DMEM tubes in ice box.
2. Bring ice box back to 6th floor CW lab. Begin defrosting CM, leupeptin, trypsin inhibitor in hand(s) on the way.
3. For each explant experiment:
    a. ***Prepare a 6-well culture plate, label lid.***
    b. ***Ensure CM is*** fully defrosted.
    c. Add 660ml each Trypsin Inhibitor, leupeptin into CM. Invert gently 7-8 times to mix.
    d. Using 1ml micropipette, pipette 1.9ml of complete media into each well (1ml + 0.9ml).
4. Pipette bile acid/agonist *into wells (10µl of 10mM = 50µM in 2ml well) – ensure this matches with what is labelled on the plate lid.*
5. *Finish defrosting all other eppendorfs. Vortex and ensure that no ice or crystals remain in solution.*
6. *Mix each well by pipetting (1mL tips). Change tips after each well.*
7. *Take out Bx, separate with narrow sucking pipettes to ensure there are 12 pieces, and place 2 in each well. Aim to have the same amount of tissue in each well.*
8. Place lid on plate.
9. Put into sealed container.
10. Gas with 95% O_2 for 30 seconds.
    a. For first use of the day you will need to turn O_2 on with spanner – see diagram, turn off with spanner at the end of the day



11. Spray hands and sealed container with alcohol spray.
12. Place into top shelf of top incubator.
13. Repeat steps 11-13 once every two hours for the next 6hrs.

After 6hrs:

1. For each experiment:
    a. Label 6 eppendorfs with experiment number and conditions, one for each well, as labelled on lid (e.g. Ex123 con, Ex123 C50…)
    b. Label 6 more eppendorfs as above. Pipette 100*µl RNAlater into each.*
2. Put Bx in appropriate RNAlater tubes using narrow sucking pipettes. Ensure Bx is adequately submerged in RNAlater.
3. Pipette all of the supernatant into appropriate eppendorf (approx 900-1000µl per eppendorf).
4. When all experiments are finished, remember to turn close off oxygen tank with the wrench.
5. Put RNAlater eppendorfs into a labelled grey plastic cartridge and place in cold room overnight.
6. Put eppendorfs with the supernatant in -80 freezer.

Next morning:

1. Move RNAlater tubes from cold room to -80 freezer.

A1.5. Explant RNA extraction (Written by J. Nolan, updated by R. Appleby)

This protocol requires:

- RNeasy Mini Kit (Qiagen)
- QIAshredders (Qiagen)
- RNase-free DNase Set (Qiagen cat no 79254).
- VWR Pellet Mixer (Fauzia's drawer)
- VWR pestles and 1.5ml microtubes (cat no: 431-0098)
- Standard microcentrifuge
- 96-100% ethanol
- RNaseZap
- Gloves
- Forceps
- Pipettes
- Sterile RNase-free pipette tips
- Sterile round-bottomed microcentrifuge tubes

SAFETY INFO: BUFFER RLT & BUFFER RW1 ARE NOT COMPATIBLE WITH BLEACH.

Before extraction

1. Make up the buffers as below, prepare in 15ml Falcon tubes, for each sample you will need (scale up as necessary, with extra):
    a. Buffer RLT 600µL
    b. Buffer RW1 700µL
    c. Buffer RPE 1000µL
    d. 70% Ethanol 600µL
    e. 100% ethanol (for cleaning forceps) 10mL

f. RNaseZAP (for cleaning forceps) 10mL

2. Add 4 volumes of ethanol (96-100%) to Buffer RPE as indicated on bottle.
3. Prepare DNase I stock solution using a RNase-free DNase Set:
    a. Open the DNase I vial very carefully, makesure not to lose any from the lid.
    b. Inject 550µl of the RNase-free water provided into the vial using a pipette, replace lid
    c. Do NOT vortex. Mix gently by inverting the vial.
    d. Remove the stock solution from the vial and divide into six-sample-use aliquots of 66µl each. Store at -20°C for up to 9 months.
4. Add 20µl of 2M dithiothreitol (DTT) per 1 ml Buffer RLT. The stock solution of 2M DTT in water should be prepared fresh or frozen in single-use aliquots. Buffer RLT containing DTT can be stored at room temperature for up to 1 month.
5. Make up 70% ethanol from 96-100% ethanol.

Things to bear in mind during extraction

1. Do NOT cough, sneeze, lean over, touch, or breathe over anything that should be RNase-free.
2. RNaseZap liberally, whenever you feel the urge.
3. Change gloves regularly.
4. WORK CAREFULLY, WORK QUICKLY.

Procedure

1. Get a foam box and fill up with wet ice (from 3rd floor).
2. Get one DNase I stock solution aliquot per tissue RNA extraction (from -20°C freezer). Let this slowly defrost on wet ice.
3. Get a foam box and fill it up with dry ice (from 2nd floor).
4. Get tissue samples from -80°C freezer and transfer over (no need for ice if in RNAlater).

5. RNaseZap everything (benchtop, pipettes, pipette tip boxes, forceps, marker pen, etc.).
6. Change gloves.
7. Label 1x1.5ml microtube (packaged with pestle) per sample
8. Label 1x QIAshredder spin column in 2ml tube per sample
9. Get pestle motor from Fauzias' drawer
10. Clean forceps with 100% ethanol and then RNaseZap.
11. Do not use more than 30mg of tissue.
12. Place tissue in the labelled 1.5ml microtube.
13. Add 600µl of Buffer RLT to the tube. Ensure that β-ME or DTT has been added to Buffer RLT before use.
14. Immediately homogenise tissue with pestle attached to pestle motor for 5-8 mins until it is uniformly homogenous. Minimise foaming by keeping the tip of the homogeniser submerged, and holding the immersed tip to the side of the tube.
15. Repeat cleaning steps (steps 11b-h & step 12) before homogenising other tissue samples.
16. Pipette each sample into labelled QIAshredder
17. Centrifuge the lysate for 3 min at full speed.
18. Change gloves.
19. While waiting label the RNesay spin columns
20. Remove the QIAshredder column and discard, use only the supernatant in the 2ml tube in the subsequent steps.
21. Add 1 volume of 70% ethanol to the cleared lysate. Mix IMMEDIATELY by pipetting.
22. Transfer up to 700µl of the sample, including any precipitate, to a RNeasy spin column placed in a 2ml collection tube. Close lid gently.
23. Centrifuge for 15s at ≥8000 x g (≥10,000 rpm).
24. Discard the flow-through, but keep the collection tube for re-use in the next step.

25. If the sample exceeds 700µl, centrifuge successive aliquots in the same RNeasy spin column. Discard the flow-through after each centrifugation.
26. Add 350µl of Buffer RW1 to the RNeasy spin column. Close the lid gently.
27. Centrifuge for 15s at ≥8000 x g (≥10,000 rpm).
28. Discard the flow-through, but keep the collection tube for re-use.
29. In a new microcentrifuge tube make up the DNase I incubation mix: add 10µl of DNase I stock solution (from defrosted aliquots) and 70µl of Buffer RDD (supplied with RNase-free DNase Set). Scale up as appropriate depending on how many samples you are using (6 samples: 60µl of DNase I stock + 420µl of Buffer RDD)
30. Mix gently by inverting the tube. Do NOT vortex.
31. Briefly centrifuge (5 secs) to collect residual liquid from sides of tube.
32. CHANGE GLOVES.
33. Add the DNase I incubation mix (80µl) **directly** to the RNeasy spin column **membrane**.
34. Place on the benchtop for 15mins. Set a countdown alarm. Quick coffee break (bring alarm with you).
35. After 15min, add 350µl of Buffer RW1 to the RNeasy spin column. Close the lid gently.
36. Centrifuge for 15s at ≥8000 x g (≥10,000 rpm).
37. Discard the flow-through, but keep the collection tube for re-use in the next step.
38. Add 500µl of Buffer RPE to the RNeasy spin column. Close the lid gently. Ensure that ethanol has been added to Buffer RPE before use.
39. Centrifuge for 15s at ≥8000 x g (≥10,000 rpm).
40. Discard the flow-through, but keep the collection tube for re-use in the next step.
41. Add 500µl of Buffer RPE to the RNeasy spin column. Close the lid gently.
42. Centrifuge for **2 mins** at ≥8000 x g (≥10,000 rpm).
43. Whilst waiting, label some **new 2ml collection tubes** (supplied) ready for step 47.
44. Change gloves.

45. Carefully remove the RNeasy spin column from the collection tube so that the column does not contact the flow-through.
46. Place the RNeasy spin column in a **new 2ml collection tube**. Discard the old collection tube with the flow-through. Close the lid gently.
47. Centrifuge for **1 min** at full speed.
48. Whilst waiting, label some **new 1.5ml collection tube**s (supplied) ready for the next step.
49. Change gloves.
50. Place the RNeasy spin column in a **new 1.5ml collection tube** (supplied). Discard the old collection tube with the flow-through.
51. Add 50µl of RNase-free water **directly** to the spin column **membrane**. Close the lid gently.
52. Centrifuge for **1 min** at ≥8000 x g (≥10,000 rpm) to elute the RNA. Ensure 1.5ml tube lids are facing away from the direction of spin of the centrifuge.
53. If the expected yield of RNA is >30µg, repeat steps 52-53 using another 50µl of RNase-free water, or using the eluate from step 53 (if high RNA concentration is required). Reuse the collection tube from the previous step. If using the eluate from step 53, the RNA yield will be 15-30% less than that obtained using a second volume of RNase-free water, but the final RNA concentration will be higher (this is the default option).
54. Keep the eluate. Discard the column. Close the lids of the 1.5ml tubes gently.
55. Put samples on wet ice.
56. Microdrop the samples. (optional)
57. Return the pestle motor to Fauzia's draw.

Clear up Fauzia's bench. Return anything else you've borrowed.

A1.6. cDNA Reverse Transcription from mRNA

Before you start you need:

- SuperScript™ First-Strand Synthesis System for RT-PCR from Life Technologies (Cat. No: 11904-018)
    a. Stored in -20 freezer.
    b. Superscript II and RNAout maybe ordered independently of the kit.
        i. RNAout is at the same strength as the kit (40 U/µl)and can be used as per the protocol
        ii. Superscript II RT is at 4x the concentration (200 U/µl) of that supplied with the kit (50 U/µl), dilute 3:1 with molecular grade water before use)

Procedure:

1. Mix and briefly centrifuge each RNA sample before use.
2. Label a sterile 0.2-ml tube for each sample:
3. To each tube add:
    a. RNA 8 µl
    b. 10 mM dNTP mix 1 µl
    c. Random hexamers (50 ng/µl) 1 µl
4. Incubate the RNA/primer mixture at 65°C for 5 minutes, then 4°C (or place on ice) for at least 1 minute.
5. In a separate tube, prepare the following 2X reaction mix, adding each component in the indicated order (scale up according to number of samples):
    a. 2 µl 10X RT buffer
    b. 4 µl 25 mM MgCl2
    c. 2µl 0.1M DTT
    d. 1 µl RNaseOUT™ (40 U/µl)

6. Add 9 µl of the 2X reaction mix to each RNA/primer mixture
7. Collect by brief centrifugation.
8. Incubate at room temperature (~25°C) for 2 minutes.
9. Add 1 µl of SuperScript™ II RT to each tube.
    a. (optional: if doing a minus RT control, replace the RT with 1µl but follow the rest of protocol)
10. Transfer to PCR machine and program for the following settings:
    a. Volume 20µl
    b. 25°C C for 10 minutes.
    c. 42°C for 50 minutes.
    d. 70°C for 15 minutes.
    e. 4°C for infinity (or chill on ice)
11. (Optional, may increase purity) Collect the reaction by brief centrifugation. Add 1 µl of RNase H to each tube and incubate for 20 minutes at 37°C.
12. The reaction can be stored at -20°C or used for PCR immediately

A1.7. PT-PCR protocol (written by J. Zhang, updated by R. Appleby)

Before you start:

1. Original protocol (V1.0) required the user to dilute the cDNA by half, this is not required, it makes no difference to the PCR protocol, it just meant that less cDNA was needed. When comparing results, or doing PCRs on old cDNA (<EX120), be aware that this sample may have been diluted.
2. Dilute your primers
    a. When they arrive, they are made up with a specified quantity of to make 100µM stock solution
    b. Dilute 1:10 with molecular grade H_2O to make a working solution for PCR
3. **For a 384 well plate:**
    a. Plan the plate, use the template in the 384 plate calculator excel document.
    b. You will need to dilute the cDNA (0.32µl in 3.04µl H_2O per well), use the 384 plate calculator excel document to calculate dilutions and amounts.
    c. Follow order below, but use the amounts from the excel sheet, a 384 well PCR machine block and SDS template.
4. **For a 96 well plate**:
    a. Plan out your plate.

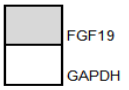


Plate _____												
Column	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

b. Calculate MM volumes (fill in table in next section)

Procedure:

1. Defrost SYBR Green, primers and cDNA. Defrost cDNA on ice.
2. Make up a MM for each primer and tick off as you go along. Ensure everything is thoroughly defrosted and vortex before adding to the MM.

