The role of FGF19 in the
diagnosis and
pathophysiology of primary
bile acid diarrhoea

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Statement of Originality

The work contained in this thesis is my own and any other work is acknowledged and referenced.

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Abstract

Introduction: PBAD is a common cause of watery diarrhoea. It is diagnosed by the SeHCAT test and is characterized biochemically by elevated bile acid biosynthesis and low fasting FGF19. FGF19 is a gut hormone produced in the terminal ileum which negatively feeds back on hepatic BA synthesis.

Aims: To assess the role of FGF19 in the pathogenesis of PBAD through characterization of fasting and postprandial FGF19 levels and through identification of associations with other biochemical and genetic factors. To show an increase in the fasting level of FGF19 in PBAD patients through the use of an FXR agonist.

Methods: Subjects with PBAD and idiopathic diarrhoea were recruited and their data added to a previously recruited cohort. Fasting FGF19, BA, lipids, B12, vitamins A and D were measured. A subgroup was characterized using post-prandial levels of these factors. SNP genotyping was performed in candidate genes. A subgroup had BA species characterized by UPLC-MS. 10 PBAD subjects entered a study investigating changes in FGF19 following administration of the FXR agonist obeticholic acid.

Results: Low fasting and post-prandial levels of FGF19 have been demonstrated in PBAD with lowest levels in those with low SeHCAT retention. Fasting and postprandial FGF19 correlates with total BAs. No strong associations have been found between diagnosis or FGF19 with vitamins A and D or with genetic variants. Approximately 30% of PBAD subjects have been found with elevated triglycerides and normal or high FGF19. Significant improvement in FGF19 and symptoms has been shown in PBAD subjects with the use of OCA.

Conclusion: Abnormalities in FGF19 levels have been explored with biochemical phenotypes described. A subset of PBAD subjects with elevated triglycerides has been identified. A central role for total BAs in the aetiology has also been shown. Potential therapeutic benefit of OCA has been found warranting further investigation.
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### Abbreviations

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<th>Definition</th>
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<tbody>
<tr>
<td>µl</td>
<td>micromolar</td>
</tr>
<tr>
<td>ASBT</td>
<td>Apical Sodium-linked Bile salt Transporter</td>
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<tr>
<td>BA</td>
<td>Bile Acids</td>
</tr>
<tr>
<td>BAD</td>
<td>Bile Acid Diarrhoea</td>
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<tr>
<td>BAM</td>
<td>Bile Acid Malabsorption</td>
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<tr>
<td>BMI</td>
<td>Body Mass Index</td>
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<tr>
<td>BSC</td>
<td>Bristol Stool Chart</td>
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<tr>
<td>BSEP</td>
<td>Bile Salt Export Pump</td>
</tr>
<tr>
<td>C4</td>
<td>7α-hydroxy-4-cholesten-3-one</td>
</tr>
<tr>
<td>CCK</td>
<td>Cholecystokinin</td>
</tr>
<tr>
<td>cDNA</td>
<td>complemetary Deoxyribonucleic acid</td>
</tr>
<tr>
<td>CI</td>
<td>95% confidence intervals</td>
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<tr>
<td>CT</td>
<td>Computerised Tomography</td>
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<tr>
<td>CYP27A1</td>
<td>sterol 27-hydroxylase</td>
</tr>
<tr>
<td>CYP7A1</td>
<td>cholesterol 7α-hydroxylase</td>
</tr>
<tr>
<td>EC</td>
<td>Enterohepatic Circulation</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic Acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-Linked Immunosorbent Assay</td>
</tr>
<tr>
<td>FGF15/19</td>
<td>Fibroblast Growth Factor 15/19</td>
</tr>
<tr>
<td>FGFR4</td>
<td>Fibroblast Growth Factor Receptor 4</td>
</tr>
<tr>
<td>FXR</td>
<td>Farnesoid X Receptor</td>
</tr>
<tr>
<td>g</td>
<td>grams</td>
</tr>
<tr>
<td>HPLC</td>
<td>High Pressure Liquid Chromatography</td>
</tr>
<tr>
<td>IBABP</td>
<td>Ileal Bile Acid Binding Protein</td>
</tr>
<tr>
<td>IBS</td>
<td>Irritable Bowel Syndrome</td>
</tr>
<tr>
<td>IBS-C</td>
<td>Constipation predominant Irritable Bowel Syndrome</td>
</tr>
<tr>
<td>IBS-D</td>
<td>Diarrhoea predominant Irritable Bowel Syndrome</td>
</tr>
<tr>
<td>ICP</td>
<td>Intrahepatic Cholestasis of Pregnancy</td>
</tr>
<tr>
<td>LRH1</td>
<td>Liver Receptor Homologue 1</td>
</tr>
<tr>
<td>MAF</td>
<td>Mean Allele Frequency</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>mg</td>
<td>milligrams</td>
</tr>
<tr>
<td>mmol/l</td>
<td>millimol/litre</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger Ribonucleic acid</td>
</tr>
<tr>
<td>mSv</td>
<td>milliSieverts</td>
</tr>
<tr>
<td>n</td>
<td>number</td>
</tr>
<tr>
<td>NAFLD</td>
<td>Nonalcoholic Fatty Liver Disease</td>
</tr>
<tr>
<td>ng</td>
<td>nanograms</td>
</tr>
<tr>
<td>ng/l</td>
<td>nanograms/litre</td>
</tr>
<tr>
<td>NTCP</td>
<td>sodium taurocholate co-transporting polypeptide</td>
</tr>
<tr>
<td>OR</td>
<td>Odd Ratios</td>
</tr>
<tr>
<td>OSTα &amp; β</td>
<td>Organic solute transporter heterodimer α and β subunits</td>
</tr>
<tr>
<td>p</td>
<td>p value</td>
</tr>
<tr>
<td>PBAD</td>
<td>Primary Bile Acid Diarrhoea</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>pg/ml</td>
<td>pictogram/millilitre</td>
</tr>
<tr>
<td>r</td>
<td>Spearman’s rank correlation</td>
</tr>
<tr>
<td>SeHCAT</td>
<td>Selenium-Homocholic Acid Taurine</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard Error Mean</td>
</tr>
<tr>
<td>SHP</td>
<td>short heterodimer partner</td>
</tr>
<tr>
<td>SNP</td>
<td>Single Nucleotide Polymorphism</td>
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<tr>
<td>Tc&lt;sup&gt;99&lt;/sup&gt;</td>
<td>Technetium-99</td>
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1. Introduction

1.1 Background
It has been known for over four decades that bile acids can cause diarrhoea. Alan Hofmann first described the condition as cholerheic enteropathy in 1967(1) and much work has been done in the intervening years to further understand bile acid absorption, malabsorption, and to unravel the underlying causes(2)(3). In recent years advances have been made to determine the pathophysiology of the so-called idiopathic type of primary bile acid malabsorption(4)(5). Furthermore the potential for future therapeutics is being explored to augment today's existing modalities. Frustratingly however, it remains under recognized in the medical community, including within the specialty of gastroenterology(6)(7).

In health over 95% of bile acids are reabsorbed at the terminal ileum. In patients with terminal ileal resection or with impaired terminal ileal function secondary to disease such as Crohn’s, bile acids are absorbed to a lesser extent, spilling over into the colon where they cause chloride and water secretion resulting in symptoms. This has been termed secondary, or type 1 bile acid malabsorption (BAM)(8). A similar picture of bile acid malabsorption occurs in patients with a histologically normal terminal ileum(9)(10), and has been called type 2 or idiopathic bile acid malabsorption (IBAM) or primary bile acid diarrhoea (PBAD). The nature of this disorder will be discussed further in the light of recent findings. BAD is also well described in patients with a variety of other gastrointestinal disorders, such as following gastric surgery, post cholecystectomy or in coeliac disease; this is termed type 3 BAD(8).

Although bile acid malabsorption is not life threatening, it can lead to copious watery diarrhoea, the passage of mucus, urgency and to faecal incontinence significantly impairing quality of life. Symptoms of diarrhoea are usually continuous and may last years and often decades. Other symptoms include bloating and abdominal pain, mean this is often diagnosed as diarrhoea-predominant irritable bowel syndrome (D-IBS).

1.2 The Enterohepatic Circulation of Bile Acids
Historical perspective: It was noted in 1883 that following the administration to fistula dogs of beef bile coloured green due to biliverdin, the dog bile changed from brown to green suggesting a possible circulation of bile pigments from the gut to the biliary system(11). The first clear evidence that the enterohepatic circulation may exist was provided by Broun in his 1923 paper examining the output or yield of biliary fistulae in dogs(12). Sheep’s bile was instilled into the stomachs of the dogs by tube. Bile from the dog underwent chloroform extraction and spectroscopy. The bile composition was noted to include choloahaematin which was previously not noted but was rich in sheep bile. Over the coming days the
spectroscopic line representing chlohaematin disappeared thus providing the first 
objective evidence that bile pigments are reabsorbed from the gut.

In 1927 the Nobel committee awarded the Chemistry prize to Henry Wieland and in 1928 to 
Adolf Windaus for their work on elucidating the chemical structure of bile acids. Their use of 
basic chemistry techniques had advanced understanding but arrived at the wrong 
conclusion regarding the steroid structure(13). Desmond Bernal developed techniques in 
the use of x-ray diffraction to study organic compounds and these were applied by Wieland 
and Dane in Munich and Rosenheim and King in London to bile acids. The steroid nuclear 
structure was thus discovered and published in 1934 (14).

In Sweden Jan Sjorvall developed mass spectrometry for the measurement of bile acids 
leading to advancement of the understanding of BA metabolism(15). His research group at 
the Karolinska Institute identified primary and secondary BAs as well as intermediates in the 
biosynthesis of BAs. Volunteers were intubated to identify sites of absorption in the gut and 
it was found that the BA pool circulates twice per meal, work published in 1957(16). The 
éarly 1960s saw advances made in the understanding of conjugated BA absorption in animal 
models and it was shown in 1961 that BAs are transported by active transport against a 
concentration gradient in the distal quarter of ileum in guinea pigs. This was achieved using 
the everted gut sack technique, in which the gut is effectively turned inside out (17). It was 
shown that the distal ileum was also likely the site of conjugated BA absorption in man (18). 
Ileal absorption of conjugated bile acids was thought to be active for a number of reasons. 
Firstly, absorption occurred against a concentration gradient, secondly individual BAs inhibit 
absorption of other BAs, thirdly the absorption kinetics demonstrated saturation features 
and finally absorption was dependent on sodium ions in the lumen(19)(20)(21).

**Biochemistry:** Cholesterol is converted into bile acids in hepatocytes via pathways which 
involve 17 different enzymes. Bile acids consist of a steroid ring which is hydroxylated at 
different positions to give it different biochemical properties and a side chain which maybe 
conjugated with the amino acids glycine and taurine. The principal steps involved in the 
synthesis of BAs are the 7α-hydroxylation of sterol precursors, modification of the ring 
stucture, oxidation and shortening of the side chain and finally conjugation(22).

90 to 95% of human BAs are synthesized via the classical route, initiated by the enzyme 
cholesterol 7α-hydroxylase (CYP7A1). This has been found to be the rate limiting enzyme in 
the production of BAs. The final step in BA synthesis is the conjugation of taurine or glycine 
to carbon 24 of the side chain.

BA are secreted into the biliary tract and stored in the gall bladder. When food enters the 
duodenum, bile is secreted into the small bowel under the effect of cholecystokinin. When 
the bile reaches the terminal ileum over 90% of the BA are absorbed(23). Daily production 
of BA is approximately 600mg per day, with the entire BA pool cycling 6 – 10 times per 
day(24). The bile acid pool size is approximately 2 – 4g.
The terminal ileum
The ileum is the section of small intestine between the jejunum and the ileocaecal valve. Anatomically the ileum is attached to the posterior abdominal wall by its mesentery. Opposite the mesentery are Peyer’s patches which are aggregated lymphatic nodules, numbering about 30 in adulthood. Peyer’s patches are most prominent in the distal ileum. The ileum differs from the jejunum in that it has a smaller diameter and muscular circular folds are reduced or absent in the terminal portion(25).

The clinical implications of the terminal ileum functioning as the site of bile acid reabsorption were realised when Alan Hofmann and Johann Poley published work examining the effect of different resection lengths on individuals symptoms and response to cholestyramine(26). Patients with diarrhoea with ileal resections of different lengths were examined. Stool weight, volume and fat content were measured. Of 12 patients with some remaining ascending colon and with terminal ileal resection lengths of less than 100 cm it was found that 10 responded to cholestyramine. Of 8 patients with resections of more than 100cm none responded to bile acid sequestrant therapy. It was also found that faecal fat content was predictive of response to cholestyramine. All 8 subjects with less than 20g fat responded versus none of five subjects with greater steatorrhoea. This work confirmed the clinical importance of the role of the terminal ileum and importantly established the degree of resection that could be tolerated without a reduction in the bile acid pool size sufficient to cause fat malabsorption.

Genetic influence of GI differentiation has been explored in recent years using animal models. The functional diversity of the GI tract depends on the on-going production of stem cells at the base of intestinal crypts. These differentiate into the main intestinal cell type: enterocytes, goblet cells, enteroendocrine cells and Paneth cells. Gene expression at different regions reflect the physiology of the site; genes encoding proteins for digestion and absorption of macronutrients are mainly jejunal whereas specific transporters for B12 and BAs are expressed in the distal ileum(27). Wnt, Hedgehog and Notch signalling have been found to be important regulatory pathways for differentiation through the use of gene knockout and over expression models(28). Gata4 is a zinc finger transcription factor that is necessary for a range of developmental pathways. It is expressed highly in duodenum and jejunum of adult mice, but not of ileum. In Humans it is highly expressed in jejunum but not in ileum. In experiments using a transcriptionally inactive Gata4, mutant mice were found to have greatly reduced expression of enterocyte genes in jejunum but not in ileum. Furthermore, in the mutant mice there was expression of ASBT in jejunum at a similar level to ileal expression on control mice. These findings show that Gata4 is central to the differentiation of jejunal and ileal tissue(29).

1.3 Bile acid transporters in liver and intestine
Bile acids are secreted into the bile by the bile salt export protein (BSEP) at the canalicular membrane. It is highly expressed in hepatocytes and efficiently pumps conjugated bile acids.
Mutations of its gene ABCB11 have been shown in patients with progressive familial intrahepatic cholestasis type 2, supporting its role in BA export (30). At the basolateral membrane of hepatocytes, BAs are transported from the portal circulation by the sodium taurocholate co-transporting protein (NTCP), gene SLC10A1.

In the intestine, BAs are absorbed actively and passively. Passive absorption occurs down the length of the small intestine (31). Within the ileum the absorption of BA involves the uptake into the enterocyte across the apical surface by active transport. This is performed by the apical sodium linked bile acid transporter (ASBT) which is encoded by the gene SLC10A2. It transports all the major BAs, with very limited uptake of other solutes. Studies have shown expression within the intestine limited to the terminal ileum. Interestingly, ASBT-null mice demonstrate features of BAD and certain cases of idiopathic or PBAD have been shown to have a loss of function mutation of this transporter (30)(32). This mutation appears not to be the cause in most patients (33).

BAs are then bound by ileal bile acid binding protein (IBABP, gene FABP6) within the cytoplasm and transported across the cell. At the basolateral membrane, they are then transported out of the cell by the heterodimer organic solute transporter α and β (OSTα, OSTβ). There has been significant experimental support for the role of OST in BA efflux from ileal enterocytes (30)(34). The expression of these transporters broadly follows that of ASBT with highest concentration in the terminal ileum. They are also found at other sites of bile acid efflux including hepatocytes, and are up regulated on exposure to higher concentrations of BA.

1.4 Regulation of Bile Acid synthesis
The bile acid pool size is maintained within limits by the precise control of BA synthesis through positive and negative feedback mechanisms. When BA concentration increases, the expression of cholesterol 7α-hydroxylase and sterol 12α-hydroxylase is decreased. In circumstances of cholesterol accumulation, cholesterol 7α-hydroxylase expression is increased.

Central to the control of BA synthesis is the Farnesoid X Receptor (FXR). This nuclear receptor binds BAs and activates expression of genes involved in BA metabolism (35). Genes controlled by FXR include those encoding short heterodimer partner (SHP), IBABP, OSTα & β, ASBT and FGF15/19. BAs vary in their potency as FXR agonists, with studies showing that some such as chenodeoxycholic acid act as powerful ligands with other BAs such as cholic acid are less potent (36).

There are two mechanisms responsible for the repression of CYP7A1. Within the liver, SHP transcription is activated by the action of FXR. SHP represses BA synthesis by binding to and inhibiting Liver Receptor Homologue 1 (LRH-1) which is required for the expression of CYP7A1 and sterol 12α-hydroxylase (a cytochrome P450 required for production of cholic acid). It was found that a second pathway of negative feedback must exist as removing
elements of the SHP pathway did not abolish the ability of BAs to self-regulate themselves (22).

It had been suspected for some time that the intestine plays a role in the negative feedback regulation of BA, following studies showing that bile duct ligation in mice led to an up regulation of CYP7A1. This is now know to be a function of fibroblast growth factor 19 (FGF19) and its orthologue in mice FGF15 (37). FGF15 and FGF19 are atypical members of the fibroblast growth factor family which consists of 22 known members. It has been demonstrated that FGF15 is expressed in terminal ileum following activation of FXR in response to BAs. FGF15 is then carried in the portal circulation to the liver where it interacts with fibroblast growth factor receptor 4 (FGFR4) leading to the inhibition of CYP7A1. β klotho is a protein which modulates FGFR4 function. Recent work has shown that β klotho is an endoplasmic reticulum (ER) resident protein that interacts with FGFR4. It is thought that the activity of FGFR4 depends upon its form of glycosylation, and it is through modulating this glycosylation that β klotho exerts its effect (38).

It is now thought of the two pathways that regulate BA synthesis, the FGF19 – FGFR4 axis physiologically predominates. Tissue-specific FXR-null mouse models were treated with the FXR agonist GW4064. In mice lacking liver FXR, CYP7A1 was significantly repressed, but this did not occur in intestinal FXR-null mice, indicating the predominance of the FGF19 regulatory mechanism (39). In man, FGF19 displays a diurnal variation related to peaks in serum BAs. The peaks in FGF19 occurred 90 – 120 minutes after the post-prandial rise in BAs. It was also found that FGF19 levels reduced when patients were given a BA sequestrant and rose following feeding with chenodeoxycholic acid (40).

Work by Schmidt et al has revealed an unexpected regulator of BA metabolism. Experiments on mice have shown that vitamins A and D induce FGF15 expression, leading to reduced BA synthesis. Induction of FGF15 by vitamin D occurs via its receptor, whereas induction by vitamin A occurs through FXR and its heterodimer, Retinoid X Receptor (RXR). This effect on FXR/RXR is independent of BAs suggesting a function as a Vitamin A receptor (41).
1.5 Bile acids and the microbiome

The microbiome: There are 500 – 1000 separate subspecies of bacteria in the GI tract, with anaerobic species predominating. Composition varies between individuals; even identical twins have been shown to have less than 50% of phylotypes in common. There is huge genetic diversity contained in the microbiome with many genes playing metabolic roles including uptake of lipids, vitamin synthesis, metabolism of carbohydrate and modulation of the intestine’s absorptive function. The microbiota also plays a role maintaining health through protective functions such as production of antimicrobial peptides and inhibiting attachment sites. Immunomodulation occurs by induction of T cells and through the activation of dendritic cells resulting in IgA production from plasma cells(42).

BA transformation: BAs which pass into the colon undergo transformation by bacteria through deconjugation, epimerisation, oxidation, hydroxylation and dehydroxylation (43). The result of this biotransformation is the production of secondary BAs including lithocholic acid and deoxycholic acid. Bile salt hydrolases (BSH) which catalyse the breakdown of the C24 N-acyl amide bond in conjugated BAs are thought to be a crucial gateway enzyme to allow further metabolism of BAs by the microbiota. Metagenomic analyses have identified functional BSH in all major bacterial phyla in the gut. Free BA are then made available to other pathways of BA modification generating secondary and tertiary forms(44).

BA signalling: BAs and their metabolites function as signalling molecules not only through the nuclear BA receptor FXR but also through the cell surface receptor TGR5. TGR5 is agonised mainly by secondary BAs. It is widely expressed on endocrine tissue, adipocytes, muscle cells, immune organs, spinal cord and the enteric nervous system. The activation of TGR5 plays a role in energy expenditure and basal metabolism. Its activation may also lead to lower levels of proinflammatory cytokines(45). FXR activation in addition to its direct role
in BA homeostasis plays a role in protecting terminal ileum from bacterial invasion through its induction of enteroprotective genes(46). The degree of activation of these receptors depends on not only the BA quantity but also on the BA profile which is dependent on the microbiome. Diet has also been shown to affect BA composition. Mice fed a high fat diet were found to have a proportional decrease in UDCA versus DCA. Physiologic concentrations of DCA have been shown to increase intestinal permeability(47).

**Antimicrobial properties of BAs:** BAs are directly toxic to cell membranes; this was shown first in experiments with erythrocytes. BAs may dissolve membrane lipids at high concentrations and damage integral membrane proteins. The effect on membranes is determined by the type of BA. Conjugated BAs which are ionised in water exist mainly in the outer part of the phospholipid bilayer whereas unconjugated BAs will diffuse through passively. BAs also affect other bacterial components inducing structural changes in RNA and leading to direct DNA damage through toxic effects to DNA repair proteins. Detergent effect can also lead to denaturation of bacterial proteins. Other effects include oxidative stress, osmotic effects and direct effects from low pH(48).

**Microbiome and IBS:** It is known that in Irritable Bowel Syndrome there is a different proportional composition of bacterial phyla compared with healthy controls with increased Firmicutes and reduced level of Bacteroidetes. There is in general a lower diversity of gut microbiota in IBS (49)(50)(51). In a study of 47 IBS patients mucosal associated microbiota was examined using rectal biopsies. Comparison was made between IBS-C and IBS-D. Bifidobacteria were lower in the diarrhoea group than in the constipation group. Importantly, no attempt was made to diagnose PBAD as no SeHCAT or other test of BA turnover was conducted(52). No work has yet been carried out looking at the microbiome in bile acid diarrhoea.

1.6 Diagnosing Diarrhoea due to Bile Acid Malabsorption
BAD causes symptoms related to diarrhoea due to excess levels of unabsorbed BAs in the colon. This has a number of effects. BAs induce secretion in the colon by activating intracellular secretory mechanisms, increasing mucosal permeability, inhibiting Cl-/OH-exchange and by detergent effects causing mucus secretion. Colonic water secretion depends on the type of bile acid, with micellar concentrations of 3-10mmol leading to secretion (53). Increased colonic permeability can also result in increased motility worsening urgency and diarrhoea (54).

Measurement of total and specific BA in the stool is the absolute standard for defining this disorder. This is not available outside of research settings and is unpopular with patients and laboratory staff. Alternative tests were developed, including C14-labeled glycocholate or taurocholate excretion, but these again relied on stool measurements (55)(56). The development of the gamma emitter, selenium-75-homocholic acid taurine (SeHCAT), was a major advance (57).
SeHCAT remains the mainstay diagnostic tool in diagnosing BAD. A SeHCAT capsule is administered orally, and measured by whole body counting using a standard gamma camera at 3 hours and at 7 days. The usual way to express the result is as percentage retention at 7 days although half-life can also be calculated (58). The labelled BA is absorbed and excreted at the same rate as cholic acid and is resistant to deconjugation and dehydroxylation. It has been shown to have good clinical applicability and predicts reliably a response to BA sequestrants (10)(59)(60). A 7d-retention under 15% is abnormal, with values less than 10% highly predictive of a response to therapy (10). Studies have shown it to have a sensitivity of 100% and specificity of 94% (61). It has been shown that SeHCAT values in the same patient remain remarkably stable over time (62).

There has been discrepancy between North America and Europe in the recognition of primary BA diarrhoea patients. A study using the 14C-taurocholate test demonstrated that although most patients with chronic idiopathic diarrhoea have some degree of bile acid malabsorption, treatment with cholestyramine made no significant difference to stool weight. This paper concluded that BAD was most likely secondary to increased transit rather than being the primary cause of diarrhoea (56). However, other studies have failed to show a correlation between transit and the SeHCAT result, and SeHCAT has also been shown to discriminate accurately between different causes of diarrhoea, providing evidence that BAD is not an inevitable result of diarrhoea (10)(63).

BAD is now thought to be much more common than previously appreciated. For instance, a study at Rotherham General Hospital UK reviewed 304 patients with chronic diarrhoea and divided them into 4 categories: Crohn’s disease with resection, Crohn’s in remission with no resection, post vagotomy/ pyloroplasty, and diarrhoea predominant IBS (64). It found positive SeHCAT results in 97%, 58%, 54% and 33% respectively. Although the proportion of D-IBS patients was the lowest, due to the high numbers of patients with D-IBS this represents the largest group of patients.

A systematic review of 18 studies reporting SeHCAT tests for BAD in chronic diarrhoea found that 32% of patients had SeHCAT retention values of less than 10% (60). It is calculated that the adult population prevalence is 1%; many millions in the world could benefit from having a firm diagnosis established.

An alternative to the SeHCAT test is measurement of serum 7α-hydroxy-4-cholesten-3-one (C4, also known as 7αHCO or 7αC4), the precursor in BA synthesis. This compound has been shown to be a reliable measure of CYP7A1 activity and as such parallels bile acid malabsorption. In comparison with SeHCAT it has a sensitivity of 87-90% and specificity of 79-86% (65)(66). The disadvantage of this test is that C4 is relatively unstable, and specialized laboratory expertise is required in its measurement using high performance liquid chromatography (HPLC) (65). With the addition of tandem mass spectrophotometry, C4 has been used in recent studies of BA and bowel function (67)(68)(69)(70).
It has recently been suggested that measurement of FGF19 maybe prove to be a useful marker in the diagnosis of BAD. FGF19 has an inverse relationship with C4 and reduced levels have been found in patients with PBAD (4)(71). Initial data suggest a specificity of 89% and sensitivity of over 75%, although work on greater numbers is required. In a paper by Lenicek et al examining BAD in inflammatory bowel disease the inverse relationship between C4 and FGF19 was again demonstrated and FGF19 was found to be a useful marker of BAD (72). FGF19 has the advantage of being a simple ELISA.

In many centres, a trial of therapy with a BA sequestrant remains the most common approach (6). This has the advantage of not requiring specialist investigations; however, as treatment can be unpalatable and response variable, requiring titration and alteration (73), this method is far from straight forward for the patient and is likely to lead to a false negative diagnosis. Furthermore a diagnosis likely to require lifelong treatment is best made definitively to ensure patient compliance and for reassurance.

A recent study has shown potential wider application of the SeHCAT scan. 141 IBS patients of all 3 sub-types underwent SeHCAT testing and it was found that retention less than 10% correlated with faster colonic transit time. Stool form, colonic transit and SeHCAT retention was similar between IBS alternating type and IBS diarrhoea type indicating a role for BAs in both these conditions (74).

### 1.6 Treatment

Treatment of bile acid malabsorption has been with anion exchange resins since the 1960s (26). Cholestyramine (RIN colestyramine) binds BAs and prevents them from exerting their effect in the colon. Unfortunately cholestyramine and colestipol are provided as a powdered resin and are unpalatable, leading to reduced patient compliance. The dose required can be variable between individuals and dose should be increased gradually to avoid side effects. Commonly, they can lead to constipation and cramping discomfort which again limits their tolerability.

A newer agent, colesevelam, has more recently become available which comes in tablet form. This has been shown to be better tolerated by patients (73). It is important to remember that these anion exchange resins are capable of binding other compounds and therefore can impair absorption of other drugs and lipid-soluble vitamins. Therefore, bile acid sequestrants should be taken at a different time from other medications.

In patients with type 1 BAD secondary to Crohn’s disease, it has been shown that the transporter ASBT is upregulated by glucocorticoids (75). Treatment with prednisolone can improve symptoms of BAD possibly acting by this mechanism in Crohn's disease. In microscopic colitis, where a considerable proportion of patients have abnormal SeHCAT
tests indicating BAD (76)(77), treatment with budesonide has been shown to be effective in 96% of patients (78), probably through a stimulation of BA absorption (79). Other potential future treatments to increase BA absorption or reduce BA production may become relevant.