	Vol for 1 well (µl)	**Vol for half a 96 well plate (µl)**	**FGF19 MM**	**GAPDH MM**
SYBR Green	12.5	700		
Forward primer	1	56		
Reverse primer	1	56		
DEPC-treated water	9.5	532		

3. Put SYBR Green and primers back in freezer (or fridge if planning to use SYBR Green again in the next 24hrs).
4. Vortex MM.
5. Pipette 24µl of FGF19 MM into Rows A, C, E and G.

6. Pipette 24µl of GAPDH MM into Rows B, D, F and H.
7. Centrifuge the plate.
8. Follow your plate plan:
    a. Add 1µl of cDNA into the appropriate wells (triplicates).
    b. Add 1µl of RT-negative sample into the appropriate wells (triplicates).
    c. Add 1µl of DEPC-treated water into the appropriate wells (triplicates).
9. Centrifuge the plate.
10. Put adhesive cover on.
11. Take plate to 2nd floor 7900 PCR machine.
12. Set up program:
    a. Open up 96-well template in 'Justine + Jonathan' folder.
    b. Under Setup, input sample names.
    c. Instrument > Real-time > Connect to Instrument > Open/Close
    d. Change plate holder to a 96 well Fast plate holder.
    e. Change block to a 96 well block.
    f. Put plate in.
    g. Instrument > Real-time > Open/Close
    h. Instrument > Real-time > Run > Save file when prompted.
    i. Wait for *time for run to be completed* to show up.
13. Go back and clear up lab.
14. Email SDS file to yourself once finished. (it will save automatically when run is finished)

Appendix 2. Study protocols

All study protocols here are the regional ethics committee approved versions, with non-procedural parts removed in the interests of space (investigator details, introduction and adverse event reporting procedures). For both the A3384 studies the brief version of the protocol is given. Only the prevalence of bile acid diarrhoea and low FGF19 NALFD protocol is authored by myself.

A2.1. Prevalence of Bile Acid Diarrhoea and low FGF19 in NAFLD

Inclusion Criteria

Aged 18 or over at study inclusion and attending the NAFLD clinic at St Marys' Hospital.

Exclusion criteria

Patients will be excluded from the study if they are unable to consent, decline or withdraw consent. Participation in other studies will not affect eligibility. Otherwise patients with known diarrhoeal conditions or evidence of a liver pathology other than NAFLD such as:

- Chronic pancreatitis or inflammatory bowel disease
- Hepatitis B or C whether active or inactive
- Alcohol consumption above the recommended weekly limits (21 units for women, 25 for men)
- Raised total iron binding capacity or diagnosis of haemachromatosis
- Clinical suspicion by hepatologist of another pathology contributing to liver disease

Recruitment and Visit 1 (15minutes)

While waiting to see the doctor in clinic patients will be consented for entry into the study and asked a series of screening questions (reported diarrhoeal symptoms, alcohol consumption, past medical and medication history, from a proforma). Consenting patients will be given a symptom diary for 1 week and instructed on its use. It is anticipated that this consultation will take a maximum of 15 mins. If the patient is fasted, the blood test for FGF19/C4 and DNA may be performed at the same time. If not the patient will asked to return for the blood test at their convenience (5 mins). The patient will offered a letter to inform their GP (GP letter before diary V1.0), at this stage it is not essential.

On the initial visit the following data is collected routinely during the clinic will be collected from the medical notes :

- Age
- Ethnic group
- BMI

- Platelet count
- Albumin
- AST/ALT ratio
- Fasting lipid profile
- Fasting blood glucose
- Fibroscan score
- Alcohol consumption
- Medication history
- Relevant medical history
- Viral hepatitis markers, Serum ferritin/ total iron binding capacity (these will checked, but not recorded)

Visit 2 fasting blood sample (5mins)

Some patients will need to return to hospital for a fasting FGF19/C4 and DNA. Where possible this blood test will be done at the same time as clinically indicated tests. The total blood required for both tests will be 12mls

GI Symptom Diary (1 week)

If the patient reports subjective diarrhoeal symptoms on the first visit they will be given a daily symptom to record their symptoms daily. This symptom diary will record the frequency and Bristol Stool Form Scale of each stool, which is a validated surrogate of GI transit time.[178] As well as 3 1-5 Likert scale questions on abdominal pain, bloating and urgency which are part of the Frequency of Digestive Symptoms Questionnaire which has been validated in healthy volunteers and individuals with IBS.[179, 180]

Telephone Consultation 1 (10 mins)

After 1 week the patient will be contacted by telephone or email (according to the patients' specified preference) for the results of the symptom diary. An appointment the fasting FGF19 blood test may also be made (if not performed already) (10 mins). This is the last intervention as part of the trial. If diary reports $\geq$21 stools/week with 50% $\geq$type 5 the patient will be asked to undergo a SeHCAT test.

SeHCAT testing and follow up (as per clinical guidelines)

If indicated, a SeHCAT test will be booked through the NHS system. The results will be reviewed in the gastroenterology clinic and the patient counselled appropriately. Treatment will be started or further investigations requested if necessary. At this point the patient will have no further contact with the study, but the clinical progress of the patient will be followed, and data (inflammation/fibrosis scores) from any liver biopsies performed will be collected for analysis. A Letter will be sent to the GP informing them of the referral.

Analysis

Sample storage

Blood samples for FGF19 and C4 will be centrifuged at >8000rpm for 10 mins, the separated sera will be stored at -80C within 1 hour of collection and stored until transfer to King College. Blood for DNA will be frozen at -20C until analysis.

FGF19/C4

These samples will be sent the biochemistry lab at Kings College on a commercial basis. FGF19 will be measured by a commercially available ELISA kit and C4 by gas chromatography.

Genomic DNA

A known polymorphism for a DIET1 SNP will be detected using qualitative PCR in our laboratory.

NALFD fibrosis scoring system

Will be calculated for each patient using clinical parameters (age, BMI, albumin, glucose, platelets, AST/ALT ratio). Histological scoring systems will also be used for those patients undergoing liver biopsy.

Statistical analysis

The odds ratio of chronic diarrhoea in patients with NAFLD will be calculated and the variance assessed by chi-square test. Demographic data will be assessed for variance using ANOVA. Non-parametric data will be assessed for variance using a Mann-Witney U test. Correlations will be calculated for SeHCAT and FGF19 values against NAFLD fibrosis score, ALT and fibroscan score.

Regulatory Issues

REC

This study will be submitted for REC (NRES East Midlands – Leicester) before starting. Most procedures apart from the extra blood test are performed within current clinical guidelines, therefore it is expected that it will be appropriate for a proportionate review and not a complete REC review.

JRCO

This study will be submitted for JRCO approval before being submitted to the REC. During this process it will be submitted for level 3 peer review.

Confidentiality

All patient identifiable information will be stored on Imperial NHS trust computers in a password protected database. This file will be destroyed 5 years after the study end. Upon study entry, a consent form will be signed and will also have patient identifiable information. These will be stored in a locked cupboard in the NHS offices in Hammersmith Hospital. All other documents and blood samples will be anonymised and only be labelled with the study number. Analysis will be of fully anonymised data and no individual data will be presented or published.

As participation in the study will not affect the patient's medical management or treatment, their GP will not be routinely informed unless the patient requests (at this point GP letter before diary V1.0 should be used). However if they report significant diarrhoea and are referred for a SeHCAT test, the GP will informed if the patient has consented to allow this. (GP letter after diary 2.0)

Tissue Storage

Human sera and DNA will be stored label in anonymised format with the study number only in the -80C freezer within Imperial College. The storage of these sample will be registered with the Imperial College Tissue Bank.

Indemnity

Imperial College London holds negligent harm and non-negligent harm insurance polices which apply to this study.

Sponsor

Imperial College London will act as the main sponsor for this study. Delegated responsibilities will be assigned to Imperial College Healthcare NHS Trust.

Funding

Funding for this project is provided by Imperial College as part of tuition costs.

Audit/Inspection

The study may be subject to inspection and audit by Imperial College under their remit as sponsor and the study co-ordination centre and other regulatory bodies to ensure adherence to GCP.

A2.2. A4250 and A3385 in healthy volunteers

Methodology:

This is a single centre, 2-part, double-blind, placebo-controlled study in healthy subjects. Part 1 is a single ascending dose assessment and Part 2 is a multiple ascending dose assessment.

Part 1 – single dose

Up to 7 parallel cohorts of 8 subjects will participate in the single dose assessment. In each cohort, subjects will be randomly assigned in a 3:1 ratio to receive either active A4250 or matching A4250 placebo. It is planned that for Cohort 1 (starting dose) a staggered 'sentinel' dose design will be used.

Dose Escalation:

The planned dose levels are 0.1, 0.3, 1 and 3 mg for the first 4 cohorts. The actual doses will be selected based on emerging data. To allow for the possibility that additional dose levels may be tested, up to 3 further cohorts may be enrolled in Part 1. Each dose increment will be no more than 3.33-fold and the maximum dose, beyond which further dose escalation cannot proceed, will be 20 mg. Following completion of Cohort 1, and after each subsequent cohort, interim safety analysis will be performed and these data will be used to determine the appropriate dose escalation. Following review of the data after each dose level, the next planned dose level to be investigated may be increased, reduced, adjusted to an intermediate dose or dose progression may be stopped.

Part 2 – multiple dose

Up to 8 cohorts of 8 subjects will participate in the multiple dose assessment. Part 2 will be initiated once the safety and tolerability data for the third cohort of Part 1 have been reviewed, and will then run in parallel with Part 1, such that the selected daily dose for multiple dosing will not exceed the highest well tolerated single dose. Up to 3 cohorts of subjects will receive A4250 monotherapy. In addition, the highest tolerated dose from the multiple dosing of A4250 monotherapy will also be evaluated in combination with Questran (1 g b.i.d.) in 1 cohort. Further, up to 3 cohorts will receive A4250 in the highest tolerated dose in combination with CRC (1 g twice daily [b.i.d.]) and the same dose of CRC will be evaluated as monotherapy in 1 cohort. In Cohorts 1 to 3, subjects will be randomly assigned in a 3:1 ratio to receive either active A4250 or matching A4250 placebo. In Cohort 4, 8 subjects will be randomly assigned in a 3:1 ratio to receive either active A4250 in combination with Questran (6 subjects) or matching A4250 placebo in combination with Questran (2 subjects). In Cohort 5, 8 subjects will be randomly assigned in a 3:1 ratio to receive either active A4250 in combination with CRC (6 subjects) or matching A4250 placebo in combination with CRC placebo (2 subjects). In Cohort 6, 8 subjects will be randomly assigned in a 3:1 ratio to receive either CRC (6 subjects) or CRC placebo (2 subjects). For all cohorts, doses will be administered over 7 days, ending with final dosing on Day 7 in the morning for once daily (qd) dosing of A4250 and in the evening for b.i.d. dosing of A4250 or Questran/CRC. The dosing frequency for A4250 is anticipated to be qd for all cohorts, but may be modified to b.i.d. based on the results of the data review from the single dose assessment. Two additional cohorts (Cohorts 7 and 8) with the same randomisation as Cohort 5 may be added to the study; the difference between cohorts will be the separation in time between the dosing of A4250 and CRC. For Cohort 4, for qd dosing of A4250 administered with b.i.d. Questran, A2450 dosing will be at approximately 08:00 and breakfast within 15 min post-dose (at approximately 08:15). Questran will be dosed at both 4 h post-dose (approximately noon) and in the evening at 14 to 15 h post-dose (approximately 22:00 to 23:00; not before 22:00) with dinner provided at approximately 17:00. If b.i.d. dosing of A4250 is decided for Cohort 4, A4250 will be

dosed at approximately 08:00 and breakfast within 15 min post-dose (at approximately 08:15). Questran will be dosed at 4 h post-dose (approximately noon). The A4250 evening dose will be 12 h after the A4250 morning dose (at approximately 20:00) and dinner within 15 min post-dose (at approximately 20:15). The Questran evening dose will be at 14 to 15 h post-morning A4250 dose (approximately 22:00 to 23:00; not before 22:00). For Cohort 5 for qd dosing of A4250 administered with b.i.d. CRC, CRC will be administered 30 min before dosing (at approximately 07:30), with dosing of A4250 at approximately 08:00 and breakfast within 15 min post-dose (at approximately 08:15). Evening dosing of CRC will be 30 min before dinner (approximately 16:30) at approximately 17:00. If Cohort 5 utilises b.i.d. dosing of A4250 administered with b.i.d. CRC, CRC will be administered 30 min before dosing (at approximately 07:30), with dosing of A4250 at approximately 08:00 and breakfast within 15 min post-dose (at approximately 08:15). Evening dosing of CRC will be 30 min before dosing (at approximately 19:30), with A4250 dosing 12 h post the morning dose (at approximately 20:00) and dinner within 15 min post-dose (at approximately 20:15). For Cohort 6, CRC will be administered b.i.d., with morning dosing of CRC at approximately 07:30 and breakfast within 45 min post-dose (at approximately 08:15). Evening dosing of CRC will be at approximately 19:30 and dinner within 45 min post-dose (at approximately 20:15). The time schedules for the administration of CRC in the optional cohorts (Cohorts 7 and 8) will be defined when data from the first cohorts have been evaluated. A4250 will be administered according to the same schedule; however, the timing of the CRC administration may be changed.

Dose Escalation

The planned dose levels are 1 and 3 mg per day for the first 2 cohorts which may be a single qd dose or a b.i.d. divided dose. The actual doses will be selected based on emerging data. To allow for the possibility that an additional dose level and/or an alternative dosing regimen may be tested, up to 1 further cohort (Cohort 3) may be enrolled in Part 2. Each dose increment will be no more than 3-fold and the total daily dose level will not exceed the highest dose already dosed and tolerated in Part 1. Following completion of each of Cohorts 1 to 3, interim safety analysis will be performed and these data will be used to determine the appropriate dose escalation for the following cohort. Following review of the data after each of Cohorts 1 to 3, the next planned dose level to be investigated may be increased, reduced, adjusted to an intermediate dose or dose progression may be stopped. Following Cohorts 1 to 3, a decision will be made on the dose level to be used for Cohort 4.

Study Design:

Part 1 – single dose

Subjects will receive the formulations in the morning (Day 1) following an overnight fast.
Subjects will be admitted to the clinical unit at 08:00 on the morning prior to product administration (Day -1) and will remain on site until 24 h post-dose. A follow-up visit will be performed at 5 to 7 days after the final dose for each subject to ensure the on-going wellbeing of the subject. If a subject reports any adverse events (AEs) which represent a cause for concern they will be followed up until resolution.

Part 2 – multiple dose

Subjects will receive the first dose in the morning of Day 1 following an overnight fast and subsequent doses will follow at appropriate intervals depending on the dosing frequency (qd dosing for A4250 = 24 h interval for A4250 and 9 h interval between am and pm dose on Days 1 to 7 for CRC; b.i.d. dosing for A4250 and CRC = 12 h interval for both). The last dose will be administered on the morning of Day 7 for qd dosing of A4250 or the evening of Day 7 for b.i.d. dosing of A4250 or CRC/Questran. Questran will be administered b.i.d. at both 4 h and 14 to 15 h post-dose (at approximately noon and in the evening [approximately 22:00 to 23:00]). Subjects will be admitted to the clinical unit at 08:00 on the morning 48 h prior to product administration (Day -2) and will collect

all stool samples up to dosing. Subjects will remain on site until 24 h post-last dose of investigational medicinal product. A follow-up visit will be performed at 5 to 7 days after the final dose for each subject to ensure the on-going wellbeing of the subject. If a subject reports any AEs which represent a cause for concern they will be followed up until resolution.

Number of Subjects Planned:

In Part 1 - single dose, 8 subjects will be enrolled in each cohort (ie 6 subjects will receive active A4250 and 2 subjects will receive matching A4250 placebo).
In Part 2 - multiple dose, 8 subjects will be enrolled in each of Cohorts 1 to 3 (ie 6 subjects) will receive active A4250, and 2 subjects will receive matching A4250 placebo). In addition, at the MTD of the A4250 monotherapy, 1 group of subjects will receive active A4250 in combination with Questran (6 subjects) or matching A4250 placebo in combination with Questran (2 subjects). One group will receive active A4250 in combination with CRC (6 subjects) or matching A4250 placebo in combination with CRC placebo (2 subjects). One group of 8 subjects will also be administered CRC 1 g b.i.d. as monotherapy (6 subjects) or CRC placebo (2 subjects). Optionally, 2 cohorts (8 subjects in each) with different timings for the administration of CRC in conjunction with A4250 may be added to the study. Subjects withdrawn due to a drug related AE will not be replaced but will be included as evaluable subjects. In all parts of this study, subjects who are withdrawn for non-drug related AEs may not be included as evaluable subjects so additional replacement subjects may be enrolled to ensure sufficient evaluable subjects. The maximum number of subjects that may be dosed in Part 1 is 63, comprising 56 subjects with 7 cohorts of 8 (with a total of 42 subjects planned to receive active A4250 and 14 subjects planned to receive matching A4250 placebo) and up to 7 replacement subjects. The maximum number of subjects that may be dosed in Part 2 is 76, comprising 64 subjects with 8 cohorts of 8 (with a total of 18 subjects planned to receive active A4250 only, 18 subjects planned to receive active A4250 and CRC, 6 subjects planned to receive CRC only, 6 subjects planned to receive active A4250 and Questran, 2 subjects planned to receive matching A4250 placebo and Questran and 14 subjects planned to receive placebo [6 subjects to receive matching A4250 placebo, 6 subjects to receive a combination of matching A4250 placebo and CRC placebo and 2 subjects to receive CRC placebo]) and up to 12 replacement subjects.

Duration of Study:

In Part 1, single dose administration on a single occasion.
In Part 2, multiple dose administration over 7 days.

Main Inclusion Criteria:

Healthy males or non-pregnant, non-lactating healthy female subjects aged 18 to 60 years.
Body mass index 18 to 32 kg/m2.

Investigational Medicinal Product, Dose and Mode of Administration:

Oral administration of:

Part 1

Planned doses of 0.1, 0.3, 1 and 3 mg A4250 as immediate release (IR) capsule(s)
Matching A4250 placebo

Part 2

Planned doses of 1 and 3 mg A4250 as IR capsule(s), 1 g b.i.d. CRC (A3384) as capsules and 1 g b.i.d. Questran as sachet powder
Matching A4250 placebo and CRC placebo
Products will be administered with 240 mL water.

Pharmacokinetic Assessments:

Part 1 – single dose

11 venous blood samples will be withdrawn via an indwelling cannula or by venepuncture at regular intervals until 24 h post-dose.

Part 2 – multiple dose

22 venous blood samples will be withdrawn via an indwelling cannula or by venepuncture at regular intervals until 24 h post-first dose and until 24 h post-last dose of A4250. If b.i.d. dosing is required, PK samples will be taken at designated times post both am and pm A4250 b.i.d. doses. The plasma concentration data for A4250 will be provided by Quotient Bio Analytical Sciences - an LGC business. Separate analyses will be performed for Part 1 – single dose and Part 2 – multiple dose. The concentration vs time data will be analysed by Quotient Clinical using non compartmental techniques (WinNonlin v6.1 or a more recent version) to obtain estimates of the standard parameters. Samples for possible CRC (A3384) PK analysis will be collected at the same time points as samples for A4250 PK analysis. These will be stored for up to 1 year in the event that a bioanalytical method becomes available and PK analysis is required.

Pharmacodynamic Assessments:

Part 1 – single dose

Assessment of bile acids and bile acid synthesis markers such as 7α-hydroxy-4-cholesten-3-one (C4) and fibroblast growth factor 19 (FGF19) Venous blood samples will be withdrawn via an indwelling cannula or by venepuncture at designated time points until 24 h post-dose, and at the follow-up visit.

Part 2 – multiple dose

Assessment of bile acids and bile acid synthesis markers such as C4 and FGF19 Venous blood samples will be withdrawn via an indwelling cannula or by venepuncture at designated time points until 24 h post-first dose, 24 h post-last dose of A4250 and at the follow-up visit. If b.i.d. dosing is required, PD samples will be taken at designated times post both am and pm b.i.d. A4250 doses.

Faecal bile acids

During the 48 h baseline period, and during the 24 h following morning dosing on Day 7, stool samples will be collected. PD concentration data will be provided by Quotient Bio Analytical Sciences – an LGC business, and the corresponding homogenised faecal weight data will be provided by Quotient Metabolism. These data will be analysed by Quotient Clinical.

Safety Assessments:

The results of Day -1 assessments will be required prior to dosing to confirm eligibility. The planned safety assessments may be amended based on emerging safety data. Any changes will be documented.

Part 1 – single dose

The safety assessments to be conducted are:

- Clinical chemistry, haematology and urinalysis assessments
- Vital signs (blood pressure and heart rate) and temperature assessments
- Electrocardiogram (ECG)
- Physical examination. A system selective physical exam may be performed if any subject reports baseline symptoms as per the judgement of the investigator.
- Screening for drugs of abuse and alcohol and carbon monoxide breath tests.
- Female subjects will be tested for pregnancy (urine test)
- Subjects will be tested for hepatitis B, hepatitis C and human immunodeficiency virus (HIV).

- Bowel Habit Diary (BHD) / Bristol Stool Form Scale (BSFS)
- Training on completion of the BHD and BSFS will be completed on Day -1.
- During the 24 h following dosing, subjects will record whether they have had a bowel movement (BM). For each BM, consistency will be recorded on the BSFS.

Part 2 – multiple dose

The safety assessments to be conducted are:

- Clinical chemistry, haematology and urinalysis assessments
- Vital signs (blood pressure and heart rate) and temperature assessments
- ECG
- Physical examination. A system selective physical exam may be performed if any subject
- reports baseline symptoms as per the judgement of the investigator.
- Screening for drugs of abuse and alcohol and carbon monoxide breath tests
- Female subjects will be tested for pregnancy (urine test).
- Subjects will be tested for hepatitis B, hepatitis C and HIV.
- BHD / BSFS
- Training on completion of the BHD and BSFS will be completed on Day -1.
- During the 24 h following dosing on Days 1 to 7, subjects will record whether they have had BM. For each BM, consistency will be recorded on the BSFS.

Statistical Methodology:

Part 1

Formal statistical analysis will be performed on the PK parameters AUC(0-last), AUC(0-inf) and Cmax to assess dose proportionality using a power model approach. In addition, formal statistical analysis will be performed on the plasma PD variables C4, FGF19 and each of the bile acids to compare A4250 placebo with each active dose of A4250 via pairwise treatment comparisons.

Part 2

Formal statistical analysis will be performed on the PK parameters AUC(0-last), AUC(0-inf) and Cmax on Days 1 and 7. Analysis of variance techniques will be used to obtain the following pairwise treatment comparisons, and the comparisons will be between subject comparisons:

- MTD of A4250 combined with CRC (ie all active subjects in Cohort 5) vs MTD of A4250 on Days 1 and 7 separately
- MTD of A4250 combined with Questran (ie all active subjects in Cohort 4) vs MTD of A4250
- MTD of A4250 combined with CRC (ie all active subjects in Cohort 5) vs MTD of A4250 combined with Questran (ie all active subjects in Cohort 4)
- No formal assessment of dose proportionality will be performed as a minimum of 3 dose levels administered at the same dose frequency (ie the same number of daily doses) is required.
- Formal statistical analysis will be performed on the plasma and faecal PD variables C4 (plasma only), FGF19 (plasma only) and each of the bile acids to compare:
    - Each active dose of A4250 vs all A4250 placebo subjects combined on Days 1 and 7 separately (ie all actives subjects in Cohorts 1 to 3 vs all placebo subjects in Cohorts 1 to 3);
    - MTD of A4250 combined with CRC (ie all active subjects in Cohort 5) vs MTD of A4250 on Days 1 and 7 separately;
    - MTD dose of A4250 combined with CRC (ie all active subjects in Cohort 5) vs CRC (ie all active subjects in Cohort 6) on Days 1 and 7 separately;
    - MTD of A4250 vs CRC (ie all active subjects in Cohort 6) on Day 1 and 7 separately

- MTD of A4250 combined with Questran (ie all active subjects in Cohort 4) vs MTD of A4250;
- MTD of A4250 combined with CRC (ie all active subjects in Cohort 5) vs MTD of A4250 combined with Questran (ie all active subjects in Cohort 4);
- MTD dose of A4250 combined with CRC (ie all active subjects in Cohort 5) vs A4250 placebo subjects and CRC placebo subjects (ie all placebo subjects in MTD of A4250 cohort combined with Cohorts 5 and 6);
- MTD dose of A4250 (eg all active subjects in Cohort 2) vs A4250 placebo subjects and CRC placebo subjects (ie all placebo subjects in MTD of A4250 cohort combined with Cohorts 5 and 6);
- CRC (ie all active subjects in Cohort 6) vs A4250 placebo subjects and CRC placebo subjects (ie all placebo subjects in MTD of A4250 cohort combined with Cohorts 5 and 6);
- MTD dose of A4250 combined with Questran (ie all active subjects in Cohort 4) vs A4250 placebo subjects and CRC placebo subjects (ie all placebo subjects in MTD of A4250 cohort combined with Cohorts 5 and 6 combined);
- Pairwise treatment comparisons will be used and the analyses will be performed separately on Days 1 and 7 for the plasma PD data, and on Day 7 for the faecal PD data only. Further formal statistical analysis will be performed on the plasma PD variables C4, FGF19 and each of the bile acids to compare Day 7 with Day 1 for each active dose of A4250.