1.7 Pathogenesis of primary bile acid diarrhoea

The cause of primary BA diarrhoea has been debated since the characterization of the condition in the 1970s. It seemed reasonable to look for malabsorption as in the patients with secondary causes. Research has focused on three main possible pathogenic mechanisms; mutations in BA transporters in the terminal ileum, reduced length of ileum expressing transporters and increased small bowel transit, with reduced time spent in the ileum. Recently a novel explanation for the pathophysiology of the condition has been postulated in which there is disordered negative feedback of BA production, resulting in over production of BAs which overwhelm the ability of the ileum to absorb them.

There is some evidence that a transporter defect is responsible for a small fraction of cases of PBAD. In one study, a family with congenital PBAD was identified and 4 polymorphisms were identified in SCL10A2, the gene encoding ASBT. In transfected cells, it was shown that although transporter protein expression and trafficking to the membrane were not affected, transport of BAs was abolished (32). A further study looked at 13 patients with PBAD, screening for these polymorphisms. ASBT polymorphisms were found in 5 patients; however these polymorphisms were also found in control subjects and did not appear to affect ASBT function (33). Polymorphisms have also been sought in FABP6 encoding IBABP, but frequencies were found to be similar between patients and controls (80). In the same study transcript expression was analysed for ASBT, IBABP, OSTα and OSTβ in terminal ileal tissue in patients and controls, with no significant difference demonstrated.

Interestingly no impairment of BA transport has been demonstrated in studies of terminal ileal tissue. In fact, active bile acid transport was found to be increased in patients compared to controls in an in vitro study of brush-border membrane vesicles from terminal ileal biopsies in 10 patients (58). In another study, taurocholate uptake into terminal ileal biopsies was also found to be increased in patients (81).

It has also been suggested that fast small bowel transit maybe responsible for reduced BA malabsorption, however the evidence is conflicting. Sciarretta et al failed to demonstrate any significant correlation between SeHCAT result and small bowel transit time, measured by hydrogen breath test after lactulose or by Tc99m-HIDA cholescintigraphy (61). Another study showed significant differences in small bowel transit between controls and those with PBAD in women, although in men the difference was less pronounced (82). Furthermore, the migrating motor complex phase III induced jejunal secretion may be upregulated in BAD patients which may overload reabsorption capacity in the colon (83).

1.8 FGF19 in BA diarrhoea
With research failing to demonstrate clear abnormalities in BA transport and with conflicting evidence with regard to the role of transit, other possible causes of primary BA diarrhoea were considered.

Van Tilburg et al published data in 1992 where, as expected, the BA content of stools was significantly raised in patients with primary bile acid malabsorption. Counterintuitively, their data also showed that the BA pool size was in fact increased (7.0mmol in patients versus 3.7mmol in controls) (84). It has been known for many years that BA synthesis, as measured by C4, is increased in PBAD. This previously had been explained as a compensatory mechanism for increased BA loss.

When these data are interpreted together with evidence of normal or increased BA mucosal transport and lack of transporter defect in the majority of patients with primary bile acid diarrhoea, it becomes apparent that there may be no true malabsorption of BAs. It has been suggested that the disorder may best be explained by a mechanism in which there is uncontrolled BA synthesis due to a lack of negative feedback control, thereby overwhelming the ability to absorb BA (4).

As discussed above, the FGF19 – FGFR4 axis has been shown to be the primary means of negative feedback controlling BA synthesis (37)(39). Our group has postulated that primary BA diarrhoea results from impaired negative feedback secondary to disordered signalling by FGF19. Work on FGF15-knockout mice has shown that they develop diarrhoea similar to the patients. In a drug safety study looking at the use of anti-FGF19 antibody in cynomolgus monkeys it was found that they developed severe diarrhoea in addition to weight loss and liver toxicity (85). In research published in 2009, 17 patients with chronic watery diarrhoea, 13 of whom had a SeHCAT test all with retention of less than 8% at one week, were compared with controls. Fasting FGF19 and C4 were measured. Median C4 was significantly higher in the patient group, (51 versus 18 ng/l). There was a negative correlation between C4 and FGF19, and median FGF19 was found to be significantly lower in patients compared with controls, (120 versus 231pg/l) (4). A much larger prospectively recruited study has confirmed these data and importantly has shown a significant difference in FGF19 between patients with primary BA diarrhoea (diagnosed by abnormal SeHCAT tests) and those with chronic diarrhoea but with a normal SeHCAT result. This indicates that the reduced FGF19 is not just a function of diarrhoea per se (86)(87).

Recent work has demonstrated the importance of genetic variation in the control of BA levels and in turn on bowel transit time. Rao et al have recently looked at the use of chenodeoxycholate in females with IBS-C (69). They found that colonic transit and ascending colon emptying was significantly increased compared with placebo. Stool frequency was increased and stools were looser. There was a significant association between fasting C4 level and bowel transit, however no association with FGF19 was found. It was found that a SNP in the FGFR4 gene was associated with increased transit with the 2 different doses of CDCA administered. The authors suggest that patients with this genotype may be more
susceptible to the effects of endogenous BAs. An association was identified with a SNP in KLB (encoding klotho β), with a different effect of the two doses on the participant subtypes. Another paper has shown that a functional variant of KLB mediating protein stability is associated with colonic transit in IBS-D (70). This work reveals a possible role of genetic variation in individual’s response to endogenous BAs. Such genetic variation may be involved in IBS-D and BAD in determining patient’s clinical response to perturbation of intraluminal BAs and warrants further investigation.

A further connection between low FGF15/19 levels and diarrhoea has recently been uncovered. In a 2010 study mice were given ampicillin or a vehicle. Treated mice were found to have significantly lower FGF15 mRNA and increased levels of primary bile acids in stool (88). These results may shed some light on the causes of antibiotic associated diarrhoea and raise the possibility of some bacterial involvement in primary BA diarrhoea.

Following the discovery of the central role played by FGF19 in the pathogenesis of primary BA diarrhoea, interesting possibilities regarding possible novel therapeutic options have arisen. As mentioned previously, vitamins A and D have been found to self-regulate their absorption in mice through induction of FGF15 and hence repression of BA synthesis. There may be a therapeutic benefit in PBAD in taking exogenous vitamins A or D. In a similar vein, FXR activation through the use of synthetic agonists may have a beneficial effect. Obeticholic acid (OCA) is a semisynthetic FXR agonist developed for use in liver disorders. OCA, (also known as 6a-ethyl-CDCA and INT–747) is about 100-fold more potent an FXR agonist than CDCA. Clinical trials of OCA in primary biliary cirrhosis (PBC) and non-alcoholic steatohepatitis (NASH) have been published or are in progress, with treated patients showing apparent dose-dependent increases in FGF19 (89)(90)(91).

1. 9 Conclusions
Great advances have been made in recent years in exploring the regulation and metabolism of bile acids and this in turn has led to a greater understanding of bile acid malabsorption. The body of research contained in this thesis will focus on the FGF19 - FGFR4 axis in primary BA diarrhoea. As our understanding increases, so will therapeutic options. It has become apparent that there is a large population of patients with this disorder and the means to diagnose them needs to be more available.
2. Hypothesis and Aims

2.1 Hypothesis
The hypothesis is that abnormal signalling by FGF19 is the key abnormality underlying the condition of Primary Bile Acid Diarrhoea. It is hypothesised that differences in fasting, post prandial and/or stimulated serum levels of FGF19 will be detected between individuals with PBAD and those with idiopathic chronic diarrhoea. It is further hypothesised that levels of fat soluble vitamins will differ between subjects and controls and that there are genotypic differences between subjects and controls. Finally it is hypothesised that the low FGF19 levels seen in PBAD will be increased through the administration of the FXR agonist obeticholic acid to patients.

2.2 Aims

Study 1
1. To recruit subjects with unexplained chronic diarrhoea.
2. To characterise subjects as primary bile acid diarrhoea, secondary bile acid diarrhoea or idiopathic diarrhoea on the basis of SeHCAT result and other investigations.
3. To measure serum FGF19, bile acids, lipids, vitamin B12, vitamin A and vitamin D in this group and to describe associations between these factors and other parameters including SeHCAT retention.

Study 2
4. To extract DNA from blood and explore genetic polymorphisms in candidate genes involved in BA homoeostasis in the subject subgroups.

Study 3
5. To characterise BA species in a smaller groups of age and sex matched subjects with PBAD and ID

Study 4
6. To recruit subjects with PBAD and ID into a further study measuring changes in FGF19 and BA over the course of 6 hours in response to meals.
7. To describe different patterns of FGF19 and BA fluctuation in response to meals in patients with PBAD.

Study 5
8. To measure fasting FGF19 in a previously recruited cohort of healthy individuals of different ages and to describe any relationships between age and FGF19.
Study 6

9. To set up a drug study exploring the use of the FXR agonist obeticholic acid (OCA) in subjects with BAD and ID: ‘Obeticholic acid treatment in patients with bile acid diarrhoea: an open-label, pilot study of mechanisms, safety and symptom response’

10. To recruit subjects to the study and investigate changes in fasting FGF19 before and after OCA treatment.

11. To explore changes in meal stimulated response of FGF19 and BAs after OCA treatment.

12. To investigate effect of OCA on symptoms in the subgroups.
3. Materials and methods

3.1 Study 1

3.1.1 Recruitment

Ethical approval for the study was obtained from the Hammersmith and Queen Charlotte's & Chelsea Research Ethics Committee ref 2000/5795.

Patients were recruited prospectively at Imperial College Healthcare NHS Trust, Charing Cross Hospital and Hammersmith Hospital sites. Patients were approached at routine gastroenterology outpatient clinics and when attending for SeHCAT scanning at the Nuclear Medicine Department.

Verbal information and a patient information sheet was provided to patients and opportunity provided to ask any questions. Written consent was obtained.

3.1.2 Patient characterisation

Patients with a history of 3 or more month’s diarrhoea were recruited. diarrhoea was defined as 3 or more stools per day of Bristol Stool Form type 6 or 7. Patient information was recorded on a proforma (Appendix 1). Demographics were noted including ethnicity. Height and weight were recorded and the body mass index (BMI) calculated. Symptoms including stool type, frequency, duration and presence of nocturnal symptoms were noted. Other symptoms including the presence of abdominal pain, weight loss, rectal bleeding, steatorrhoea, urgency and incontinence were also recorded. Past medical History and drug history were taken down. Further clinical information was accessed by interrogating Trust clinical IT systems and accessing medical notes where necessary.

Investigation results were recorded including:

• Full blood count, routine biochemistry, C reactive protein, thyroid function tests and tissue transglutaminase.
• Stool MC&S, Ova, cyst and parasite examination and faecal elastase.
• Colonoscopy, Flexible sigmoidoscopy and colonic +/- ileal biopsy
• Cross sectional imaging if available

When SeHCAT retention was calculated subjects were classified as:

1. Type 1 bile acid diarrhoea
2. Type 2 bile acid diarrhoea or Primary Bile Acid Diarrhoea (PBAD)
3. Type 3 bile acid diarrhoea
4. Idiopathic diarrhoea (ID)
5. Other cause of diarrhoea
6. No diarrhoea
A cut off value of 15% SeHCAT retention at 7 days was used to define normal bile acid retention.

A fasting blood sample was taken in a 4ml serum separator vacutainer and immediately centrifuged at 3100rpm for 10 minutes. 0.5ml was then pipetted into 4 x 2ml vials and stored at -80c. 10ml of blood was also taken in an EDTA vacutainer and was stored at -20c for later DNA preparation.

The recorded information was then pseudanonymised and transferred to a database in accordance with the Data Protection Act.

### 3.1.3 Serum Analyses

#### FGF19

**Reagent preparation**

1. Wash buffer: 20 mL of Wash Buffer Concentrate diluted into deionized water to prepare 500 mL of Wash Buffer.
2. Substrate Solution: 10ml Color Reagent A and 10ml reagent mixed together in equal volumes. 200μL of mixture is used per well.
3. Calibrator Diluent RD5P (1X): 10 mL of Calibrator Diluent RD5P diluted into into 20 mL of deionized water to prepare 30 mL of Calibrator Diluent RD5P (1X).
4. FGF-19 Standard: FGF-19 Standard reconstituted with 1.0 mL of deionized water. Resulting in stock solution at concentration 10,000 pg/mL. Standard and gently agitated for 15 mins. 900 μL of Calibrator Diluent RD5P (1X) pipetted into the 1000 pg/mL tube. 500 μL Calibrator Diluent RD5P (1X) pipetted into the remaining tubes. Stock solution used to produce dilution series (Figure 2). Each tube was mixed thoroughly before the next transfer.

![Figure 2 Use of stock solution to make dilution series. Image taken from R&D systems Quantikine FGF19 manual](92)
Assay procedure

1. 100 μL assay diluent added to each well.
2. 100 μL of standard, control, or sample added to each well and covered with adhesive strip. Samples were done in duplicate.
3. Samples incubated for 2 hours at room temperature
5. 200 μL FGF19 conjugate added to each well and covered using adhesive strip.
6. Incubated 2 hours at room temperature
7. Washed and aspirated 4 times as before
8. 200 μL substrate solution added to each well.
9. Incubated 30 mins.
10. 50 μL stop solution added to each well
11. Optical density of each well measured using microplate reader

[Diagram of assay procedure]

Figure 3 Assay procedure. Taken from R&D systems Quantikine FGF-19 manual(92)
Results calculation

1. Sample readings were averaged and the average zero standard optical density subtracted.
2. Standard curve generated using optical density from standards using Elisa analysis software.
3. FGF19 concentration determined by finding the absorbance value on the Y axis and extending to the curve. Then extended down to the X axis.

Total Bile Acids

Total bile acids were measured by an enzymatic colorimetric method using 3-α-hydroxysteroid dehydrogenase, (Ref. no. 6K9001, Sentinel Diagnostics, Milan, Italy). This assay was performed by the Clinical Chemistry Department, Hammersmith Hospital.

Vitamin D

25 OH vitamin D was measured using a semi-automated method by liquid/liquid chromatography-Tandem mass spectrometry which has been previously described(93). This assay was performed by the Clinical Chemistry Department, Hammersmith Hospital.

Vitamin A

Vitamin A was calculated by reverse phase high performance liquid chromatography following extraction into iso-hexane and re-constitution into ethanol. Detected by UV absorption at 325nm (94). This was performed at Rotherham General Hospital.

Analysis

Data has been combined with a previously recruited prospective cohort of patients and analysed. Subject recruitment, characterisation, recording and assays have been conducted in an identical fashion using the same standards in the current cohort and the previously recruited cohort. It will be made clear in the results section whether the current study cohort or the combined cohort is being described.