Sample Size and Power:

No formal sample size calculation has been made. Based on experience from previous similar studies, a total of 8 subjects enrolled in each cohort (6 active, 2 placebo), to ensure 7 evaluable subjects and hence data in at least 5 subjects on active treatment in each cohort is considered sufficient for both parts. In Cohort 4, the active treatment is A4250 and Questran in combination and placebo is matching A4250 placebo and Questran combined. In Cohort 5, the active treatment is A4250 and CRC in combination and placebo is matching A4250 placebo and CRC placebo combined. In Cohort 6, the active treatment is CRC and placebo is CRC placebo. In the optional Cohorts 7 and 8, the active treatment is A4250 and CRC in combination and placebo is matching A4250 placebo and CRC placebo combined with the only difference being the separation in time between dosing of A4250 and CRC.

A2.3. A3384 in bile acid diarrhoea

OVERVIEW OF STUDY DESIGN

This is a double-blind, randomized, placebo-controlled, multiple centre study of the efficacy and safety of twice daily oral 250 mg or 1 g doses A3384 for two weeks in up to 36 patients diagnosed with BAM/BAD.

The primary efficacy endpoint for the study is "reduction in average # of BMs during the second week of treatment" compared to baseline.

The study will involve three treatment groups where two groups will be actively treated with 250 mg or 1 g A3384 BID and one group will get placebo.

Sample size estimations are based on assumptions of a 40% reduction in the # of BMs in the groups dosed with 1 g A3384 BID compared to placebo.

There will be 5 clinic visits at the investigative sites for assessments:

- Visit 1: Baseline Period Visit 1, (Day -21 to Day -24) before 1 week of symptom registrations on concurrent pre-study BAM/BAD bile acid sequestrant treatment;
- Visit 2: Baseline Period Visit 2, (Day -14 to Day -17) before 2 weeks of symptom registrations when concurrent pre-study bile acid sequestrant treatment is withdrawn;
- Visit 3: End of Baseline and Start of Treatment Period, (Day 1);
- Visit 4: End of Treatment Period, (Day 15 to Day 18);
- Visit 5: Follow-up Visit, (Day 22 to Day 25), 7 days after End of Treatment, whether the patient completes the treatment as planned or discontinues prematurely.

Additional clinic visits may be required for patients who need direct site assistance e.g., due to AE monitoring and/or safety maintenance.

The patients will receive either active treatment with A3384 in two daily doses each of 250 mg or 1 g or will get placebo for two weeks. Each patient has a 67 per cent chance of receiving active treatment and a 33 per cent chance of receiving placebo during the study.

As soon as a patient is considered for this study and prior to any other study procedures conducted by the Investigator/Investigative Staff, the patient will have the nature of the study explained to him/her and be asked to sign an Informed Consent Form (ICF). After signing these documents, patients will be evaluated for study eligibility. Informed consent must be obtained prior to any procedures that do not form a part of the patient's normal care. Randomization of patients to the treatment groups will be used to avoid bias and to guarantee an allocation independent of known and unknown patient attributes (e.g., demographics and Baseline characteristics).

At Visit 1, the Investigator/Investigative Staff will enter the patient's initials and date of birth onto an electronic CRF in order to obtain a Screening patient identification number (PID). Additional Screening baseline procedures will include a thorough review of study inclusion and exclusion criteria, medical and surgical history review, physical examination, laboratory testing, vital signs, height, weight, recording of prior and concomitant medications and daily recording of BMs and GI symptoms using a paper diary to determine confirmation of the BAM/BAD diagnosis and the baseline symptoms before the study start.

The patients will at Visit 1 to the clinic be thoroughly instructed on the daily recordings in the paper diary. This will start with recordings during 1 week when the patients continue with the bile acid sequestrant treatments taken pre-study (Baseline 1). After Visit 2 to the clinic the patients start a 2 week diary symptom registration period (Baseline 2) when pre-study bile acid sequestrant treatments are withdrawn.

At Visit 3 the pre-treatment Screening - Baseline period is completed. There will be a +3 day window for Baseline period 2 enabling up to 17 days of diary entries without pre-study bile acid sequestrant treatment. However, only the most recent 7 days will be used for eligibility and Baseline calculations. If the patient is eligible for randomization the study treatment starts. Ineligible patients due to recorded Baseline data will be reported as screen-failures.

After all applicable screening assessments have been performed patients who meet all inclusion criteria and none of the exclusion criteria will receive a unique randomization number and be randomized to one of the three treatment groups. If a patient fails to meet the study entry criteria, he/she will be reported as a screen-failure and not be randomized to the study.

On Study Day 1 (Clinic Visit 3, the day of first study medication dose) at the clinic, the patients will receive 1 of the 3 study treatments (in a 1:1:1 ratio) orally 30 minutes before breakfast: 1 capsule of 250 mg A3384 + 3 placebo capsules, 4 capsules of 250 mg A3384 or 4 placebo capsules, respectively. Study medication will be presented in dosage units containing 4 capsules to be taken at one dose occasion.

Treated patients will return for clinic visit assessments on study Day 15 (Visit 4, the end of treatment visit) and on study Day 22 (Visit 5). The allowed time windows for these visits are Day 15 to Day 18 and Day 22 to Day 25, respectively. All remaining study medication will be collected at Visit 4.

Patients should not restart original BAM/BAD treatment until after study completion but if symptoms recur after the active treatment phase, restart is accepted.

The study is completed at a follow-up visit, Visit 5 (Day 22), 7 days after study treatment completion.

At the randomization visit (Study Day 1, Clinic Visit 3), one medication packet containing 34 dose intakes (totally 136 capsules) in divided dosage units, labelled with the patient's PID and the treatment or randomization number, will be dispensed to each patient.

Beginning on the day of randomization on study Day 1 (Visit 3) and through the treatment period up to Visit 4, the patients will use a paper diary for the daily recording of the date and the time the treatment doses were taken, and for the recording of daily and weekly information necessary for the efficacy assessments (i.e., frequency of bowel movements, scales for stool consistency etc.). All diary information will be brought into the study data base.

MAIN INCLUSION/EXCLUSION CRITERIA

The **main inclusion criteria** for eligibility for study participation will be:

- Symptoms compatible with the diagnosis of Bile Acid Malabsorption/Bile Acid Diarrhoea (BAM/BAD) with onset >12 months prior to randomization;
    - SeHCAT 7 day retention of less than 10% with measurement conducted within the last 5 years and no dramatic change in symptomatology since the SeHCAT assessment
    - A history of at least 3 liquid or soft stools (BSFS ≥5) per day on average calculated from 7 days without therapy
    - The last seven days before randomization having had totally > 21 BMs of which >50% should be of BSFS ≥5 (Evaluated pre-randomization at Visit 3)
- The patient reports having understood and has signed both the ICF and is willing to comply with all study visits and assessments;

- The patient is a male or non-pregnant female ≥18 years of age and ≤80 years of age with body mass index (BMI) ≥18.5 but <35;
- Having had a macroscopically normal colonoscopy with no histological signs of microscopic colitis within 5 years of screening

The **main exclusion criteria** for eligibility for study participation will be:

- Any condition that, in the opinion of the Investigator constitutes a risk for the patient or a contraindication for participation and completion of the study, or could interfere with study objectives, conduct, or evaluations.
- An identified cause for bile acid malabsorption such as surgical intestinal resection or bypass, IBD or drug use.
- Patients with other diagnoses leading to diarrhoea, including colorectal neoplasia, ulcerative colitis, coeliac disease, chronic pancreatitis or drug-induced diarrhoea.
- The patient has a structural abnormality of the GI tract or a disease or condition that can affect GI motility.
- The patient has a known, active, clinically significant acute or chronic infection, or any major episode of infection requiring hospitalization or treatment with parenteral anti-infectives within 4 weeks of treatment start (study day 1) or completion of oral anti-infective treatment within 2 weeks prior to start of baseline period.
- The patient has unexplained and clinically significant GI alarm signals (e.g., lower GI bleeding or hem-positive stool, iron-deficiency anaemia, unexplained weight loss) or systemic signs of infection or colitis.
- The patient has rectal bleeding and/or is hem-positive in the absence of known internal or external haemorrhoids.
- The patient has a history of cancer with last date of proven disease activity/presence of malignancy within 5 years, except for adequately treated basal cell carcinoma of the skin, cervical dysplasia, or carcinoma in situ of the skin or the cervix.
- Previous biliary surgery, excluding cholecystectomy.
- Other reason for diarrhoea such as pancreatitis, celiac disease, infection etc.
- Treatment with bile acid sequestrants (cholestyramine, colesevelam, colestipol or similar) during Baseline period 2.
- Treatment with loperamide or codeine after Visit 1 and before Visit 4.
- The patient has laboratory test levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT) or alkaline phosphatase (AP) >2.5 x the upper limit of normal (ULN) or total bilirubin >1.5 x ULN
- Chronic liver or chronic kidney disease
- Active, serious medical disease with life expectancy less than 5 years
- Active substance abuse in the year before screening
- Allergy to cholestyramine
- The patient has a history of a psychiatric disorder requiring hospitalization or suicide attempt in the 2 years prior to study inclusion;
- The patient has participated in any investigational clinical study within 30 days prior to study inclusion or within 5 half-lives of the investigated compound (whichever is longer) prior to study inclusion (unless the patient was never randomized to study treatment), or plans to participate in another clinical study during the period of participation in this study;
- The patient is a pregnant, breast-feeding or lactating female.

STUDY POPULATION

During a 9 month period starting 1 February 2014 approximately 48 patients with BAM/BAD will be enrolled into this study at multiple investigational sites in order to obtain a total of 12 randomized

patients for each treatment group (for a total of 36 patients), assuming an estimated screening failure rate of 25%. The randomization will be stratified by site.

DOSAGE AND ADMINISTRATION

Capsules containing 250 mg A3384 or matching placebo capsules, provided in dose divided (dosage units) treatment packs containing totally 136 capsules enough for 34 doses, are to be taken during 14 to 17 treatment days. The capsules will be taken twice daily orally with water, 30 minutes before breakfast and 30 minutes before supper at approximately 5 to 8 pm. Four capsules from one dosage unit are taken at each dose occasion.

RANDOMIZATION AND BLINDING

This is a randomized, double-blind, placebo-controlled study of 2 different daily dose regimens of A3384 for 14 days.

To avoid bias and to guarantee a process independent of known and unknown patient attributes randomization of patients to treatment groups receiving A3384 or placebo will be done. After patients have signed the ICF and have been found to meet eligibility criteria following the screening period, they will be randomized to 1 of the 3 treatment groups in a 1:1:1 ratio for a total of 12 patients in each group.

EFFICACY CRITERIA FOR EVALUATIONS

Diary recordings during Baseline period 2 will constitute the primary reference for the treatment efficacy comparisons. Baseline values for BM endpoints will be calculated from the diary recordings during the second week (last 7 days of recording) of the 14 to 17 days of Baseline period 2 (without pre-study bile acid sequestrant treatment).

Treatment efficacy comparisons with the diary recordings during Baseline period 1 (with pre-study bile acid sequestrant treatment) will also be done.

The primary efficacy criterion will be assessed based upon patients' reports, through the paper diary, of the date and time of each bowel movement (BM) during the second treatment week (last 7 days of reporting) of treatment.

Secondary efficacy criteria will be assessed based upon patients' reports, through the paper diary during the second treatment week (last 7 days of reporting).

- Stool consistency using the Bristol Stool Form Scale
- Abdominal discomfort using rating scales
- Bloating using rating scales
- Severity of diarrhoea using rating scale
- Degree of global symptom relief using rating scales

SAFETY CRITERIA FOR EVALUATIONS

The primary safety criterion will be the incidence of treatment-emergent SAEs, based upon information from patient reports, including the description, incidence, and severity of an SAE. This information will be recorded on the eCRF.

Secondary safety criteria will be assessed based upon information in patients' diary reports; this will also be captured on the eCRF:

- Occurrence of treatment-emergent AEs (TEAE) including severity and relatedness to study drug
- Physical examinations at screening/baseline and Visit 4 (Day 15 to 18) and Visit 5 (Day 22 to 25)
- Concomitant medication at all visits

- Vital sign measurements at all visits
- Laboratory test results (including haematology, clinical chemistry and urinalysis) at all visits

EFFICACY ENDPOINTS

The primary baseline period is defined as the last 7 days of reporting during Baseline period 2 prior to the first day of study drug administration (Day 1). Efficacy endpoints will be summarized and analysed for the ITT population by treatment group (250 mg A3384 BID, 1 g A3384 BID or placebo).

The **primary efficacy endpoint** is change from Baseline in # of BMs during the second treatment week (last 7 days of reporting)

Secondary efficacy endpoints are:

- Change from Baseline in average severity of diarrhoea during the second treatment week or the last 7 days of reporting
- Change from Baseline in average severity of abdominal discomfort during the second treatment week or the last 7 days of reporting
- Change from Baseline in average severity of abdominal bloating during the second treatment week or the last 7 days of reporting
- Change from Baseline in average BSFS during the second treatment week or the last 7 days of reporting

Treatment efficacy comparisons with the diary recordings from Baseline period 1 (with pre-study bile acid sequestrant treatment) will also be done.

Exploratory endpoints:

- Change from Baseline 2 in FGF19 at Visit 4 and Visit 5
- Change from Baseline 1 in FGF19 at Visit 4
- Change from Baseline 2 in C4 at Visit 4 and Visit 5
- Change from Baseline 1 in C4 at Visit 4
- Change from Baseline 2 in total s-BA at Visit 4 and Visit 5
- Change from Baseline 1 in total s-BA at Visit 4

SAFETY ENDPOINTS

The primary safety endpoint will be the occurrence of treatment-emergent SAEs during the two treatment weeks. Description and severity of any SAE will also be reported.

Secondary safety endpoints will include:

- Occurrence of TEAEs during the two treatment weeks. Description and severity of any AE will also be reported;
- Changes in laboratory test results (including haematology, clinical chemistry and urinalysis) from baseline to the end of treatment visit (Visit 4);
- Discontinuations from treatment with reasons given.

STATISTICAL METHODS

The primary hypothesis to be tested in this study is that A3384 administered in a daily dose of 1 g twice daily for two weeks is effective in treating BAM/BAD as measured by the BM response compared with placebo.

The sample size for this study is determined to provide 90% power at a 5% significance level to detect a 40% reduction in mean # of BMs during the last week of treatment for the high dose treatment group compared with placebo (with an assumption of a mean # of BMs in the placebo

group of 4 with a SD of 1.5). Twelve (12) patients per treatment group are required to achieve the desired power under the stated assumptions if there is a subject dropout of 10%. The assumptions and method for the power calculation are described more detail in Section 10.1.

The Intent-to-treat population (ITT) will include all randomized patients with the exception of patients who had no post-baseline patient diary data (BM or GI symptoms) collected.

For all efficacy analyses, patients/data will be summarized and analysed as randomized.

The Safety Population will be used for safety evaluations and will include all randomized patients who received at least 1 dose of study treatment and for whom follow-up safety data are available.

TIME AND EVENTS SCHEDULE

Study Activity (See Sections 8.3.2 through 8.3.5 for appropriate order of activities at each visit)	Screening - Baseline Period for symptom registrations		Treatment Period		Follow-up Period
	Visit before Baseline period 1[1]	Visit before Baseline period 2[2]	START	END	
Study Days	–21	–14	1	15	22
Allowed days for the Visit	–21 to –24	–14 to –17		15 to 18	22 to 25
Visits	Visit 1	Visit 2	Visit 3	Visit 4	Visit 5
Informed consent	•				
Randomization			•		
Inclusion/exclusion criteria	•	•	•		
Demographics	•				
Medical and surgical history	•				
Physical examination	•			•	•
Vital Signs	•	•	•	•	•
GI symptom evaluation (paper diary)	•	•	•	•	
Clinical laboratory tests and blood for pharmacodynamic markers	•	•	•	•	•
Pregnancy test	•	•	•	•	•
Study medication dispensed			•		
Study medication compliance evaluated				•	
Concomitant medication documented	•	•	•	•	•

Study Activity (See Sections 8.3.2 through 8.3.5 for appropriate order of activities at each visit)	Screening - Baseline Period for symptom registrations		Treatment Period		Follow-up Period
	Visit before Baseline period 1[1]	Visit before Baseline period 2[2]	START	END	
Study Days	–21	–14	1	15	22
Allowed days for the Visit	–21 to –24	–14 to –17		15 to 18	22 to 25
Visits	Visit 1	Visit 2	Visit 3	Visit 4	Visit 5
AEs documented	•	•	•	•	•

[1] Period with pre-study bile acid sequestrant treatment
[2] Period without pre-study bile acid sequestrant treatment

PROHIBITED MEDICATIONS

From the Visit 1 up to Visit 4, drugs with effects on bile acid concentrations in the gastrointestinal tract or drugs with known effects on GI motility are not allowed. **NB:** During Baseline period 1 (up to Visit 2) however treatment with bile acid sequestrants in fixed doses are allowed to be continued.

These drugs include but are not limited to:

- Bile acid sequestrants such as cholestyramine, colesevelam, colestipol
- Sucralfate
- Opioid derivatives such as loperamide, codeine

Other drugs/natural products with possible effects on GI motility (SSRIs, TCAs, dietary fibre supplementation, yoghurt variants etc.) are allowed providing a stable usage of at least 4 weeks before screening commence and during the whole study period from Visit 1 up to Visit 5.

Appendix 3. Data tables

A3.1. Explant Series

A3.1.1. Unstimulated SeHCAT Series

Table A3.1: mRNA expression of genes in arbitrary units (2^-dCt)x100.

	SeHCAT (%)	FGF19	ASBT	IBABP	OSTα	OSTβ	SHP	FXR	SIRT1	SREBP2
BD51	4.40	0.11	111.03	387.45	29.50	42.99	1.24	18.33	48.63	244.53
BD06	5.40	0.46	217.95	324.90	10.27	33.40	1.68	22.61	106.22	481.99
BD25	7.30	0.05	17.54	498.99	22.41	27.72	2.86	13.04	7.41	12.18
BD52	8.80	2.95	270.01	334.04	18.34	55.06	4.49	36.65	14.26	43.53
BD87	18.90	0.17	166.90	368.33	21.87	5.53	3.31	25.38	33.13	189.21
BD10	23.00	1.46	496.92	1142.40	41.78	17.28	9.94	66.07	59.63	116.72
EX45	26.40	0.11	196.43	215.70	14.16	38.66	1.27	32.00	60.67	105.19
BD21	39.00	0.15	37.27	178.51	17.96	25.24	1.08	14.47	7.16	35.70
BD84	48.00	3.84	798.89	801.67	41.29	17.36	7.22	95.33	71.85	246.06
BD13	59.00	1.57	478.66	799.45	27.49	105.85	2.71	53.63	112.74	341.29

A3.1.2. Stimulated SeHCAT Series

Table A3.2: Relative Quotient (RQ) of FXR target genes in ileal explants after 6 hours incubation with 50 uM CDCA

Sample	SeHCAT (%)	IBPAP	FGF19	SIRT1	SREBP2	SHP	OSTα	ASBT
Ex103 a	0.3	1.358486	15.0741	0.604578	0.604578	3.087	7.916	0.565
EX72a	1.40	1.01	16.01	0.85	0.94	0.94	1.33	1.04
Ex111a	3.00	2.85	204.36	2.06	3.83	5.39	2.76	5.22
Ex112 a	4.00	1.41	22.32	0.76	0.79	1.86	2.88	2.29
Ex59 a	7.70	1.66	8.59	1.08	0.47	2.88	1.69	1.26
EX69a	14.40	2.52	409.58	0.77	1.53			
Ex104 a	17.00	2.30	161.68	0.93	0.55	3.17	4.73	0.99
Ex70 a	20.00	1.97	230.40	1.03	1.07	1.70	1.99	0.84
EX62a	22.00	0.94	73.31	0.83	1.68	10.84	3.21	1.59
EX04	22.80	2.05	416.94	0.40	0.87			
EX100 a	24.00	2.64	110.58	0.77	0.62	2.81	2.48	1.03
EX45	26.40		52.93	0.38		2.16	4.42	0.59
EX02	31.00	2.56	266.66	0.57	0.54	2.28	6.25	0.72
EX33 a	33.00	4.79	182.28	1.58	1.06	1.92	2.42	1.04
EX06	51.00		477.60	1.21		5.09	3.09	1.13
Ex113 a	75.00	2.30	83.34	1.40	2.48	0.86	1.27	0.57

A3.1.3. Resveratrol, Cafestol, LCA and UCDA

A3.1.3.1. *Resveratrol*

Table A3.3: FGF19 expression relative to controls in resveratrol stimulated ileal explants

Con	C50	RSV12.5	RSV25	RSV50	RSV100	RSV50+c50	RSV100+c50
1		1.437	0.881	0.515	0.637		
1	128.915	1.359	1.251		2.414		
1	23.396	0.421			0.155		
1	199.396	1.783	0.959	1.638	1.123		
1	28.695		1.852	4.433	3.585		
1			0.933	1.219			
1	87.796		0.746		0.376		
1	40.937	0.429		0.285	0.333		
1	520.283				2.083	246.428	184.715
1					1.526	21.895	23.015
1	8.203			0.499	0.932	16.447	8.168

Table A3.4: SIRT1 expression relative to controls in resveratrol stimulated ileal explants

Controls	C50	RSV12.5	RSV25	RSV50	RSV100
1.		2.711	1.891		1.059
1.	1.660	1.789	1.612	2.161	1.459
1.	1.312	1.858	2.132	1.704	3.181
1.	5.124	4.107	1.880		3.166
1.	1.417	1.815	2.126	1.063	1.338
1.	1.227	2.477	1.007	1.664	1.470
1.	1.221	1.916	1.411	1.229	1.092
1.	0.340	0.477	0.506	0.327	0.373

Table A3.5:ASBT expression relative to controls in resveratrol stimulated ileal explants

Controls	C50	RSV12.5	RSV25	RSV50	RSV100
1.	0.497				
1.		3.878	2.177	3.050	2.566
1.	5.354			1.594	
1.	1.111		0.989	0.893	
1.	0.821				1.748
1.		0.476	0.608	0.916	0.782

Table A3.6: Table G: FXR expression relative to controls in resveratrol stimulated ileal explants

Controls	C50	RSV12.5	RSV25	RSV50	RSV100
1.	0.083	1.610	0.603		0.636
1.				0.365	
1.	2.845	5.775	3.914	2.343	1.568
1.	0.927	1.727	1.915	1.793	1.102
1.	0.655	1.367	1.061	1.065	0.761
1.	0.865	1.721	1.353	1.057	0.672
1.	0.329	0.289	0.355	0.198	0.198

A3.1.3.2. *Cafestol*

Table A3.7: FGF19 expression relative to controls in cafestol stimulated ileal explants relative to controls

CON	C50	CAF 12.5	CAF25	CAF50	CAF100	CAF 200	CAF50+C50	CAF100+C50
1.	268.298		2.074					
1.	110.655	0.418	0.161		0.199			
1.	158.682	4.110	3.500					
1.	122.828		3.204	4.379	1.248	0.732		
1.	117.481		1.139					
1.	290.971			1.861	2.582		311.554	32.600
1.	43.307	0.985	1.202	6.945	2.716			
1.	102.947	27.700	3.466	4.390	5.100			
1.	360.506			3.558	1.200			
1.	92.773				0.089			
1.	17.953	0.679	1.407	0.452	2.534			
1.	577.075			1.554	1.220		566.876	371.669
1.	28.232			3.178	3.011		49.800	15.157
1.	39.526			3.009	2.038		15.073	
1.	71.251			3.400	0.376		25.702	5.340
1.	73.026			8.179	6.204		79.543	25.073

Table A3.8: FGF19 expression relative to C50

C50	CON	CAF50	CAF100	CAF50+C50	CAF100+C50
1.	0.003	0.006	0.009	1.071	0.112
1.	0.002		0.644	0.003	0.982
1.	0.035	0.107	0.537	0.113	1.764
1.	0.025	0.052		0.076	0.381
1.	0.014	0.005	0.075		0.361
1.	0.014	0.085	0.343		1.089
1.	0.057				0.646

A3.1.3.3. *LCA*

Table A3.9: FGF19 with LCA relative to controls and C50

	CON	L50	C50	C50+L10	C50+L50
Relative to control	1.000	1.158	41.245	12.963	18.381
Relative to C50			1.000	0.314	0.446

A3.1.3.4. *UCDA*

Table A3.10: FGF19 fold change relative to controls with UCDA Values with a SE<0.3 have been removed

CON	UR12.5	UR25	UR50	UR100	C50	C50+UR100	C50+UR50	OCA5	OCA5+UR100	OCA5 +UR50
1.		0.743	0.633	0.905	6.370					
1.	7.481		5.748		280.918					
1.	1.117	2.193	0.840		115.183					
					20.965		16.311			
			0.873		15.113		13.471			
1.			18.374	11.361				1392.346	3092.596	3037.636
1.				13.229					1729.036	
1.				18.352				71.953	57.598	122.176
1.			11.759	9.075						
1.								1273.172	1005.041	575.302
1.				1.940	48.899	116.251		55.542	310.885	
1.					44.524	70.307		19.531	82.873	
1.				16.316	552.269	228.524		759.985	2931.039	
1.		3.991			243.981					
1.				2.726	15.973	4.101			36.981	
1.					83.393			78.395	72.328	
1.	2.850		10.445		621.846					
1.			1.763	5.861	67.248	154.069	112.231	62.244	165.512	152.256

Table A3.11: FGF19 fold change relative to OCA5 with UCDA Values with a SE<0.3 have been removed

CON	UR50	UR100	OCA5	OCA5+UR50	OCA5+UR100
0.009	0.003		1.000		0.003
0.002		0.021	1.000		2.706
0.014	0.065	0.255	1.000	1.698	0.800
0.239	2.813	2.171	1.000		5.468
			1.000	0.452	0.789
0.031		0.084	1.000		1.145
		0.035	1.000		5.597
0.051			1.000		4.243
0.244			1.000		0.923
0.001		0.021	1.000		3.857
0.016	0.028	0.094	1.000	2.659	2.446