Statistical analyses and Power Calculations

GraphPad Prism was used for all data analysis in each of the sub studies. Statistical differences in FGF19, Total BA, vitamins A and D was achieved using non-parametric Mann-Whitney or Wilcoxon paired tests with significance defined as p < 0.05. 2 tailed statistical testing was performed in calculations.

Spearman Rank coefficient was used to seek significant relationships between variables.
3.2 Study 2: Genetic Polymorphisms in PBAD and ID

3.2.1 DNA Extraction

Genomic DNA was extracted from whole blood using a commercial DNA extraction kit (QiAAMP DNA mini kit, Applied biosystems, Foster City, CA). Leukocytes in the whole blood were lysed during the freeze thaw cycle involved with storage of the samples. The minikit works on the principle of DNA binding to a silica membrane, which then undergoes 4 washing steps using mini spin columns and a microcentrifuge, before the DNA is eluted using elution buffer (95).

Procedure

The following DNA extraction was carried on 10 samples at a time.

1. 20 μl QIAGEN Protease pipetted into the bottom of 1.5 ml microcentrifuge tube.
2. 200 μl blood sample added to the microcentrifuge tube.
3. 200 μl Buffer AL added to the sample. Pulse-vortexed for 15 s until mixed to a homogeneous solution.
4. Incubated at 56°C for 10 min.
5. 1.5 ml microcentrifuge tube centrifuged 2s to remove drops from the inside of the lid.
6. 200 μl ethanol (96–100%) added to the sample, and mixed again by pulse-vortexing for 15s. After mixing, the 1.5 ml microcentrifuge tube was centrifuged for 2s to remove drops from the inside of the lid.
7. Mixture pipetted to the QIAamp Mini spin column in a 2 ml collection tube without wetting the rim. Cap closed, and centrifuged at 6000 x g (8000 rpm) for 1 min. QIAamp Mini spin column placed in a clean 2 ml collection tube, filtrate tube discarded. Each spin column closed. Centrifuged at 6000 x g (8000 rpm).
8. Mini spin column opened and 500 μl Buffer AW1 added without. Cap closed and centrifuge at 6000 x g (8000 rpm) for 1 min. Spin column placed in a clean 2 ml collection tube containing the filtrate discarded.
9. Spin column opened and 500 μl Buffer AW2 added without wetting the rim. Cap closed and centrifuged at full speed (20,000 x g;14,000 rpm) for 3 min.
10. Spin column placed in a new 2 ml collection tube, old collection tube with the filtrate discarded. Centrifuged at full speed for 1 min.
11. Spin column placed in a clean 1.5 ml microcentrifuge tube. Collection tube containing the filtrate discarded. Spin column opened and add 200 μl Buffer AE added. Incubated at
room temperature (15–25°C) for 1 min, and then centrifuged at 6000 x g (8000 rpm) for 1 min.

**Determination of DNA purity**

12. 1μl of purified DNA pipetted to optical surface of NanoDrop ND-100 spectrophotometer.

13. ‘Measure’ function selected

This instrument utilises fibreoptics and surface tension to directly measure absorption at 2 wavelengths, 260nm and 280nm. Samples with values outside the ratio 1.7 to 1.9 were extracted again and retested. Samples were diluted with buffer to achieve concentrations of 3 – 25 ng/μl.

**3.2.2 Genotyping**

SNP genotyping was performed on the samples using TaqMan assays (Applied Biosystems, Foster City, CA). This technique utilises commercially produced primers specific to a DNA region to amplify the allele. Chromophore labelled probes specific to the SNP hybridise to the polymorphism allowing allele discrimination. The probe chromophore is at the 5’ end and a quencher is at the 3’ end. If the probe hybridises perfectly with the polymorphism the 5’ nuclease cleaves the 5’ end releasing the chromophore from the quencher, which creates a signal detectable by the StepOnePlus thermal cycler (Applied Biosystems).

![Figure 4 Showing genotyping work flow](96)
Procedure

A genotyping mix was made containing 7.5μl TaqMan genotyping master mix, 0.375μl SNP assay, 6.125μl DNase free water per assay to be performed. 96 well plates were used, 2 wells had no DNA for control purposes.

Genotyping mix procedure

1. TaqMan Universal PCR Master gently swirled
2. 20× SNP Genotyping Assay vortexed and centrifuged briefly.
3. 750μl TaqMan master mix, 37.5μl SNP assay and 612.5μl DNase free water pipetted into sterile microcentrifuge tube.
4. Tube capped and inverted several times to mix.
5. Tube centrifuged briefly
6. 2 wells of a 96 well reaction plate had 1μl water pipetted as controls.
7. 1μl DNA was pipetted into each of up to 94 wells. Careful note was made of sample idetifiers and reaction well allocation.
8. Reaction plate centrifuged 10s
9. 14μl of the prepared reaction mix pipetted into each of the wells
10. Adherent optical cover sealed onto plate.
11. Plate vortexed 2s then briefly centrifuged

Thermal cycling

StepOne software was programmed to cycle according to either Standard Protocol or Alternate Protocol depending on the SNP assay.

Standard protocol: 10minutes at 95°C followed by
40 cycles of: denature period - 15secs at 92°C
               anneal/extend period - 60secs at 60°C.

Alternate Protocol: 10minutes at 95°C followed by
50 cycles of: denature period - 15secs at 92°C
              Anneal/extend period - 90secs at 60°C.
Data was plotted automatically by the StepOne software, with calls assigned to each sample depending on the chromophore signal detected.

3.2.3 Statistical analysis and power calculations

Minor allele frequency (MAF) was calculated for subjects with PBAD and for subjects with ID. Fisher’s exact test was used to compare MAFs using GraphPad Quickcalc software (GraphPad Software Inc., La Jolla CA). A p value of <0.05 was deemed significant. A genetic dominance effect was sought by comparing number of homozygote wild types with combined heterozygous and homozygous mutants, again using Fisher’s exact test. The data was reanalysed comparing MAFs of disease and controls within ethnic groups. SeHCAT and FGF19 values were compared between subjects with wild type and mutant alleles to detect differences using Mann-Whitney rank sum test. Power and sample size calculations were performed using an online calculator (http://osse.bii.a-star.edu.sg/calculation1.php).

Power calculations were performed for the polymorphisms. Quoted MAFs varied widely between SNPs from 0.16% to 48%. In this study a 2 fold difference was sought between disease subjects and controls with a power of 80%. 6 of the 9 SNPs had a MAF of 18% or more. For these SNPs a sample size of 94 was calculated to be necessary. One SNP had a MAF of 9.4%, one of 4.4% and one of < 1 %. For these polymorphisms using the criteria above the samples would be underpowered however it is important to emphasise the hypothesis generating nature of this study and the SNPs with lower MAFs are investigated for the possibility of a much stronger association with the disease phenotype.
3.3 Study 3: Bile Acid Species Characterisation

If abnormalities in FGF19 signalling are the underlying abnormality responsible for the clinical condition of PBAD then it would be expected that there would be an effect on BA concentration and composition. In this sub-study we have in collaboration with colleagues at the Department of Biomolecular Medicine, South Kensington Campus sought to describe serum BA species composition through the use of Ultra Performance Liquid Chromatography – Mass Spectrometry. From the previously recruited cohort described in 3.1, fasting serum samples from 20 patients, 10 with PBAD and 10 with ID were analysed. All the following experimental methodology was performed by colleagues of the Department of Biomolecular Medicine.

3.3.1 Subjects

Subject characteristics are shown in the table. PBAD subjects with SeHCAT of < 10% and ID subjects with SeHCAT > 20% were selected. No formal randomisation or matching process was used however attempts were made to make the 2 groups comparable in terms of clinical characteristics.

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<thead>
<tr>
<th>Subject</th>
<th>Diagnosis</th>
<th>Age</th>
<th>Sex</th>
<th>BMI</th>
<th>Movements/24hrs</th>
<th>Stool Type</th>
<th>Duration yrs</th>
<th>FGF 19</th>
<th>SeHCAT</th>
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<td>F</td>
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3.3.2 Sample preparation protocol

Samples have been prepared and analysed according to the method validated by Want et al and described elsewhere (97). Serum samples were prepared for UPLC-MS analysis by methanol protein precipitation. Cold methanol (150 μL) was added to 50 μL of serum, vortexed for 30 s, incubated at -20 °C for 20 min, centrifuged at 16089g for 20 min, and the supernatant transferred to a clean tube. This supernatant was dried down in an Eppendorf vacuum evaporator, reconstituted in 100 μL of water/methanol 1/1 mixture, and transferred into 350 μL volume 96-well plates. The reconstitution solvent has been modified compared to Want et al. to improve solubilisation of bile acids by addition of methanol. Quality control (QC) samples were prepared by pooling 15 μL of each extract and were used for column conditioning and system stability assessment.

3.3.3 UPLC-MS Analysis

Metabolite extracts (5 μL) were injected onto a 2.1 X 100 mm (1.7 μm) HSS T3 Acquity column (Waters Corp., Milford, MA) and eluted using a 25 min gradient of 100% A to 100% B (A: water, 0.1% formic acid; B: acetonitrile, 0.1% formic acid), with the last 4 min as column re-equilibration. Samples were analyzed using a UPLC system (UPLC Acquity, Waters Ltd., Elstree, U.K.) coupled online to a Q-TOF Premier mass spectrometer (Waters MS Technologies, Ltd., Manchester, U.K.) in negative electrospray mode with a scan range of 50-1000 m/z. MSE mode has been used to distinguish glycine (m/z 74) and taurine conjugates (80, 106 and 124) by their fragmentation pattern. Low energy acquisition has been performed with collision energy of 5 eV while it was set up at 50 eV for the high energy acquisition. Leucine enkephalin was used as the Lockmass.

3.3.4 Data processing and analysis

Chromatograms were processed in both a targeted and untargeted way using QuanLynx and XCMS respectively.

Targeted analysis

BA were annotated based m/z ratio and retention time reported by Want et al. and updated from data collected from the analysis of a mixture of 18 authentic BA standards. Extracted ions chromatograms were created with a mass window of 100 mDa and retention time were specified with a 2 second tolerance. A 1 X 2 smoothing was applied before peak integration. Peak areas were determined by Apex3D algorithm and systematically reviewed manually.

Areas of BA chromatographic peaks were compared by a two-tailed unpaired t-test after variance has been assessed in the control and PBAD groups (Origin Pro 8.6). A p-value of less than 0.05 was taken to be significant.
Untargeted analysis

Peak picking was performed by the centWave algorithm optimised for high mass accuracy UPLC-MS data. Detected features were grouped and chromatograms aligned. Features not detected in one or more sample were kept and background integrated to avoid missing values in the dataset which is then exported.

Multivariate statistical analysis (MVA) was performed with SIMCA P+ v12 (Umetrics, Umeå, Sweden). Principal Component Analysis (PCA) was used to identify outliers and trends in the dataset. A supervised MVA was then performed by introducing sample group knowledge in the model. Partial Least Squares – Discriminant Analysis (PLS-DA) was used to relate experimental data to sample classification. To improve classification of samples and make interpretation easier, orthogonal PLS-DA model (OPLS-DA) was created to separate the (orthogonal components).

3.4 Study 4: Meal Response

See Appendix 2 for full schedule of events.

Subjects were recruited and characterised as described in 3.1.1. Bile acid sequestrants were omitted in the week leading up to the testing day. Subjects were asked to fast from 9pm the night before and the following morning attended the research ward, The Sir John McMichael Centre at 08.30. Consent was confirmed. A peripheral cannula was then inserted and 4ml blood drawn for processing. Blood was centrifuged and decanted as described above. At 9.00am breakfast consisting of cornflakes, 2 slices of toast with butter and jam, a banana and tea/coffee was provided. Total weight 395g, total energy 2153KJ, protein: carbohydrate: fat energy ratio 9%:71%:20%. Peripheral venous blood was collected every 90mins at 10.30am, 12.00pm, 1.30pm and 3.00pm. Lunch was given immediately after the 12.00pm sample collection and consisted of a cheese and lettuce sandwich, crisps, yoghurt and a drink. Total weight 690g, total energy 2860KJ, protein: carbohydrate: fat energy ratio 11%: 58%: 31%. Serum samples were labelled and stored at -80°C immediately for later processing. Serum FGF19 and total bile acids were measured as described at a later date.
3.5 Study 5: Ageing study
In collaboration with Prof Gary Frost’s team of the Nutrition and Dietetics Research Group at Imperial College we have explored fasting serum FGF19 levels in individuals of different ages. Subjects were recruited by the Nutrition and Dietetics Group as part of their research project ‘Comparison of gut appetite hormones in healthy older and young adults’. Ethical approval was provided by the Riverside Research Ethics Committee (REC No 08/H0706/128). Serum samples and data were made available to our group for analysis.

The subjects were all healthy with no acute or chronic disease, and having undergone no surgery or taking any medication known to interfere with gut peptide secretion. They were matched to sex and BMI (within ±3Kg/M²).

Subjects with the following were excluded:

- History of alcoholism or substance abuse within the last 5 years.
- History of major haematological, renal, gastrointestinal, hepatic, respiratory, cardiovascular or psychiatric disease or any other illness or use of any medications including over the counter (OTC) products, which, in the opinion of the investigators, would either interfere with the study or potentially cause harm to the volunteer.
- Medical or psychological condition or social circumstances which could interfere with ability to participate reliably in the trial.
- Women who are pregnant, breastfeeding or unable to maintain adequate contraception for the duration of the study and for one month afterwards.
- Treatment with an investigational drug within the preceding 2 months.
- Volunteers who have donated, or intend to donate blood within 3 months before or following study completion.
- Smokers.

Those on statins, antihypertensives and analgesics were not excluded.

Full study details are described elsewhere but briefly, subjects were admitted for 4 hours to the research ward at 8.30am after an overnight fast from 9pm. A peripheral cannula was sited and blood drawn. This was centrifuged and pipetted into vials and frozen at -20°C. These samples were made available to our group, thawed and assayed for FGF19. Further samples were taken and a test meal provided – these samples were not used as part of our study.
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3.6 Study 6: Obeticholic acid treatment in patients with bile acid diarrhoea: an open-label, pilot study of mechanisms, safety and symptom response (Obadiah 1)

3.6.1 Introduction

Bile acid diarrhoea is the condition in which excess bile acids reach the colon where they exert their toxic effect on colonic mucosa and the enteric nervous system leading to water and chloride secretion and motility changed, resulting in symptoms of diarrhoea and urgency for patients. We have shown lower fasting levels of serum FGF19 in patients with both primary BAD and secondary BAD. Lower levels of FGF19 are associated with bile acid dysregulation with excess bile acid synthesis as reflected by elevated levels of C4 in those with BAD when compared with diarrhoea controls.