Table A3.12: FGF19 fold change relative to C50 with UCDA Values with a SE<0.3 have been removed

CON	UR50	UR100	C50	C50+UR100	C50+UR50
0.048			1.		0.778
0.066	0.058		1.		0.891
0.002			1.		
0.063			1.	0.257	
0.020		0.040	1.	2.377	
0.012			1.		
0.002		0.030	1.		
0.015	0.026	0.087	1.	2.291	1.669

A3.2. Colonic release cholestyramine

A3.2.1. CRC Healthy Volunteer Study

Table A3.13: FGF19 (pg/mL) in healthy volunteers (A3850 study)

Day	1	1	1	7	7	7	7	7	7	7	7	7	7	14
Hour	Base line	4	24	0	0.5	1	1.5	2	3	4	6	8	12	24	Follo w up
CRC 1g BD															
F705	162	122	183	95.5	63.6	60	87.7	124	176	218	335	236	444	101	
F717	47.6	62	54.7	61.9	66.7	84.1	111	85.4	60.1	85.4	244	245	201	78.3	258
F709	172	55.7	198	243	143	79	63.8	70.5	61.7	43.4	178	244	280	158	425
F704	152	62.1	185	127	119	122	97.8	68	49.6	50.3	101	268	357	106	140
F711	113	124	68	50.1	34.8	33.9	25.8	26.6	51.8	62.7	197	173	191	95.2	456
F722	109	30.1	43.5	95.5	46.3	30.9	30	36.3	31.5	51.7	160	291	170	42.9	98.4
Questran 4g BD															
232	134	124	33.1	50.3	55.2	48.9	52.2	50.6	36.7	82.7	891	296	344	87.6	449
232	199	164	111. 55	90.6 5	101. 6	82.9 5	76.6	84.8	97.3 5	105. 35	551	266. 5	265	143. 3	328. 5
CRC Placebo BD															
234	118	50.4	43.9	67.5	63.4	70.5	124	109	65.5	111	400	277	163	108	179
240	53.4	75.0	27.5	34.7	34.3	78.5	93.0	95.7	75.9	94.7	115	199	75.8	33.5	27.0
243	264	204	190	131	148	117	101	119	158	128	211	237	186	199	208
247	109	30.1	43.5	95.5	46.3	30.9	30.0	36.3	31.5	51.7	160	291	170	42.9	98.4

Table A3.14: C4 (mmol/L) in healthy volunteers (A3850 study)

Day	1	1	1	7	7	7	7	7	7	7	7	7	7	7	7
Hour	0	4	24	0	0.5	1	1.5	2	3	4	6	8	12	24	Follow up
CRC 1g BD															
241	10.1	16.2	15.2	32.6	31.4	35.9	36.8	30.2	35	31.4	20.9	12.6	11.1	35.3	17.6
242	25	24.3	47.3	25.1	21.2	17.7	27.3	23.9	21.8	29.8	30.4	22.2	11.1	26.7	12.6
244	9.45	39.2	6.84	31.2	24.2	21.7	23.7	24.8	33.9	52.1	50.8	31.7	17.4	40	BLQ
245	5.29	11.2	10.5	6.79	5.4	4.44	5.89	5.43	9.27	14.4	19.6	14.7	6.03	14.6	8.35
246	19.4	32.4	9.38	39.1	37.9	39.1	48.3	48.1	67.9	74.5	60.2	31.7	39.9	24.9	10.1
248	37	64.6	41.5	80.2	70.7	65.6	64.5	61.4	64.2	82	95.7	47.1	28.3	56.7	20
Questran 4g BD															
228	21.4	13.1	64	52.8	42.3	26.8	25.3	29.6	26.2	40.6	33.3	39.1	33.4	58.3	20.1
232	14.9	58.3	118	132	133	111	112	111	132	174	139	65	35.8	124	3.96
Placebo															
234	19.4	36.7	14.6	28.3	22.5	16.6	20.4	23.4	37.9	52.4	20.5	16.4	11.9	39.8	5.73
240	45.2	48.1	52.2	41.6	33.7	28.8	29	37.8	49.2	48.2	30.8	32.9	26.6	57.6	55.4
243	3.92	7.31	11.1	21.8	17	14.9	12	13.8	13.7	13.7	19.4	15.6	9.86	20.3	2.99

247	5.49	16.2	16.3	25.1	18	20.3	18.9	18.7	22.5	23.8	16	12.1	8.43	12.5	6.91

Table A3.15: Serum total BA (means, ng/mL) in healthy volunteers

	Questran			**Placebo**			**CRC**		
	Mean	SD	n	Mean	SD	n	Mean	SD	n
Baseline	2314.43	1818.34	2	3224.6	2816.25	2	2785	2562.17	6
4	1085.84	172.021	2	2699.09	1182.75	2	1635.18	518.62	6
24	880.506	880.506	2	1682.77	1718.65	2	906.601	627.589	6
0	870.725	179.004	2	1476.33	1539.13	2	1188.67	817.716	6
0.5	1530.32	511.068	2	1774.36	1439.41	2	1179.74	507.394	6
1	767.82	1.711	2	2174.87	1033.61	2	1434.18	887.587	6
1.5	701.855	113.993	2	1592.08	887.497	2	1345.39	383.491	6
2	1054	554.858	2	1491.86	806.163	2	1457.11	737.375	6
3	1226	351	2	2556.01	1341.88	2	1526.23	743.691	6
4	2010.56	324.86	2	2578.02	762.374	2	1472.79	540.418	6
6	1729.73	964.673	2	3091.95	1153.24	2	2498.91	669.621	6
8	2077.85	495.01	2	4324.59	535.648	2	2465.62	589.45	6
12	2177.45	112.953	2	4046.96	1302.72	2	3862.38	1371.06	6
24	1626.62	1002.51	2	2407.13	278.897	2	1334.08	1084.8	6
Follow Up	2478.12	2478.12	2	4170.36	3776.45	2	2211.1	1152.8	6

Table A3.16: Faecal total BA (means, ng/24 hours) in healthy volunteers

	CRC			**Questran**			**Placebo**		
	Mean	SD	n	Mean	SD	n	Mean	SD	n
baseline	6967163	7252845	6	6163925	1218321	2	5015114	2402681	4
Treatment	26913050	31086560	6	36300060		1	12895510	12921740	3

Table A3.17: Serum CDCA and its conjugates (means, ng/mL) in healthy volunteers

		Serum								
		CDCA			TCDCA			GCDCA		
		median	SD	n	median	SD	n	median	SD	n
CRC	Baseline	183	721.598	6	19.13	40.3287	6	76.25	153.7786	6
	Treatment	56.2	76.96386	6	11.585	28.46554	6	173.5	152.3685	6
Placebo	Baseline	82.35	166.2898	6	30.15	47.3909	6	127	415.1182	6
	Treatment	59.1	36.53711	6	11.4	11.33646	6	160	181.5944	6
Questran	Baseline	348.5	223.5	2	88.32	85.68	2	705.5	634.5	2
	Treatment	87.5	35.5	2	8.915	3.785	2	272	166	2
		Faeces								
		CDCA			TCDCA			GCDCA		
		median	SD	n	median	SD	n	median	SD	n
CRC	Baseline	121.65	306.7952	6	76.25	153.7786	6	19.85	50.00861	4
	Treatment	570	1032.249	6	173.5	152.3685	6	35.6	130.0928	4
Placebo	Baseline	60.3	36.9944	5	58.75	47.42016	5	15.8	7.436995	3
	Treatment	64.2	503.8192	5	169	135.5668	5	17.1	13.92488	3
Questran	Baseline	98.9	34.1	1	62.15	28.95	1	45.3		0
	Treatment	68		0	16.6	0	1			

A3.2.2. A3384 in BAD Study

Table A3.18: A3384 Weekly and daily symptom scores

number	PLACEBO	lg	250mg	W3 total BMs	W3 Ave BMs / day	W3 ave type 5/6/7 /day	W3 ave type 6/7	W5 total BMs	W5 Ave BM /day	W5 ave type 5/6/7	W5 ave type 6/7 day	Daily Difference no	Daily difference No	Daily difference no	% reduction weekly total BMs	>40% reduction
BAM101	1	0	0	35	5.00	3.43	1.71	28	4.00	3.57	1.57	-1.00	0.14	-0.14	20.00	0
BAM102	1	0	0	41	5.86	5.29	2.29	35	5.00	2.86	0.57	-0.86	-2.43	-1.71	14.63	0
BAM103	0	1	0	48	6.86	6.86	6.86	33	4.71	4.71	4.71	-2.14	-2.14	-2.14	31.25	0
BAM104	0	0	1	74	10.57	10.57	9.43	24	3.43	3.00	1.57	-7.14	-7.57	-7.86	67.57	1
BAM105	0	1	0	43	6.14	5.71	3.71	42	6.00	4.86	2.14	-0.14	-0.86	-1.57	2.33	0
BAM106	0	0	1	29	4.14	4.00	3.43	10	1.43	0.14	0.14	-2.71	-3.86	-3.29	65.52	1
BAM201	0	1	0	39	5.57	5.57	5.43	49	7.00	7.00	6.86	1.43	1.43	1.43	-25.64	0
BAM202	0	0	1	33	4.71	4.71	4.71	46	6.57	6.57	6.00	1.86	1.86	1.29	-39.39	0
BAM301	0	0	1	46	6.57	6.43	5.71	44	6.29	6.14	5.86	-0.29	-0.29	0.14	4.35	0
BAM302	0	1	0	23	3.29	3.14	2.00	12	1.71	0.86	0.00	-1.57	-2.29	-2.00	47.83	1
BAM303	0	0	1	26	3.71	3.57	3.43	25	3.57	2.00	0.57	-0.14	-1.57	-2.86	3.85	0
BAM304	0	1	0	26	3.71	3.71	3.00	26	3.71	3.71	2.86	0.00	0.00	-0.14	0.00	0
BAM305	1	0	0	23	3.29	3.29	3.29	23	3.29	3.14	0.86	0.00	-0.14	-2.43	0.00	0
BAM306	1	0	0	32	4.57	4.57	4.57	32	4.57	4.43	4.43	0.00	-0.14	-0.14	0.00	0
BAM307	0	1	0	30	4.29	4.29	4.14	23	3.29	2.86	2.00	-1.00	-1.43	-2.14	23.33	0
BAM308	1	0	0	33	4.71	2.43	2.14	36	5.14	4.71	3.57	0.43	2.29	1.43	-9.09	0
BAM309	0	0	1	21	3.00	1.57	0.57	22	3.14	0.43	0.14	0.14	-1.14	-0.43	-4.76	0
BAM310	0	1	0	29	4.14	4.14	4.14	9	1.29	0.43	0.29	-2.86	-3.71	-3.86	68.97	1
BAM311	1	0	0	27	3.86	2.57	3.86	13	1.86	0.43	0.29	-2.00	-2.14	-3.57	51.85	1

A3.3. BAD in NAFLD

Table A3.19: BAD in NALFD Master data table (overleaf).