Obeticholic acid is a potent Farnesoid X receptor agonist which has been shown in clinical studies to lead to elevated serum FGF19 levels in patients with other medical conditions and healthy volunteers. Its 100 times more powerful a FXR ligand than the natural ligand chenodeoxycholic acid.(89)(90)(91). It is hypothesised that administration of OCA to individuals with primary and secondary BAD will increase their fasting FGF19 level, which may in turn lead to reduced bile acid synthesis with lower C4 levels. If the markers of bile acid biosynthesis are normalised by OCA this may lead to clinical improvement in symptoms.

This study is designed to test this hypothesis by taking subjects with primary BAD and examining the effects of OCA on FGF19, bile acid biosynthesis markers, clinical symptoms and other safety markers.

NB. The OBADIAH study is designed to examine the effects of OCA in 3 groups of patients, those with ID and SBAD in addition to PBAD. Only the PBAD cohort will be discussed in detail in this thesis.

3.6.2 Study design

The study was an open label with no placebo controlled arm. It was a pilot study investigating mechanisms of BAD as well as symptom and safety response to the treatment. The study was sponsored by Imperial College London and Imperial College Healthcare NHS Trust. The study was registered with the Europeans Medicine Agency through the trial register EudraCT (registration number 2011-003777-28). The trial was also registered through the US clinical trials register ClinicalTrial.gov (registration number NCT01585025). Ethical approval was provided by the National Research Ethics Service Committee (REC reference 12/LO/0123). Obeticholic Acid was provided free of charge by Intercept Pharmaceuticals, Inc (San Diego, CA). The use of OCA was approved by the Medicines and Healthcare products Regulatory Authority (MHRA, LONDON, UK).
All subjects had the study explained to them orally and were provided with a patient information sheet (PIS) for further information. Written informed consent was obtained in all cases. Subjects were free to withdraw from the study at any point. The study was conducted in accordance with the principles of Good Clinical Practice. An independent Data and Safety Monitoring Committee reviewed study progress and safety criteria at regular intervals.

Each participant attended for a screening visit to ensure that inclusion and exclusion criteria were met, for screening bloods and for consent purposes. The study lasted 6 weeks for each subject. Any usual bile acid sequestrants were stopped at the start of the study and not permitted throughout. They continued with their other usual medication. Rescue therapy of up to 16mg of loperamide was allowed daily. A symptom diary was kept by the subject throughout the study period (Appendix 2). Every day the time and type of stool (according to the Bristol Stool Form Scale) was recorded. Hours of abdominal pain per day and pain severity on a scale of 0 – 10 were recorded. Additionally urgency and bloating were assessed also on a scale of 0 – 10. Number of loperamide per day was recorded. Patients were asked to record any adverse events on the diary.

Weeks 1 and 2 were a ‘wash out’ phase to allow the effects of any bile acid sequestrants on bile acid physiology to normalize. On the first day of week 3 and the first day of week 5 subjects attended the Sir John McMichael Centre at 8.30am having been nil by mouth from 9pm the night before. On arrival routine observations were performed and bloods were taken for Liver Function Tests, lipids,
total serum bile acids and a sample for centrifugation for later analysis of FGF19 and C4. At 9am OCA 25mg was given immediately followed by a standard breakfast consisting of cornflakes, 2 slices of toast with butter and jam, a banana and tea/coffee was provided. Total weight 395g, total energy 2153KJ, protein: carbohydrate: fat energy ratio 9%:71%:20%.

Subjects had a peripheral cannula inserted and venous blood was collected every 60mins at 10am, 11am, 12pm, 1pm, 2pm and 3pm. Lunch was given immediately after the 12.00pm sample collection and consisted of a cheese and lettuce sandwich, crisps, yoghurt and a drink. Total weight 690g, total energy 2860KJ, protein: carbohydrate: fat energy ratio 11%: 58%: 31%.

3.6.3 Subjects

35 patients with PBAD were recruited from the Nuclear Medicine departments and Gastroenterology outpatient departments of Hammersmith and Charing Cross Hospitals. Subjects were aged 18 to 80 and all had had chronic diarrhoea prior to recruitment. Chronic diarrhoea was defined as 3 or more Bristol Stool type 6 or 7 stools per day for at least 3 months. Patients had had standard investigation for chronic diarrhoea including standard blood tests including TTG and CRP. All had endoscopic assessment of the lower bowel with a flexible sigmoidoscopy or colonoscopy plus biopsy to exclude microscopic colitis. Other causes of diarrhoea were excluded including neoplasia, inflammatory bowel disease, enteric infection, coeliac disease, lactose intolerance and drug induced diarrhoea.

All patients in the Primary BAD group had a SeHCAT scan performed and had a 7 day retention value of less than 10%. All subjects in the idiopathic diarrhoea group also had a SeHCAT performed and had retention of more than 15% at 7 days. Those in the Secondary BAD group all had either had terminal ileal resection or had evidence of overt terminal ileal disease. A SeHCAT scan was not deemed necessary for this group as it is known that >90% of those individuals with resected TI with chronic diarrhoea have a SeHCAT retention of less than 10%. There were 2 subjects in the secondary BAD group with Crohn’s disease who had not had resections. These 2 patients had SeHCAT tests with 7 day retention values of 8% and 10%. The ileal resection lengths ranged from 15 to 71cm with a median of 34cm.

3.6.4 Statistical analysis

The primary endpoint was change in fasting serum FGF19 before and after 2 weeks administration of obeticholic acid. The serum level of FGF19 on D0 immediately before the first dose of OCA and the fasting FGF19 level on D14 immediately before the final dose of OCA. Secondary outcomes were changes in fasting serum total bile acids, C4, lipids and liver function tests. Further outcomes were analysis of areas under the curve of FGF19 and bile acids with comparison of AUC between the first and final days of OCA, D0 and D14.

Clinical symptoms were analysed. Comparison was made between clinical symptoms in week 2, the second run in week in which no bile acid sequestrant or OCA was taken and
week 4, the second week of OCA treatment. Stool frequency was calculated as total stool numbers/week. Stool consistency was calculated as average BSFS for weeks 2 and 4.

A stool index was devised as follow:

\[
\text{Stool index} = (\text{Total stool number} \times \text{mean BSFS}) + 3 \times \text{total weekly loperamide use in mg.}
\]

This index provides a composite score of frequency and consistency whilst allowing for individual’s variable use of loperamide. The score equates one 2mg tablet of loperamide to one type 6 stool.

Data are usually reported as medians and interquartile ranges (IQR). Nonparametric tests were used to look for treatment effects including Kruskal-Wallis comparisons between the three groups, Mann-Whitney U unpaired or Wilcoxon paired rank tests and Spearman rank correlations. Statistical analyses were performed using Winstat for Excel (R. Fitch Software, Bad Krozingen, Germany). P values <0.05 were considered significant.

### 3.6.5 Sample analysis

At each time point blood was drawn into 3 x 4ml gold top serum separator tubes using the BD vacutainer system. 2 serum separator tubes were centrifuged at 3100rpm for 10 minutes. 1ml of serum was pipetted into 4 x vials. These were labelled and stored at -80c awaiting later processing. FGF19 was measured by quantitative sandwich enzyme immunoassay technique using a commercially available kit as previously described (Quantikine catalogue 1900, R&D systems, Minneapolis).

Serum C4 was measured at the Department of Clinical Chemistry, Western General Hospital, Edinburgh using High Performance Liquid Chromatography (HPLC) following solid phase extraction. Total bile acids were measured by an enzymatic colorimetric method using 3-α-hydroxysteroid dehydrogenase (Ref. no. 6K9001, Sentinel Diagnostics, Milan Italy). Liver function tests and lipids were measured by standard techniques by Clinical Chemistry at Hammersmith Hospital.
4. Results

4.1 Study 1

95 patients were prospectively recruited from out-patient clinics and at Nuclear Medicine at Imperial College Healthcare NHS Trust. 3 subjects did not attend one or both nuclear medicine appointments and were thus excluded. 3 further subjects were excluded as they did not fit criteria for diarrhoea on closer questioning. Another 3 subjects were excluded because another cause of diarrhoea was found, 1 idiopathic ulcerative colitis, 1 microscopic colitis and 1 with radiation colitis. 86 subjects were left for analysis.

There were 51 females and 35 males. 41 subjects had SeHCAT retention of > 15% and were classified as idiopathic diarrhoea. 2 subjects were classified as type 1 BAD, 1 with previous right hemicolecction and 1 with evidence if of ileal inflammation on TI biopsy. 34 subjects were classified as type 2 or primary BAD. 8 subjects had type 3 BAD, 6 with a history of cholecystectomy and 2 with coeliac disease.

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Combined data

The data have been combined with a previously recruited prospective cohort of patients. The previous cohort was prospectively recruited by the same criteria, with identical recording and analysis methodology and the same assay standards between October 2008 and October 2010.

In total 197 subjects were classified as either Idiopathic diarrhoea, type 1 BAD, type 2 BAD or type 3 BAD. There were 93 with ID, 60 female and 33 male, median age 48. 35 subjects had secondary BAD (types 1 and 3 combined). 24 female and 11 male with median age 53. There are 69 subjects with primary BAD, 36 female, 33 male. Median age for those with primary BAD is 48.
The focus of this research is primary bile acid diarrhoea. The results and discussion will reflect this with the dataset containing only those without other significant GI disease, that is PBAD and ID.

**General characteristics**

**Age**

The median age for PBAD is 47 and 48 for ID. There is no significant age difference between those with PBAD and those with ID. There were also no differences when PBAD was split by SeHCAT into mild (10.1 – 15%), moderate (5.1 – 10%) and severe (0 – 5%) groups. The median ages for these groups were 48, 45 and 47, respectively.

**Sex**

More subjects with ID are female than those with PBAD. The female to male ratio for ID as a whole is 1.8:1. The ratio for PBAD is 1.1:1. This difference does not meet significance (p=0.15 Fishers). Those with SeHCAT retention of less than 5% the F:M ratio is = 0.8:1; 5.1-10% = 1.4:1, 10.1-15% = 1.2:1. There are no significant differences between any of these groups.

**Ethnicity**

The ethnicity make up of each group and sub group were similar. For ID most (74.2%) were white, followed by Asian (12.9%) with Afro-Caribbean accounting for (4.3%). There were 8 of other race (8.6%). PBAD proportions were white (68.1%), Asian (13.0%) Afro-Caribbean (4.3%) and other (14.5%). No significant differences were found between the groups and subgroups.
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Figure 6 Subject clinical characteristics according to subgroup defined by SeHCAT retention

BMI

The median body mass index of those with PBAD is 27.00kg/m² and 23.95 kg/m² in ID. This is significantly different p = 0.009. Between subgroups the only significant difference is between mild PBAD and ID (p=0.03).

All PBAD and ID subjects were analysed together. BMI correlates with age (R=0.24 p=0.001). This correlation is lost in the PBAD but is present in the ID group (R = 0.37 p = 0.0002)
BMI also correlated with fasting serum triglyceride level in ID and PBAD combined (R = 0.35 p = 0.0001), and in the separate diagnoses in (PBAD: R = 0.35 p = 0.006, ID: R = 0.38 p = 0.07).

BMI correlated negatively with SeHCAT retention (R = -0.18, p = 0.008). This correlation did not reach significance in the ID or PBAD groupings. FGF19 tended to be lower in those with higher BMI (R = -0.11, p = 0.09). There was no significant correlation between BMI and FGF19 in ID or PBAD groups when analysed separately.

**Symptoms**

Symptoms are similar between those with PBAD and those with ID. The median number of bowel motions per day is 5.0 for both PBAD and ID. Similarly the median Bristol stool form is not significantly different between the 2 groups, 6.5 for PBAD and 6.0 for ID.

Those with PBAD tend to report more steatorrhoea, 39% of those with PBAD and 26% with ID (p=0.08). 55% of those with PBAD and a SeHCAT of < 5% report steatorrhoea which is significantly different compared with the whole ID group (p = 0.02).

57% of PBAD and 63% of ID subjects experience abdominal pain. Bloating is 74% and 68% for PBAD and ID respectively. Faecal incontinence is experienced by 36% with PBAD and 39% with ID. All symptoms tend to be worse in PBAD with SeHCAT < 5% than in PBAD with higher SeHCAT retention or compared with Idiopathic diarrhoea.

Those with PBAD have had symptoms for a similar length of time to those with normal SeHCAT retention, 2.00 versus 1.83 years. In those with SeHCAT retention of less than 5% however, individuals have had symptoms significantly longer than those with retention of 5 – 10% (2.00 years) or 10 – 15% (1.5 years).

**FGF19**

Median fasting FGF19 levels were compared between PBAD and ID. PBAD have significantly lower fasting FGF19 than ID (148 pg/ml vs 235 pg/ml, p = 0.0005) (Figure 7).
When PBAD subjects are analysed as subgroups by SeHCAT (mild 0 – 5%, moderate 5.1-10%, severe 10.1-15%) each subgroup has a significantly lower fasting FGF19 than the ID group. Medians: ID 236.0 pg/ml, severe PBAD 95.83 pg/ml, mod PBAD 190 pg/ml, mild PBAD 173.2 pg/ml (Figure 8).

Difference in medians between the different severities of PBAD neared significance between severe and moderate (p = 0.06); and severe and mild (p = 0.08)

---

**Figure 7 Median fasting FGF19**

**Figure 8 Median fasting FGF19 in PBAD subgroups and ID**
Spearman rank non-parametric correlations have been performed, seeking associations between FGF19 and other variables. As has been demonstrated before (87) there is a strong correlation between FGF19 and SeHCAT retention ($R = 0.3345$, $p < 0.0001$; Pearson linear regression $p = 0.0013$ $R = 0.06$).

![Graph showing correlation between SeHCAT and FGF19. Pearson linear regression shown](image)

Figure 9 Correlation between SeHCAT and FGF19. Pearson linear regression shown

There were associations between fasting serum lipids and FGF19. Strongest association was found between FGF19 and total cholesterol ($r = 0.33$, $p = 0.009$) and LDL cholesterol ($r = 0.34$, $p = 0.01$). There is a non-significant association between FGF19 and HDL cholesterol ($r = -0.14$, $p = 0.27$).