% 5/6/7				42.9		100.0		52.9			
no type5/6/7				3		33		9			
stool/week				7		33		17			
Liver Bx	Steatosis	steatohepati	Steatosis					Fibrosis			Fibrosis
Fibroscan	7.7	6.7	5.6	11.5	6.3	6.8	5.9	7.2	5.5	n	4.6
NAFLD score	-1.27	-2.65	-2.12	2.54	-0.91	-2.00	-0.77	-0.83	-0.82	-0.74	-2.14
Plt	274	281	220	184	160	274	176	301	248	216	277
Alb	41	37	40	38	44	40	45	39	39	33	42
Br	14	7	15	8	16	11	22	13	9	14	10
GGT	24	269	139	68		34			17	77	24
ALT	52	47	47	30	34	71	51	37	59	50	27
AST	30	31	49	29	23	31	32	34	44	46	28
TGs	1.54	2.7	1.41	2.53	1.95	1.41	1.71	3.15	2.16	2.74	0.94
LDL	2.26	1.95	3.44	1.64	2.62	1.44	2.02	0.69	1.86	3.78	1.77
HDL	0.94	1.12	1.22	1.21	1.29	0.89	1.28	1.38	2.16	1.27	1.3
Total chol	3.9	4.3	5.3	4	4.8	3.2	3.9	3.5	3.9	4.9	3.5
Glc	7.1	8	5	11.2	5.1	8.7	6.1	11.9	8.5	6	6.2
BMI	31.99	22.69	27.48	47.27	29.22	26.23	25.25	29.18	36.69	32.08	26.73
Metformin	1	0	0	1	0	1	1	1	1	0	0
DM	1	0	0	1	0	1	1	1	1	0	0
ETOH Units	1	5	1	0	0	0	0	4	0	0	1
Gender	M	M	M	F	M	M	M	F	F	F	F
Age	53	63	39	70	63	50	55	69	36	54	64
Diarrhoea	0	0	0	1	0	1	0	1	0	0	0
C4	40.0	14.2		179.5	76.7	85.7		56.5	53.5		56.2
FGF19	76.0	197.3		54.9	165.3	43.0		49.8	65.8		88.9
DIET1	G/G	T/T		T/T	T/T	G/T		T/T	G/T		G/T
Study No	**FL001**	**FL002**	**FL003**	**FL004**	**FL005**	**FL006**	**FL007**	**FL008**	**FL009**	**FL010**	**FL011**

				22.2							93.8
				2							15
				9							16
fibrosis		Steatosis		Fibrosis					Steatosis	Steatohepat	Fibrosis
5.3	5.4	7.1	5.5	6.7	4.8	4.9	6.2	4.4	5.3	10.4	9.4
-2.59	-1.85	-4.60	0.86	-0.98	-3.51	-0.66	-2.64	-0.32	-2.81	-0.28	-0.42
200	174	470	165	200	232	211	247	160	304	237	216
43	46	42	47	43	46	40	44	41	39	45	42
22	15	49	14	13	15	11	13	14	24	7	5
42	34	22		67	87	493	269	29	37	359	130
43	57	132	15	59	44	59	297	33	52	55	79
34	30	51	25	52	26	49	86	39	35	44	42
0.77	1.4	3.15	3.41	3.31	1.21	1.1	1.5	0.65	2.87	2.52	2.3
2.14	2.73	2.63	1.82	1.71	2.99	2.75	3.15	1.42	2.19	2.07	1.01
1.51	1.03	0.81	0.63	0.69	0.96	1.35	0.97	1.18	1.01	1.38	0.74
4	4.4	4.6	4	3.9	4.5	4.6	4.8	2.9	4.5	4.6	2.8
4.6	4.7	16.1	5.5	5.1	4.6	6.1	2.2	5.3	5	7.8	5.7
25.22	29.27	29.18	26.86	34.35	30.73	22.55	27.17	26.16	29.41	33.15	34.19
0	0	1	1	0	0	1	0	0	0	1	1
0	0	1	1	0	0	1	1	0	0	1	1
6	0	0	0	0	1	4	2	14	0	8	0
M	M	M	M	M	M	M	M	F	M	F	M
37	50	46	67	55	20	63	32	68	53	65	53
0	0	0	0	1	0	0	0	0	0	0	1
25.9	65.6	51.4	9.7	38.3	49.5	21.0			104.6	44.1	25.9
87.0	31.8	46.1	390.1	157.1	167.5	304.8			231.3	68.5	149.8
G/T	Homo G/G		T/T	T/T	G/T	T/T			T/T	G/T	G/T
FL012	**FL013**	**FL014**	**FL015**	**FL016**	**FL017**	**FL018**	**FL019**	**FL020**	**FL021**	**FL022**	**FL023**

	100.0	50.0	100.0		100.0						
	12	9	18		32						
	12	18	18		32						
Steatohepat			Steatohepat		Fibrosis			Fibrosis			
4	3.5	13.6	n	n	4.3	13	6	7	n	4.3	n
-1.81	-1.73	-0.21	0.86	-3.22	-0.49	1.98	1.46	-1.11	0.33	-1.99	-0.41
241	209	198	210	275	247	188	188	247	231	207	80
38	43	38	27	44	43	39	40	44	39	41	37
8	29	23	6	12	39	9	10	13	10	10	9
	32	43	45	35	26		75	269		433	39
38	37	53	42	83	40	26	34	297	73	64	27
31	28	36	37	43	26	28	38	86	53	35	23
3.81	1.46	2.53	0.87	1.19	1.98	1.77	1.86	1.5	2.2	2.84	2.17
3.57	3	1.42	3.66	4.06	0.91	1.13	2.24	3.15	2.06	0.96	2.68
0.9	0.94	0.73	1.24	1.2	0.99	1.37	0.91	0.97	1.04	1.35	0.63
6.2	4.6	3.3	5.3	1.19	2.8	3.3	4	4.8	4.1	3.6	4.3
4.3	4.7	14.9	6.9	5.1	6.5	12.1	7.4	2.2	18	4.6	6
33.89	31.65	34.34	34.81	30.52	38.05	42.17	37.66	40.89	40.14	29.39	
0	0	1	1	0	1	1	1	0	1	0	1
0	0	1	1	0	1	1	1	0	1	0	1
0	15	1	0	0	0	0	1	2	0	14	0
M	M	M	F	M	M	F	M	M	F	M	F
41	48	41	48	42	51	68	66	69	53	48	75
0	1	1	1	0	1	0	0	0	1	0	0
46.5	49.7	45.3	112.6	53.9	88.6	28.4		22.0		23.3	69.6
178.5	368.9	118.7	173.0	69.3	67.5	104.8		74.1		224.4	262.3
T/T	T/T	T/T	G/T	T/T	T/T	T/T		T/T		T/T	T/T
FL024	**FL025**	**FL026**	**FL027**	**FL028**	**FL029**	**FL030**	**FL031**	**FL032**	**FL033**	**FL034**	**FL035**

		0.0							100.0	52.6	
		0							10	10	
		16							10	19	
Cirrhosis		Cirrhosis	Steatohepat		Steatohepat				Fibrosis	Steatohepat	
13.5	n	11.9	n	6.1	4.4	5.3	n	8	5.4	7.4	29.5
-0.40	2.12	-0.52	-4.52	-0.38	-3.34	-0.14	-3.42	0.63	0.06	-3.03	-0.68
232	113	187	323	175	206	247	336	183	209	263	176
41	34	42	40	39	46	35	41	41	43	43	43
9	23	26	15		18	5	4	34	13	5	15
94	60	242	53	26	227	426	57	85	62		337
50	31	97	74	30	202	89	73	65	46	92	272
33	37	62	39	25	91	50	37	39	31	46	129
1.31	0.81	1.36	1.33	2.71	2.67	2.21	1.15	2.35	1.02	11.8	1.17
2.32	2.27	2.21	1.82	2.69	2.62	1.89	2.84	1.09	1.35	2.79	5.88
1.28	1.36	0.97	1.58	1.18	1.27	1.61	0.94	4.4	1.19	0.73	1.99
4.2	4	3.8	4	3.1	5.1	4.5	4.3	5.5	3	6.1	8.4
15.3	8.4	10.3	4.6	5.2	4.9	6.9	5.4	5.5	7.8	5.4	5.3
29.09	28.16	27.27	21.19	30.18	23.72	35.13	32.11	34.64	35.56	29.55	27.99
1	1	1	0	0	0	1	0	1	1	0	1
1	1	1	0	0	0	1	0	1	1	0	1
0	0	0	1	3	0	0	0	0	1	0	1
F	F	M	M	M	M	F	M	F	M	M	M
67	69	55	40	67	37	56	49	65	58	44	51
0	0	1	1	0	0	0	0	0	1	1	0
	25.2	39.1			45.7	9.5	25.4		56.8	185.3	
	130.5	85.2			103.9	201.4	61.1		89.9	55.4	
	T/T	G/T			T/T	T/T	G/G		T/T	G/T	
FL036	**FL037**	**FL038**	**FL039**	**FL040**	**FL041**	**FL042**	**FL043**	**FL044**	**FL045**	**FL046**	**FL047**

Steatosis	fibrosis		Fibrosis	Fibrosis		Fibrosis	Fibrosis	Steatohepatit			Steatohepat
10.4	n	8.4	12.2	n	6.3	13.1	13.1	4.4	6.2	8.8	5.5
-1.40	#VALUE!	0.11	0.59	0.71	-2.81	5.13	-0.13	-3.68	-0.93	-1.27	-3.86
220	243	257	216	197	237	96	247	238	229	183	271
40	38	43	43	41	42	31	39	45	40	46	43
8	6	5	18	13	10	29	10	13	15	20	20
82	25	42	83	32	80	117	26		66	25	91
16	37	38	73	34	94	48	57	39	77	35	103
19	n	27	95	26	46	87	32	17	35	26	35
1.42	6.16	3.34	3.75	1.99	2.48	0.94	1.93	1.6	0.84	2.9	3.25
2.92	n	1.33	1.61	2.3	3.23	1.5	2.3	2.22	2.72	3.49	2.62
1.53	1.04	0.75	0.79	1.2	0.94	1.07	0.72	0.95	1.3	0.89	0.8
5.1	5.7	3.6	4.1	4.4	5.3	3	3.9	3.9	5.2	5.7	4.9
3.8	n	4.5	12.1	8.7	5	4.8	6.4	5.2	6	5.9	5.2
25.67	30.30	38.81	41.08	39.68	28.85	47.57	32.09	22.88	40.06	30.83	29.07
0	0	1	0	0	0	0	1	0	0	0	0
0	0	1	1	1	0	1	1	0	0	0	0
0	2	0	0	0	0	0	0	0	1	2	0
F	F	M	M	F	M	F	M	M	M	M	M
59	18	67	44	55	41	73	71	40	58	59	30
0	0	0	1	0	0	1	0	0	0	0	0
140.1	16.8	94.1	74.9	25.8			69.3		14.7	29.4	47.0
105.5	242.9	73.1	180.8	215.5			98.4		95.2	154.9	72.5
T/T	G/T	G/T	T/T	T/T			T/T		T/T	T/T	G/T
FL048	**FL049**	**FL050**	**FL051**	**FL052**	**FL053**	**FL054**	**FL055**	**FL056**	**FL057**	**FL058**	**FL059**

57.1		100.0									
4		24									
7		24									
	Fibrosis	Fibrosis				Cirrhosis				fibrosis	
3.3	8.3	9	6.1	7.1	6.4	8.6	6	4.4	4.9	10.2	7.4
0.92	-2.55	-0.83	-2.29	-1.22	-5.06	0.28	-3.13	0.18	-1.57	-0.45	-1.40
183	328	332	231	161	335	135	246	297	254	160	206
41	41	39	43	40	49	39	42	40	39	42	43
8	3	9	15	8	27	14	13	12	13	30	14
20		70	24			458	102	18	37	16	73
32	34	38	116	59	81	63	148	24	24	43	118
28	18	91	63	40	57	97	59	28	31	30	59
0.64	2.95	1.94	1.08	1.83	1.93	1.65	1.12	1.23	0.75	2.26	2.15
1.39	1.28	2.2	3.17	2.15	3.29	3.51	3.5	2.06	3.06	1.14	1.67
0.82	1.28	1	0.84	0.82	0.93	1.64	1.29	1.18	1.5	0.73	0.95
2.5	3.9	4	4.5	8	5.1	5.9	5.3	3.8	4.9	2.9	3.6
11.9	12.9	5.5	5.2	3.9	5.1	5.1	6.2	6.6	4.5	5.4	10.8
30.55	30.71	39.71	32.54	31.09	25.06	26.27	22.98	36.63	30.34	27.59	27.38
1	1	1	0	0	0	0	0	1	0	0	1
1	1	0	0	0	0	0	0	1	0	1	1
0	0	22	0	0	0	0	0	2	3	0	10
M	m	f	m	m	m	f	M	F	M	M	M
76	42	44	44	43	31	62	53	71	50	45	43
1	0	1	0	0	0	0	0	0	0	0	0
49.7	35.4	25.9	9.7	30.3	26.5	10.8		86.8	54.2		59.1
43.9	193.5	72.5	229.1	134.2	57.3	329.2		79.8	64.4		72.1
G/G	T/T	G/T	G/T	T/T	G/T	T/T		G/T	T/T		T/T
FL060	**FL061**	**FL062**	**FL063**	**FL064**	**FL065**	**FL066**	**FL067**	**FL068**	**FL069**	**FL070**	**FL071**

					31.6			53.3	57.1		
					6			8	8		
					19			15	14		
		Fibrosis		Fibrosis	Steatohepat	Fibrosis		cirrhosis			Steatohepat
3.8	10.1	7.8	5.9	9.6	4.2	8.6	6.7	n	n	n	n
-1.69	-1.90	-3.99	-2.55	-1.01	-1.88	-4.59	0.53	0.92	-2.89	-1.65	-0.93
171	209	387	302	255	193	320	134	194	236	267	224
42	43	46	42	39	44	42	42	38	41	46	37
11	15	5	8	15	13	11	15	8	13	6	9
18		50	109	39	46	25	34	289	40	23	40
35	59	119	57	102	50	35	27	32	70	19	135
30	45	71	39	78	33	28	16	41			46
2.04	1.24	3.8	2.63	0.75	1.78	2.15	1.23	1.95	1.28	2.08	1.78
3.21	0.78	1.46	1.61	1.94	3.37	1.34	1.08	3.05	4.01	2.09	3.99
0.96	1.06	1.21	0.79	1.52	0.92	0.88	0.66	1.16	1.01	0.96	1
5.1	2.4	4.4	3.6	3.8	5.1	3.2	2.3	5.1	5.6	4	5.8
5.2	5.5	5.4	11.2	6	5.9	5.8	22.7	5.5	4.6	7.2	4.5
27.00	28.94	36.42	24.61	38.05	26.07	20.93	28.01	27.66	34.72	27.59	30.76
0	0	1	1	0	0	0	1	1	0	1	1
0	0	0	1	0	0	0	1	1	0	1	1
0	0	0	0	18	1	0	0	0	2	0	1
m	m	m	f	f	m	f	m	f	m	m	m
43	50	47	46	60	57	34	64	71	35	76	47
0	0	0	0	0	1	0	0	1	1	0	0
	24.4	31.9	34.9	38.0	11.4		8.7	107.0	30.8	100.7	28.4
	86.6	44.3	94.8	43.0	369.1		61.8	57.3	73.0	68.5	44.8
	G/T	T/T	G/T	T/T	G/G		G/T	G/T	T/T	G/T	T/T
FL072	**FL073**	**FL074**	**FL075**	**FL076**	**FL077**	**FL078**	**FL079**	**FL080**	**FL081**	**FL082**	**FL083**

		steatosis					Fibrosis		steatohepat	Fibrosis	cirrhosis
5.5	3.3	5.4	8.7	7.6	6.8	6.3	5.2	4.4	5.8	7.3	14.7
-2.18	-0.12	-2.14	-3.82	-1.21	-1.51	-2.46	-0.55	-2.59	-1.55	-4.66	-0.09
264	177	310	377	310	228	285	303	232	218	364	211
41	38	38	38	38	44	42	40	43	47	42	41
10	16	10	7	10	9	9	13	10	18	7	41
31	38	38	38	38	28		24	42	29	47	157
39	35	41	32	92	35	37	31	139	49	163	48
25	40	39	27	99	35	28	39	47	43	100	38
2.03	1.02	3.09	1.33	3.84	3.04	2.44	1.77	2.28	2.63	1.16	2.24
2.55	2.65	2.02	2.55	1.87	2.76	2.45	2.09	4.48	2.61	2.58	2.41
1.53	1.19	1.08	0.95	0.88	1.16	1.14	1.21	1.18	0.99	1.16	1.17
5	4.3	4.5	4.1	4.5	5.3	4.7	4.1	6.7	4.8	4.1	4.6
4.5	5	10.4	4.8	10.4	5.8	4.8	9.2	5	4.8	5	10.3
27.16	26.12	26.43	36.44	36.20	26.47	27.41	36.58	32.80	29.65	27.44	42.89
0	0	1	0	1	0	0	1	0	0	0	1
0	0	1	0	1	0	0	1	0	0	0	0
2	1	0	0	0	7	2	0	0	1	0	0
f	m	m	f	f	m	m	f	m	m	f	m
66	75	41	27	38	69	64	51	41	65	36	60
0	0	0	1	1	0	0	1	0	0	1	0
		58.1				30.6		55.5			73.4
		51.0				162.8		43.5			30.2
		T/T				G/T		G/T			G/T
FL084	**FL085**	**FL086**	**FL087**	**FL088**	**FL089**	**FL090**	**FL091**	**FL092**	**FL093**	**FL094**	**FL095**

70.6									37.5	0.0	
12									3	0	
17								0	8	11	
		steatosis				Fibrosis		Fibrosis	Steatosis	cirrhosis	Fibrosis
17.3	7.1	no	9.9	no	8	4.3	no	16.3	no	20.9	7.8
1.66	-1.67	-2.44	-3.18	#DIV/0!	-3.22	-1.44	-1.64	1.64	-2.13	-0.23	-2.69
175	186	211	261		264	164	216	123	130	221	386
38	50	43	42		43	44	43	37	43	42	38
6	10	39	30		31	17	5	10	6	11	7
67	196	186	33		43	33	243	297	52	135	44
44	56	46	80		42	43	33	96	95	38	31
40	32	38	33		31	32	29	70	40	45	19
1.28	3.94	4.6	1.89		1.87	1.28	2.65	6.67	1.41	1.95	2.48
1.41	1.71	3.18	2.93		3.63	3.28	4.23	3.09	1.92	1.3	1.8
1.01	0.8	0.97	1.01		1.12	1.14	1.07	1.23	0.94	1.01	0.97
3	4.3	4.6	4.8		5.6	5	6.5	5.5	3.5	3.2	3.9
6.7	4.8	4.9	5.6		4.8	n	4.6	9.9		9.2	15.8
32.09	35.99	24.46	25.93	26.22	31.87	27.33	32.27	31.34	27.43	29.23	27.12
1	0	0	0	0	0	0	0	1	0	1	1
1	0	0	0	0	0	0	0	1	0	1	1
4	15	0	0	2	2	0	0	14	1	0	0
f	m	m	m	f	m	m	m	m	m	f	f
83	48	46	49	25	27	53	48	69	29	55	60
1	0	0	0	0	0	0	0	0	1	1	0
57.5	32.5	22.5	47.6		11.7		13.0	113.7	76.7	93.4	24.7
69.4	986.6	246.0	63.1		145.2		362.0	53.7	66.7	127.7	204.7
T/T	T/T	T/T	G/T		T/T		T/T	G/T	G/T	G/T	T/T
FL096	**FL097**	**FL098**	**FL099**	**FL100**	**FL101**	**FL102**	**FL103**	**FL104**	**FL105**	**FL106**	**FL107**

									100.0		100.0
									14		7
									14		7
Fibrosis		fibrosis		Fibrosis	Fibrosis			Fibrosis		Fibrosis	Fibrosis
5.4	n	8.6	10.3	n	12.1	20.4	7.6	10	n	14.8	6.9
-0.71	-0.64	-0.80	0.01	-3.45	-1.92	-2.27	-0.45	-1.94	-0.37	-1.50	-1.09
203	161	195	262	250	307	143	162	213	177	233	345
40	43	43	36	47	45	46	38	44	38	39	40
15	10	12	10	31	6	32	27	12	9	12	6
26	358	35	83	46	36	79	62	206	126	31	
37	139	34	24	213	51	157	38	55	53	110	49
32	109	34	50	99	29	74	41	48	37	49	49
1.61	1.68	1.63	2.34	0.65	2.05	1.49	1.36	4.05	1.32	1.03	1.52
3.43	1.79	3.61	3.65	2.7	3.39	5	3.06	2.34	2.77	1.6	2.17
1.75	0.75	1.25	0.79	1.1	0.98	0.82	1.22	0.92	1.53	0.73	0.94
5.9	3.3	5.6	55	4.1	5.3	6.5	4.9	5.1	4.9	2.8	3.8
4.9	11.8	4.8	4.9	4.8	10.8	6.5	4.7	4.7	6.6	6.3	19.8
28.98	27.82	39.43	28.16	31.15	32.94	30.12	25.96	29.80	32.09	30.37	36.98
0	1	0	1	0	1	0	0	0	1	1	1
0	1	0	1	0	1	0	0	0	0	0	1
0	0	15	0	0	4	0	13	1	0	1	0
f	m	m	f	m	m	m	m	m	f	m	f
72	39	42	44	32	52	27	63	47	65	67	57
0	0	0	0	0	0	0	0	0	1	0	1
48.5	27.5	57.9		76.0	41.9	29.2	11.0		114.4	13.0	171.2
83.9	31.6	97.0		149.8	77.1	32.0	184.7		73.9	113.0	60.9
T/T	G/T	T/T		T/T	T/T	G/G	T/T		G/G	G/T	T/T
FL108	**FL109**	**FL110**	**FL111**	**FL112**	**FL113**	**FL114**	**FL115**	**FL116**	**FL117**	**FL118**	**FL119**

							83.3
							10
							12
fibrosis	cirrhosis	Fibrosis		cirrhosis	Steatohepat	Fibrosis	
6.1	29.1	5.6	6.1	12.3	10	n	5.3
-2.37	-1.55	-1.73	-1.21	-2.46	-0.52	-1.26	-0.12
175	353	306	298	361	196	212	212
42	38	39	41	40	42	43	39
16	6	9	15	11	11	16	6
43	191			86	33	403	
99	45	39	16	46	22	147	38
39	52	33	18	37	17	91	29
1.56	1.2	1.7	2.1	2.11	1.84	4.09	1.75
2.19	2.25	3.52	1.92	1.28	1.71	4.06	2.85
1.2	0.9	1.41	1.23	0.76	0.85	0.78	1.45
4.1	3.7	5.7	4.1	3	3.4	6.7	5.1
5.2	9.4	6.4	8.3	3.5	6.4	4.6	5.4
26.84	28.61	31.47	27.03	27.54	28.70	25.70	39.65
0	1	0	1	1	0	1	0
0	1	0	1	1	1	1	0
25	1	2	0	0	0	24	0
m	f	m	f	m	m	m	f
39	61	73	61	55	51	50	65
0	0	0	1	0	0	0	1
40.9	17.3	35.5	149.8	106.5	135.0	16.2	124.1
136.0	280.4	53.3	83.4	34.2	81.1	149.8	68.0
T/T	G/T	T/T	T/T	G/T	T/T		T/T
FL120	**FL121**	**FL122**	**FL123**	**FL124**	**FL125**	**FL126**	**FL127**

Appendix 4. Publications and presentations

A4.1. Original Research

Effects of conventional and a novel colonic-release bile acid sequestrant, A3384, on fibroblast growth factor 19 and bile acid metabolism in healthy volunteers and patients with bile acid diarrhoea. Appleby RN, Bajor A, Gillberg P-G, Graffner H, Simren M, Unh KA, Walters JRF. United European Gastroenterology Journal. 2016 July, e-publication ahead of print

Characterising the differences in ileal FGF19 production in patients with primary bile acid diarrhoea. Johnston IM, Nolan JD, Pattni S, Appleby RN, Zhang JH, Kennie SL, Madhan GK, Pathmasrirengam S, Lin J, Hong A, Dixon PH, Williamson C, Walters JRF. American Journal of Gastroenterology, 2016; 111(30) 423-32

A4.2. Reviews

The role of bile acids in functional GI disorders. Appleby, R. Walters, J. Review, Neurogastroenterology and Motility, 2014 Aug; 26(8): PMID 24898156.

A4.3. Editorials

A variant of FGF19 for treatment of disorders of cholestasis and bile acid metabolism. Walters J, Appleby R. Editorial. Annals of Translational Medicine, 2015 May;3(Suppl 1):S7.

Colesevelam effects on faecal bile acids in IBS-diarrhoea. Walters J, Appleby R. Editorial. Alimentary Pharmacology and Therapeutics, 2015 Apr;41(7):696-7.

A4.4. Oral conference presentations

Bile acid diarrhea and low fibroblast growth factor 19 (FGF19) are associated with non-alcoholic fatty liver disease (NAFLD) and metformin use. Moghul I, Appleby RN,′ Khan S, Yee M, Mansousou P, Walters JRF. Oral presentation, DDW, San Diego; May 2016

Primary bile acid diarrhoea is associated with non alcoholic fatty liver disease. Appleby R, Nolan J, Johnston I, Pattni S, Fox J, Walters J. Oral presentation, DDW, Washington; May 2015

A4.5. Poster conference presentations

Diarrhea and high 7-hydroxy-4-cholesten-3-one (C4), but not low Fibroblast Growth Factor 19 (FGF19) are predictors of high Non-Alcoholic Fatty Liver Disease (NAFLD) fibrosis score. Appleby RN, Moghul I, Khan S, Yee M, Mansousou P, Walters JRF. Oral presentation, DDW, San Diego; May 2016

Cafestol but not resveratrol is a partial agonist of farnesoid x receptor and stimulates FGF9 in human ileal explants. Jamie-Oskooei S, Appleby RN, Geers J, Walters JRF. Poster presentation EASL, Barcelona, April 2016

Ursodeoxycholic acid increases obeticholic acid stimulation of FGF19 in human ileal explants. Geers J, Appleby RN, Walters JRF. Poster presentation EASL, Barcelona, April 2016

Bile acid diarrhoea is associated with Gallstones. Appleby R, Nolan J, Johnston I, Pattni S, Walters J. Poster presentation, DDF, London, June 2015.

A positive faecal calprotectin does not predict bile acid diarrhoea. Appleby R, Bin Da T, Walters J. Poster presentation, DDF, London, June 2015

Stimulated expression of ileal Fibroblast Growth Factor 19 by bile acids is impaired in patients with primary bile acid diarrhea. Nolan J, Madhan G, Appleby R, Johnston I, Zhang J, Kennie L, Walters J. Poster presentation to DDW, Washington; May 2015

Farnesoid x receptor agonist induce fibroblast growth factor 19 in human ileal explants. Madhan G, Nolan J, Appleby R, Adorini L, Shapiro D, Walters J. Poster, Falk Foundation Meeting, Freiburg, Germany; October 2014

SeHCAT retention is correlated with bile acid induced expression of fibroblast growth factor 19 in human ileum. Nolan J, Madhan G, Appleby R, Zhang J, Dixon P, Williamson C, Walters J. Poster, Falk Foundation Meeting, Freiburg, Germany; October 2014