**Triglycerides**

Serum triglycerides display some interesting associations. Higher FGF19 is associated with higher serum triglycerides ($r = 0.20$, $p = 0.04$) (See Figure 10). Given that FGF19 correlates with SeHCAT retention one may also have expected triglycerides to correlate positively with SeHCAT retention, that is those with PBAD and low SeHCAT retention to have a correspondingly low triglyceride level to go along with their low serum FGF19. However, there is a significant negative association between triglycerides and SeHCAT retention ($R = -0.32$, $p = 0.0008$). As can be seen from Figure 11 there is a clear negative correlation. In particular however is a subset of subjects with a serum triglyceride level of over 2.4mmol/L the majority of whom have PBAD (12 out of 17) and of the 5 subjects with triglycerides of over 2.4 mmol/L with idiopathic diarrhoea 3 out of 5 have SeHCAT retention of under 20%. 

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These subjects with elevated triglycerides and low SEHCAT retention may represent a separate subset of disease with different underlying aetiology.

Figure 10 Correlation FGF19 vs Triglycerides
A comparison therefore has been made between FGF19 levels of those PBAD subjects with triglycerides of under 2.4 mmol/L against those with levels above 2.4 mmol/L. As can be seen from Figure 12 those with higher triglycerides have significantly higher fasting FGF19 236.9 pg/ml vs 124.7 pg/ml.
Therefore a group of patients has been identified with low SeHCAT retention, high triglycerides and higher than expected FGF19. The significance of this is that these subjects with low SeHCAT retention would be expected to have a low FGF 19, but in fact they have a fasting level slightly higher than those with idiopathic diarrhoea and normal SeHCAT retention (236.9 vs 236.0 pg/ml). Duane et al have examined gene expression of ASBT from terminal ileal biopsies in subjects with hypertriglyceridaemia and controls. Significantly lower ASBT expression was found in hypertriglyceridaemics(99). The fact that this group has normal FGF19 levels implies normal FGF19 production and signalling in a group with a primary transporter defect.

It may be that our subjects with higher triglycerides had higher SeHCAT retention and thus higher fasting FGF19 levels so the SEHCAT retention was compared between the two triglyceride PBAD groups and there was no significant difference (trigs < 2.4 SeHCAT 6.7%, trigs > 2.4 SeHCAT 7.0%, p = 0.94, see figure 13).
Figure 13 SeHCAT retention of PBAD subjects, split according to triglyceride level

Figure 14 Total BA ID vs PBAD

Cholesterol

Cholesterol was measured in 62 of 162 subjects with ID and PBAD. Fasting FGF19 correlates with total cholesterol, see Figure 15. There is no correlation between FGF19 and HDL cholesterol ($r = -0.14, p = 0.27$) however there is a correlation between FGF19 and LDL cholesterol ($r = 0.14, p = 0.03$).
cholesterol ($r = 0.33$, $p = 0.01$). 5 subjects had a cholesterol of 7.0 mmol/L or more, the highest value measured was 9.6 mmol/L. Of these 5 subjects 2 has ID and 3 had PBAD with SeHCAT ranging from 0 to 75%. They are all clinically unremarkable. At the other end of the scale, there were 6 individuals with cholesterol of under 3.5 mmol/L. 3 had PBAD and 3 ID, SeHCAT range 0 – 65%, FGF19 21.7 – 200.4 pg/ml. These patients are also clinically unremarkable.

![Graph showing correlation between total cholesterol and FGF19](image)

**Figure 15 Correlation Total cholesterol and FGF19**

**Bile Acids**

Of all the factors with which FGF19 correlates, the strongest is with serum fasting total bile acids. All ID and PBAD was analysed together. Total BA values were available for 157 of 161 patients. All had FGF19 values. As can be seen from Figure 16, Subjects with lowest fasting FGF19 had lowest BA and those with highest FGF19 had very high BA values ($R = 0.32$, $p < 0.0001$). This correlation is maintained when analysing the PBAD group separately ($r = 0.35$, $p = 0.004$).
There was no significant relationship found between BA and SeHCAT retention, however. Nor was there a significant difference between median fasting BA levels between ID and PBAD (2.25 mmol/L vs 2.1 mmol/L, p = 0.68, Figure 19). Subgroups of PBAD according to SeHCAT (0 – 5%, 5 – 10%, 10 – 15%) also had no difference in BA from each other or compared with ID. Correlation of BA with other variables does not reach significance. BA tend to increase with serum triglycerides (r = 0.19, p = 0.06).
Summary

In this part of Study 1 fasting levels of FGF19, cholesterol, triglycerides and total bile acids have been explored in subjects with ID and PBAD. As has been shown previously, subjects with PBAD have lower fasting levels of FGF19, and FGF19 correlates with SeHCAT retention as a whole and within the two diagnoses of ID and PBAD. It has been shown that triglycerides correlate with FGF19 and SeHCAT retention and that there is a subset of patients with elevated triglycerides, low SeHCAT retention and normal FGF19 who may have PBAD due to reduced ASBT expression.
It has also been shown that fasting total BA correlates strongly with fasting FGF19 across the whole cohort and within the diagnoses, however BA does not correlate with SeHCAT retention.
4.1.3 Fat soluble vitamins A and D
Serum vitamin A ranged from 0.67 – 4.8 µmol/l. Only one subject had a value below our laboratory’s normal range. The subject was a 56 year old female of Asian origin with diabetes and cirrhosis, with a SeHCAT of 1.4% and FGF19 of 260 pg/ml. Liver disease is known to lower serum vitamin A (100). 20 subjects had values above the normal range. The diagnoses were similar in proportion to those subjects within the normal range.

The median serum vitamin A level for the diarrhoea control group was 1.95 µmol/l (IQR 1.50 - 2.33). In the primary BAD group the median was 2.10 µmol/l (IQR 1.83 - 2.39) and in the secondary BAD group 1.90 µmol/l (IQR 1.60 - 2.35) (Figure 20). The differences were not significant between the diarrhoea controls and primary or secondary BAD (p = 0.116, 0.908) or between the two BAD types (p = 0.207).

Figure 20 Serum vitamin A in diarrhoea controls, PBAD and SBAD. IQR, 10 – 90 centiles, outliers.
FGF19 and vitamin A did not correlate ($r = -0.037, p > 0.06$). There was also no correlation when analysed within the diarrhoea control, primary or secondary BAD groups ($r = -0.02, -0.11, 0.30 p = 0.82, 0.45, 0.12$) (Figure 21). Vitamin A also did not correlate with SeHCAT retention ($r = -0.068, p = 0.39$). Again, there was no correlation within groups ($r = -0.14, 0.037, 0.11; p = 0.25, 0.80, 0.56$).

![Figure 21 Vitamin A and FGF19 levels in individual subjects in the 3 groups](image)

Serum 25 OH vitamin D ranged from 10 – 135 nmol/l. Using a generally recognised cut-off of 50 nmol/l, 57% of subjects had hypovitaminosis D (101). Proportions of subjects with BAD above and below 50 nmol/l were similar (51%, 55% $p = 0.55$).

Median serum 25 OH vitamin D level for diarrhoea control subjects was 45 nmol/l (IQR 26.0 – 71.5). Median level for primary BAD was 38 nmol/l (IQR 24.00 – 63.45) and for secondary BAD 47 nmol/l (IQR 27.0 – 62.5) (Figure 22). The differences between diarrhoea controls and primary and secondary BAD was not significant ($p > 0.3, 0.89$). There was no significant difference between primary and secondary BAD ($p = 0.611$).
25 OH Vitamin D did not correlate with FGF19 (r = -0.05, p > 0.55). When analysed within the diarrhoea control, primary and secondary BAD groups there was also no significant correlation groups (r = -0.14, 0.17, -0.29; p = 0.22, 0.27, 0.13) (Figure 23). There was also no correlation between 25 OH vitamin D and SeHCAT retention either as a whole, or within the groups (r = 0.02, -0.011, -0.070, -0.14; p = 0.74, 0.92, 0.62, 0.46).

Figure 22 25 OH Vitamin D in diarrhoea controls, PBAD and SBAD IQR, 10 – 90 centiles and outliers.
Significant differences in serum vitamin A and D were also sought in those with more severe BAD. PBAD with SeHCAT retention of ≤ 5% were compared with diarrhoea controls however they were not significantly different. Vitamin A median 2.08 vs 1.95 p = 0.583, vitamin D median 41.8 vs 45.0, p = 0.78.

Vitamin levels were also analysed according to ethnicity. No significant differences in serum vitamin A were found between ethnic groups. As would be expected subjects of Indian origin had significantly lower serum vitamin D levels than those of white British origin (32.5 vs 50.7 p = 0.004). There were no other significant differences found in vitamin D between ethnic groups. Among subjects of Indian origin those with type 2 BAD had lower vitamin D levels although this was non-significant (15.0 vs 34.4 p = 0.14). Significant differences within the other racial groups were not found. There were no significant differences in vitamin A between type 2 BAD and diarrhoea controls within racial groups.

Neither serum vitamin A nor 25 OH vitamin D level correlated with various other factors including age, body mass index, number of stools per day or duration of illness. Vitamin A and D levels did not correlate with each other. There was also no correlation between Vitamin A and D with total serum BAs (p > 0.7, 0.4).
4.1.4 Vitamin B12

Vitamin B12 data was available for 101 subjects, 62 with ID and 39 with PBAD. Those subjects taking supplemental hydroxocobalamin were excluded from analysis. 4 individuals on regular parenteral hydroxocobalamin were identified. 2 were elderly women aged 79 and 90, one with ID and the other with PBAD. They were clinically unremarkable with no significant co-morbidities. There was a further clinically unremarkable female aged 60 with ID on supplemental B12. There was a 46 year old male on supplemental B12 with no major illnesses but who took a proton pump inhibitor. B12 deficiency is common in the elderly and may relate to diet. Iatrogenic achlorhydria due to PPI may explain the need for supplemental B12 in the 46 year old male.

Imperial College Clinical Chemistry laboratory quotes normal reference range for B12 as 180 – 800 pg/ml (102). 14 subjects had levels < 180 pg/ml. 8 had ID, 6 had PBAD. 8 were female and 6 male. Their clinical characteristics were not significantly different to the rest of the study group. There were no individuals with a B12 level higher than the normal range.

Correlations have been sought between B12 and other biochemical parameters including FGF19, total bile acids, vitamin A and D and triglycerides. There was a trend for higher FGF19 with higher B12 level (Figure 24). The other correlations did not near significance.

![Graph showing correlation between serum B12 and fasting FGF19](image)

**Figure 24** All ID and PBAD subjects: correlation between serum B12 and fasting FGF19

Subjects with PBAD have non-significantly lower median B12 levels than ID (295 vs 375 pg/ml p = 0.15). Subgroups of PBAD divided by SeHCAT retention (0 – 4.9%, 5.0 – 9.9%, 10 –
15%) have similar B12 levels (Figure 26). Comparison between ID and each of the PBAD subgroups does not reveal significant differences in serum B12 level.

Figure 25 B12 levels in ID and PBAD (medians, 10 – 90 centile + outliers)

Figure 26 Serum B12 in PBAD subgroups of differing severity
4.1.5 Reproducibility of FGF19 data

Previous work on FGF19 stability: The short term stability of FGF19 has been investigated by my predecessor Dr Sanjeev Pattni. In studies on 3 healthy subjects blood was drawn and centrifuged. A 0 hour sample was processed for FGF19. Serum was then kept at room temperature on a lab bench. Samples were processed at 3 and 6 hours. After 6 hours the mean percentage change was 8%.

Longer term stability was also assessed. Whole blood from 4 healthy individuals was again drawn and centrifuged and a zero hour FGF19 level measured by ELISA. Samples were stored at room temperature for 6, 12, 24 and 48 hours and processed at these time points. Mean change in FGF19 was 9% at 24 hours and 6% at 48 hours (103).

The effect of freeze – thaw cycles was also determined. Serum from 3 healthy volunteers was frozen and then thawed twice over a period of 5 days with no significant changes FGF19. Mean change was +15%.

Reproducibility of Fasting FGF19: Of the 21 subjects who underwent serial sampling for FGF19 in the day series study (Study 4) 20 had previously provided a fasting sample for FGF19 analysis in Study 1. A comparison has been made of the fasting values in these 20 subjects on the 2 testing days. The time between the samples varied widely between subjects between 2 weeks and 5 months. The percentage change varied from −34 to +542%. The % change was made +ve for each individual and the median was calculated as 36.5% difference between the 2 testing days.

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Table 4 Fasting FGF19 from study 1 and at later date from study 4
Most (12/20) subjects had variation of less than 65 pg/ML between the two samples however 5 showed much greater variation with differences of > 200 pg/ML.

![Figure 27 Fasting FGF19 from study 1 and at later date from study 4](image)

4.1.6 Biological significance of results

It has been confirmed in study 1 that those with PBAD have lower fasting FGF19 than those with ID and furthermore that FGF19 correlates with SeHCAT over the whole cohort and within ID and PBAD. The biological significance of this is that it shows perturbation of the homeostatic mechanism regulating the bile acid pool. Although it is not possible to say whether the lower FGF19 level is the primary abnormality, there is increased BA synthesis and turnover due to reduced feedback from the FXR – FGF19 axis. This increased synthesis has been shown previously through higher levels of C4(86).

The finding of a subset with high triglycerides and normal FGF19 is important and its biological significance is that it may reflect reduced BA absorption at the enterocyte, possibly due to reduced ASBT expression or transport.

The finding that self-reported steatorrhoea is more common in severe PBAD is relevant as this suggests fat malabsorption. The simplest explanation would be ‘true’ BA malabsorption leading to reduction in bile acid pool size. Other than the triglyceride findings above there are no biochemical results to suggest actual BA malabsorption.
B12 levels are non-significantly lower in PBAD than ID and B12 nears significant correlation with FGF19. The significance of lower B12 is that this may reflect small intestinal bacterial overgrowth which is known to cause B12 deficiency. The near correlation of FGF19 and B12 may be due to reduced BA concentration in the TI. Lower BA would have less bactericidal properties and would lead to less FXR activation and vice versa.
4.2 Genetic polymorphisms

161 subjects had blood taken for DNA extraction and later analysis. The general characteristics of the group are described in detail in Study 1 section of results. Successful DNA extraction occurred in all 161 patients. All but 4 subjects had an A260/A280 nanodrop purity of 1.7 – 1.9. These samples were then re-extracted and then fell within range. One subject yielded no results due to accidental loss of extracted DNA. In total there were 1326 successful genotype calls from the sample set with 123 fails, a total success of 91.5%. Two batches of genotyping failed. The first affected SNP rs61755050 was due to expiration of SNP assay. Unfortunately time limitations did not permit a repeat of this experiment with fresh SNP assay. The second batch failure was the final batch run and this was due to running out of extracted DNA, due to its use in previous experiments. When these batches are excluded the call rate was 97%.

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<td>PBAD</td>
</tr>
<tr>
<td>FGF19</td>
<td>FGF19</td>
<td>rs1789170</td>
<td>G&gt;A 3' UTR</td>
<td>84</td>
<td>0.357</td>
<td>0.396</td>
</tr>
<tr>
<td></td>
<td></td>
<td>rs948992</td>
<td>A&gt;G 3' UTR</td>
<td>86</td>
<td>0.366</td>
<td>0.331</td>
</tr>
<tr>
<td>FXR</td>
<td>NR1H4</td>
<td>rs61755050</td>
<td>T&gt;C Met173Thr</td>
<td>60</td>
<td>0</td>
<td>0.020</td>
</tr>
<tr>
<td></td>
<td></td>
<td>rs56163822</td>
<td>G&gt;T Transl. start</td>
<td>80</td>
<td>0.044</td>
<td>0.030</td>
</tr>
<tr>
<td>FGFR4</td>
<td>FGFR4</td>
<td>rs376618</td>
<td>T&gt;C Leu136Pro</td>
<td>86</td>
<td>0.285</td>
<td>0.251</td>
</tr>
<tr>
<td>KLB</td>
<td>KLB</td>
<td>rs17618244</td>
<td>G&gt;A Arg728Gln</td>
<td>86</td>
<td>0.186</td>
<td>0.120</td>
</tr>
<tr>
<td>ASBT</td>
<td>SLC10A2</td>
<td>rs188096</td>
<td>C&gt;A Ala171Ser</td>
<td>86</td>
<td>0.098</td>
<td>0.113</td>
</tr>
<tr>
<td>OSTα</td>
<td>SLC51A</td>
<td>rs939885</td>
<td>G&gt;A Val86Ile</td>
<td>55</td>
<td>0.482</td>
<td>0.493</td>
</tr>
<tr>
<td>TNFSF15</td>
<td>TNFSF15</td>
<td>rs7848647</td>
<td>C&gt;T 5'-flanking</td>
<td>87</td>
<td>0.282</td>
<td>0.338</td>
</tr>
</tbody>
</table>

Table 5 Summary table

rs1789170

This is a SNP in the FGF19 gene. There were 5 fails, 156 successful results. Minor allele frequency (MAF) of 37.5%, no significant difference between patient groups and no dominance effect found.

Table 6 rs1789170

<table>
<thead>
<tr>
<th>Dx</th>
<th>AA</th>
<th>AG</th>
<th>GG</th>
<th>A</th>
<th>G</th>
<th>MAF%</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>ID</td>
<td>10</td>
<td>40</td>
<td>34</td>
<td>60</td>
<td>108</td>
<td>35.7</td>
<td>0.48</td>
</tr>
<tr>
<td>PBAD</td>
<td>13</td>
<td>31</td>
<td>28</td>
<td>57</td>
<td>87</td>
<td>39.6</td>
<td></td>
</tr>
</tbody>
</table>
rs948992
Polymorphism in FGF19. There were 3 failed samples with no significant difference in MAF% identified. No dominance effect was found.

<table>
<thead>
<tr>
<th>ID</th>
<th>AA+AG</th>
<th>50</th>
<th>34</th>
<th>0.87</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBAD</td>
<td>44</td>
<td>28</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

rs61755050
FXR SNP 518T>C has previously been shown to affect FXR expression and function (104). Unfortunately later assays failed due to expiry of the SNP assay. Only 2 subjects out of 111 successful genotypes had the minor allele CT. Both have severe PBAD with SeHCAT retention of 3% and 2% and low FGF19 levels of 59 pg/ml and 149 pg/ml respectively. Total BA were 0.9 mmol/L and 5.9 mmol/L. Comparison of genotype frequencies using Fishers exact test did not reach significance. Mann-Whitney test for comparison of SeHCAT and FGF19 between subjects of different genotype is not significant, possibly due to small sample size. For the previously recorded minor allele frequency of 0.16% the study is inadequately powered. For a significance of 0.05 and a power of 0.80 necessary sample size would have to be 494.

<table>
<thead>
<tr>
<th>ID</th>
<th>CC+CT</th>
<th>TT</th>
<th>C</th>
<th>T</th>
<th>MAF%</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBAD</td>
<td>0</td>
<td>2</td>
<td>49</td>
<td>2</td>
<td>100</td>
<td>2</td>
</tr>
</tbody>
</table>
**rs56163822**
This is a SNP in gene FXR with associations demonstrated with IBD and intrahepatic cholestasis of pregnancy. It leads to -1G>T change. There is a low reported MAF of 2.2%. No significant differences have been found between disease and control subjects; however the sample size is too low to detect small differences for this allele frequency.

Table 12 rs56163822

<table>
<thead>
<tr>
<th>Dx</th>
<th>GG</th>
<th>GT</th>
<th>TT</th>
<th>G</th>
<th>T</th>
<th>MAF%</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>ID</td>
<td>74</td>
<td>5</td>
<td>1</td>
<td>153</td>
<td>7</td>
<td>4.38</td>
<td>0.76</td>
</tr>
<tr>
<td>PBAD</td>
<td>63</td>
<td>4</td>
<td>0</td>
<td>130</td>
<td>4</td>
<td>2.99</td>
<td></td>
</tr>
</tbody>
</table>

One individual was homozygous for the minor allele. She had ID with a 7 day SeHCAT retention of 20.9%, fasting FGF19 237.0 pg/ml. There was no GI disease but the patient has bipolar disorder.

No dominance effect was detected. There were 137 successful genotype calls with 13 fails due to technical reasons.

Table 13 Dominance

<table>
<thead>
<tr>
<th>Dx</th>
<th>CC+CT</th>
<th>TT</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>ID</td>
<td>0</td>
<td>60</td>
<td>0.21</td>
</tr>
<tr>
<td>PBAD</td>
<td>2</td>
<td>49</td>
<td></td>
</tr>
</tbody>
</table>

**rs17618244**
This is a polymorphism in the KLB gene encoding klotho-β, a protein that modulates the function of FGFR4. Associations with diarrhoea predominant IBS has been shown(105). The G allele was more frequent in PBAD than in ID (p = 0.12). The G allele has previously been shown to be associated with faster gut transit time in response to oral chenodeoxycholic acid. 7 subjects had the genotype AA of whom 6 had ID and 1 had PBAD. The PBAD individual had a SeHCAT of 7.1%. The 6 individuals with ID had 7 day SeHCAT retention ranging from 19 – 51 % with a median of 21.5%. Performing Fisher’s exact test of AA genotype against AG + GG in the disease types reveals a difference in proportions p = 0.07.

There were 4 failed genotype calls.

Table 14 rs17618244

<table>
<thead>
<tr>
<th>Dx</th>
<th>AA</th>
<th>AG</th>
<th>GG</th>
<th>A</th>
<th>G</th>
<th>MAF%</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>ID</td>
<td>7</td>
<td>18</td>
<td>61</td>
<td>32</td>
<td>140</td>
<td>18.6</td>
<td>0.12</td>
</tr>
<tr>
<td>PBAD</td>
<td>1</td>
<td>15</td>
<td>55</td>
<td>17</td>
<td>125</td>
<td>11.97</td>
<td></td>
</tr>
</tbody>
</table>
**Table 15 Dominance effect**

<table>
<thead>
<tr>
<th></th>
<th>DX</th>
<th>AA+AG</th>
<th>GG</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>ID</td>
<td></td>
<td>25</td>
<td>61</td>
<td>0.37</td>
</tr>
<tr>
<td>PBAD</td>
<td></td>
<td>16</td>
<td>55</td>
<td></td>
</tr>
</tbody>
</table>

**rs939885**

This SNP in OSTα has been investigated by others for associations with IBS-D. No significant associations have been found in this study. 34 individuals could not be genotyped because of insufficient DNA due to previous analyses. With very similar MAFs in the 2 groups and a high p value, slightly higher sample size would have made no material difference to significance.

**Table 16 rs939885**

<table>
<thead>
<tr>
<th></th>
<th>DX</th>
<th>AA</th>
<th>AG</th>
<th>GG</th>
<th>A</th>
<th>G</th>
<th>MAF%</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>ID</td>
<td></td>
<td>14</td>
<td>25</td>
<td>16</td>
<td>53</td>
<td>57</td>
<td>48.2</td>
<td>0.90</td>
</tr>
<tr>
<td>PBAD</td>
<td></td>
<td>18</td>
<td>30</td>
<td>19</td>
<td>66</td>
<td>68</td>
<td>49.3</td>
<td></td>
</tr>
</tbody>
</table>

**Table 17 Dominance**

<table>
<thead>
<tr>
<th></th>
<th>DX</th>
<th>AA+AG</th>
<th>GG</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>ID</td>
<td></td>
<td>39</td>
<td>16</td>
<td>1.00</td>
</tr>
<tr>
<td>PBAD</td>
<td></td>
<td>48</td>
<td>19</td>
<td></td>
</tr>
</tbody>
</table>

**rs7848647**

This polymorphism results in a C to T change in the TNFS15 gene at position 116608867. The TNFS15 family of proteins are involved in the aetiology of inflammatory bowel disease and SNPs have been associated with both post infectious IBS and diverticulitis (106)(107).

**Table 18 rs7848647**

<table>
<thead>
<tr>
<th></th>
<th>DX</th>
<th>TT</th>
<th>CT</th>
<th>CC</th>
<th>T</th>
<th>C</th>
<th>MAF%</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>ID</td>
<td></td>
<td>5</td>
<td>39</td>
<td>43</td>
<td>49</td>
<td>125</td>
<td>28.2</td>
<td>0.33</td>
</tr>
<tr>
<td>PBAD</td>
<td></td>
<td>8</td>
<td>32</td>
<td>31</td>
<td>48</td>
<td>94</td>
<td>33.8</td>
<td></td>
</tr>
</tbody>
</table>

Genotyping was successful in 158 of 161 samples. The T allele was found to be more prevalent in the PBAD population, not reaching significance. There was no dominance effect noted. TT subjects did not differ clinically from those with other genotypes.
The FGFR4 variant rs376618 is non-synonymous (Leu136Pro) and has been linked to faster colonic transit in TT homozygotes. It has been investigated previously for a possible role in hepatic tumorigenesis. 156 assays successfully completed.

No significant difference in MAF was found but there was a lower proportion of CT heterozygotes in PBAD compared with in ID subjects (24% vs. 43%) and the frequency of TT versus CC + CT was significantly higher ($p = 0.05$). TT subjects have a significantly lower SeHCAT to those in the combined CT + CC group (11 vs. 19, $p = 0.037$) but FGF19 levels were not significantly different (214 vs. 178 pg/mL, $p = 0.33$).

The minor allele was more common in PBAD, however did not reach significance. For the minor allele frequencies found the sample size would have to be 817 to reach significance [online calculator www.stat.ubc.ca/~rollin/stats/ssize/b2.html].

When a Fisher’s exact test is used to compare frequencies of TT versus CC + CT there is a significant difference in proportions, $p = 0.05$.

Polymorphism in SCL10A2 gene encoding apical sodium linked bile acid transporter (ASBT) with a MAF of 16.0% (108). SNP leads to Ala to Ser substitution at amino acid 171. It has been found to be present in a family with the congenital version of PBAD(32).

No significant difference in MAF was found although the sample size is low for the reported MAF. 3 individuals had the AA genotype, one with ID SeHCAT of 37% and two had PBAD.
both severe with SeHCAT of less than 2%. All 3 individuals were clinically otherwise unremarkable. The ID individual had an FGF19 of 238 pg/ml which is unremarkable. The 2 PBAD individuals with extremely low SeHCAT retention has surprisingly high FGF19 levels of 260 and 313 pg/ml. Triglycerides for the ID individual was 0.76 ml/L, and 1.43 and 1.86 mmol/L for those with PBAD. One of the PBAD subjects had very elevated total BA (14.2 mmol/L) the others BA were well within the normal range.

Analysing subgroups by SeHCAT also reveals no significantly different proportions of alleles.

Table 22 RS188096

<table>
<thead>
<tr>
<th>Dx</th>
<th>AA</th>
<th>AC</th>
<th>CC</th>
<th>A</th>
<th>C</th>
<th>MAF%</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>ID</td>
<td>1</td>
<td>15</td>
<td>70</td>
<td>17</td>
<td>155</td>
<td>9.9</td>
<td>0.7148</td>
</tr>
<tr>
<td>PBAD</td>
<td>2</td>
<td>12</td>
<td>57</td>
<td>16</td>
<td>126</td>
<td>11.3</td>
<td></td>
</tr>
</tbody>
</table>

Table 23 Dominance

<table>
<thead>
<tr>
<th>Dx</th>
<th>AA+AC</th>
<th>CC</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>ID</td>
<td>16</td>
<td>70</td>
<td>1.00</td>
</tr>
<tr>
<td>PBAD</td>
<td>14</td>
<td>57</td>
<td></td>
</tr>
</tbody>
</table>
4.3 Bile Acid Species Characterisation

20 subjects had serum analysed for BA characterisation. There were 10 subjects with PBAD 4 male, 6 female with median age 37.5 years (range 26 to 70). There were 10 subjects with ID 5 male, 5 female median age 59 (range 18 to 90). For PBAD the median SeHCAT was 4.9% (range 2 to 6.7%); ID subjects median 48% (31 to 74.5%). Clinical characteristics were similar between the 2 groups.

Relative BA levels for 17 BAs are shown in Figures 28, 29 and Table 24. Values are expressed in arbitrary units. Median values in PBAD and ID are shown in the table with p values. 7 unconjugated, 4 glyco-conjugated and 6 tauro-conjugated BAs have been identified.

### Table 24 BA species in PBAD and ID

<table>
<thead>
<tr>
<th></th>
<th>aMCA</th>
<th>HCA</th>
<th>CA</th>
<th>UDCA</th>
<th>HDCA</th>
<th>CDCA</th>
<th>DCA</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBAD median</td>
<td>0.001</td>
<td>0.445</td>
<td>1.68</td>
<td>0.24</td>
<td>0.047</td>
<td>0.8805</td>
<td>4.672</td>
</tr>
<tr>
<td>ID median</td>
<td>0.001</td>
<td>0.78</td>
<td>1.982</td>
<td>0.376</td>
<td>0.138</td>
<td>1.754</td>
<td>7.385</td>
</tr>
<tr>
<td>p value</td>
<td>0.78</td>
<td>0.33</td>
<td>0.52</td>
<td>0.65</td>
<td>0.14</td>
<td>0.62</td>
<td>0.28</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>GCDCA</th>
<th>GDCA</th>
<th>GCA</th>
<th>GLCA</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBAD median</td>
<td>13.4</td>
<td>4.156</td>
<td>4.577</td>
<td>0.1895</td>
</tr>
<tr>
<td>ID median</td>
<td>9.86</td>
<td>4.577</td>
<td>4.156</td>
<td>0.1875</td>
</tr>
<tr>
<td>p value</td>
<td>0.84</td>
<td>0.83</td>
<td>0.84</td>
<td>0.89</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>aTMCA</th>
<th>TCA</th>
<th>TUDCA</th>
<th>TCDCA</th>
<th>TDCA</th>
<th>TLCA</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBAD median</td>
<td>0.01</td>
<td>1.298</td>
<td>0.01</td>
<td>1.414</td>
<td>1.34</td>
<td>0.01</td>
</tr>
<tr>
<td>ID median</td>
<td>0.328</td>
<td>1.817</td>
<td>0.0122</td>
<td>2.42</td>
<td>1.166</td>
<td>0.0385</td>
</tr>
<tr>
<td>p value</td>
<td>0.0043</td>
<td>0.47</td>
<td>0.4</td>
<td>0.24</td>
<td>1</td>
<td>0.44</td>
</tr>
</tbody>
</table>

Of the 17 BAs, the highest median level across the 20 patients is GCDCA 10.4. Lowest is aMCA (0.001). 9 of the 17 have median values of < 1.00. 6 of the 17 BA have median levels of < 0.10. There is significant variation in BA levels between subjects. BA species have been added together for each individual. Median total BA by this technique is 30.7 and 45.5 for PBAD and ID (not significant p= 0.52)
Figure 28 BA species in PBAD and ID, medians and IQRs

Figure 29 BA species in PBAD and ID, medians and IQRs
BA species levels are lower in PBAD than ID in 12 of 17. The BAs which are higher in PBAD are GCDCA, GCA, GLCA and TDCA. Median alpha-MCA is the same in both groups (0.001). The only BA in which there is a significant difference between the 2 groups is alpha-tauromuricholic acid (ID 0.33, PBAD 0.01 p = 0.0043) This is a very minor component overall with uncertain relevance in humans.
Figure 31 alpha-tauromuricholic acid in PBAD and ID

BA species and FGF19

Spearman correlations have been performed between FGF19 and individual BAs. No correlations reaching significance were found. The BAs with the strongest correlation were aTMCA ($r = 0.35$, $p = 0.13$) and TCA ($r = 0.39$, $p = 0.09$).

Correlation was also sought between FGF19 and the sum of the BAs. No significant correlation was found ($r = 0.15$, $p = 0.53$).

<table>
<thead>
<tr>
<th></th>
<th>aMCA</th>
<th>HCA</th>
<th>CA</th>
<th>UDCA</th>
<th>HDCA</th>
<th>CDCA</th>
<th>DCA</th>
</tr>
</thead>
<tbody>
<tr>
<td>r</td>
<td>-0.07</td>
<td>0.26</td>
<td>0.12</td>
<td>0.12</td>
<td>0.14</td>
<td>0.14</td>
<td>0.04</td>
</tr>
<tr>
<td>p value</td>
<td>0.75</td>
<td>0.27</td>
<td>0.63</td>
<td>0.60</td>
<td>0.56</td>
<td>0.55</td>
<td>0.86</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>GCDCA</th>
<th>GDCA</th>
<th>GCA</th>
<th>GLCA</th>
</tr>
</thead>
<tbody>
<tr>
<td>r</td>
<td>0.02</td>
<td>0.10</td>
<td>0.13</td>
<td>-0.12</td>
</tr>
<tr>
<td>p value</td>
<td>0.93</td>
<td>0.69</td>
<td>0.59</td>
<td>0.63</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>aTMCA</th>
<th>TCA</th>
<th>TUDCA</th>
<th>TCDCA</th>
<th>TLCA</th>
</tr>
</thead>
<tbody>
<tr>
<td>r</td>
<td>0.35</td>
<td>0.39</td>
<td>0.12</td>
<td>0.29</td>
<td>0.07</td>
</tr>
<tr>
<td>p value</td>
<td>0.13</td>
<td>0.09</td>
<td>0.60</td>
<td>0.22</td>
<td>0.78</td>
</tr>
</tbody>
</table>

Table 25 Correlation of BA species with FGF19
4.4 Meal stimulated response
For this section of the study 10 individuals were recruited and data gathered as described in section 3.4. Data have been combined with that previously gathered by my predecessor making a total of 21.

General Characteristics

There were 21 subjects, 19 with PBAD and SeHCATs less than 15% and 2 subjects with ID with SeHCAT retention of > 15%. There were no individuals with secondary BAD, none had evidence of other GI disease. 6 subjects had severe PBAD with SeHCAT retention of < 5%. There were 10 with SeHCAT 5 – 10% and 3 with SeHCAT 10 – 15%. The 2 ID subjects had 7 day retention of 49.2 and 53.0%.

10 subjects were female and 11 male. Age ranged from 26 to 70 years. Over the preceding 3 months, average bowel movements per day ranged from 3 to 20 times with average Bristol stool type of 6 and 7. Duration of symptoms ranged from 6 months to 27 years with a median of 3 years. Data for individuals is shown in table 23.

Changes in measured FGF19 over the course of 6 hours are shown for each individual subject in the Figure 32.
Figure 32 Individual FGF19 responses over 6 hour time course. Meals at 9.00 and 12.00
## Table 26 General characteristics of subjects in meal stimulated study

<table>
<thead>
<tr>
<th>Patient</th>
<th>Diagnosis</th>
<th>Age</th>
<th>Sex</th>
<th>Ethnicity</th>
<th>BMI</th>
<th>Movements/24hrs</th>
<th>av BSFS</th>
<th>Duration years</th>
<th>Bile Acids</th>
<th>FGF19</th>
<th>SeHCAT</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>PBAD</td>
<td>38</td>
<td>F</td>
<td>W</td>
<td>32.0</td>
<td>7</td>
<td>7</td>
<td>3</td>
<td>1.5</td>
<td>56</td>
<td>5.4</td>
</tr>
<tr>
<td>25</td>
<td>PBAD</td>
<td>38</td>
<td>F</td>
<td>W</td>
<td>22.0</td>
<td>4</td>
<td>7</td>
<td>5</td>
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Patterns of FGF19

Data analysis refers to subjects with PBAD and not the 2 subjects with ID, unless otherwise stated. There was great variation in FGF19 levels between subjects. As would be expected the time point with lowest median FGF19 was the 9am fasting sample (143 pg/ml), very close to the median fasting level of 149 pg/ml in study 1. There was significant variation in the 9am fasting sample ranging from 16.8 to 752 pg/ml. 6 of 19 PBAD subjects had levels over 235 pg/ml, the median fasting level for ID subjects in study 1. There was less variation at the second time point (10.30am) with a range of 24.8 to 402 pg/ml, median 150 pg/ml. Figure 33 shows median levels of FGF19 in PBAD subjects with interquartile ranges.

FGF19 increases after lunch (provided at 12 midday) with median level 232 pg/ml recorded at time 13.30, not significantly different to 0900 or 1030 time points (p = 0.42, 0.15). FGF19 increases further to the 1530 time point (median 296 pg/ml) which is significantly higher than at 1030 (p < 0.05).

As stated there is significant variation in the FGF19 meals response patterns seen in the 19 individuals with PBAD. We thought it might be beneficial to sub-divide these FGF19
responses into different patterns in an attempt to describe FGF19 phenotypes. This has been done according to the following classification:

**Low – low (L-L):** Serum FGF19 level < 300 pg/ml at each of the 5 time points.

**Low – high (L-H):** 09:00 sample < 200 pg/ml, 13:30 or 15:00 sample > 400pg/ml.

**High – high (H – H):** 09:00 and 12:00 sample> 300 pg/ml plus 13:30 or 15:00 sample > 300 pg/ml.

**Low – low (L-L) phenotype**

There were 8 subjects with FGF19 less than 300 pg/ml throughout the course of the study day. 6 had SeHCAT retention of 5% or less, the other 2 having retention of 6.7% and 11.6%. L-L individuals have lower SeHCAT retention than the L-H group (4.4% vs 8.1% p < 0.05) and lower than the HH group (7.4% p = 0.07).

---

**Figure 34 FGF19 for L-L individuals**
Differences have been sought between L-L subjects and L-H and H-H subjects. No significant differences were found in age, BMI, stool form, stool frequency or duration of symptoms.

There was however a difference in fasting serum Vitamin A level. Those with the L-L pattern have a higher Vitamin A level of 2.2 µmol/l compared with median 1.8 µmol/l (p = 0.03).

In mice vitamin A is required for the basal production of FGF15 (41). If differences in either vitamin A absorption or in dietary vitamin A were causative of lower FGF19 it may be expected that the L-L pattern would be associated with low serum Vitamin A. The fact that higher levels are found in those with L-L phenotype may therefore reflect increased fat soluble vitamin absorption due to differences in bile acid metabolism. It must be noted however that no correlation was found between Vitamin A and SeHCAT or FGF19 in the larger main cohort of patients see section 4.1.3. A separate analysis compared vitamin A in those with SeHCAT < 5% with ID individuals in the larger cohort and again found no
difference. It may be that differences found in these L-L subjects are due to chance. Vitamin D has also been found to be higher in L-L than L-H H-H combined (44.0 vs 19.8 nmol/L p = 0.25). No differences between L-L and the other phenotypes have been found in other biochemical parameters.

**Low – High phenotype (L-H)**

5 individuals have the described L-H phenotype. SeHCAT retention ranges from 6.8 to 9.3% and as mentioned is higher than the L-L group. It is not significantly different to the H-H group.

![Figure 37 L - H phenotype](image)

Significant differences in clinical and biochemical parameters have not been demonstrated between L – H and the other groups.

**High – High phenotype (H – H)**

4 subjects had the H – H phenotype of FGF19 with levels starting high, tending to fall until lunch before increasing to over 400 pg/ml in one of the final 2 readings.
SeHCAT retention ranged from 5.4 to 11%. Again there were no significant differences between these patients and those from the other groups.

**Area under the curve**

For each individual with PBAD undergoing the ‘day series’ the area under the curve (AUC) has been calculated expressed in pg/mlhr. 7 day SeHCAT retention correlates with the AUC $p < 0.05$, those with lower SeHCAT retention have lower AUC. Higher AUC tends to be associated with fewer bowel motions in 24 hours ($p = 0.13$) There is no correlation between the AUC and other symptoms.

AUC correlates with serum vitamin A ($r = -0.61, p = 0.016$). Those with lower AUC FGF19 have higher serum vitamin A. These findings support the findings that the L-L phenotype is associated with higher vitamin A. As mentioned earlier, the higher levels may reflect increased absorption of fat soluble vitamin due to altered BA homeostasis.
Other correlations

The only factor in the day series experiments found to be significantly associated with a clinical characteristic is the serum FGF19 taken at time point 15.00. Higher FGF19 at this time was found to be associated with fewer bowel movements per 24 hours (r = 0.53 p = 0.02).
Bile Acids

Total serum BAs were measured at each time point. Serum for those individuals who had participated in the day series prior to October 2010 were not available for further analysis and quantification of total bile acids.

The lower end of the reported range by the clinical chemistry lab was 1.00mmol/L. Some values were reported as < 1.00 which have been analysed as 0.99 mmol/L. The highest BA was 10.07 mmol/L for BD25.

Figure 41 FGF19 and BA in L-L subjects

Figure 42 FGF19 and BA in L-H subjects

Figure 43 FGF19 and BA in H-H subjects
In study 1 it was found that fasting values of FGF19 and BAs correlated. In this study a comparison has made of all measurements at all time-points of FGF19 with corresponding BA. There is a strong correlation between these values ($r = 0.46$, $p < 0.0001$).

![Figure 44 FGF19 and BA for all samples in day series](image)

The total BA and FGF19 traces for each individual follow a similar pattern. BA traces that are relatively flat have accompanying relatively flat FGF19 traces. Similarly, rises in BA tend to have the same change in FGF19 although at the following time point. An attempt has been made to statistically demonstrate a correlation between changes in BAs and changes in FGF19 for individuals over the time period. For each time point except 09:00 the percentage change from the previous time point has been calculated. If there is no change then % change is 0%, if it doubles then % change is 100%. This has been done for both FGF19 and BA. These % changes have been plotted against one another and correlated. There was no significant correlation between % change of TBA and FGF19 at the same time points (Figure 45). FGF19 was then plotted against % change in TBA at the next (t + 90 mins) time point and with the previous time point (t – 90 min). No significant correlation found with FGF19 against TBA at the next time point (t + 90 min). There was however a correlation between FGF19 and TBA at the previous time point. Changes in TBA strongly correlate with changes in FGF19 90 minutes later, see figure 46 ($r = 0.51$, $p < 0.001$).
The finding that proportional change in FGF19 correlates strongly with proportional changes in serum total bile acids 90 minutes before indicates that FGF19 is synthesised and released in direct response to BAs. The similar patterns of FGF19 and BA are present across the range of SeHCAT values and phenotypes. The fact that the FGF19 profile echoes the BA profile applies regardless of SeHCAT retention of phenotype. When the correlation is performed on the L-L, L-H and H-H phenotype there is similar correlation coefficient and significance between the groups albeit > 0.05 due to reduced sample numbers (p = 0.13, 0.23, 0.18 r = 0.46, 0.41, 0.42, respectively.)
Table 27 Genotype of each individual in day series study. F = failed genotyping

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Polymorphisms and day series

As part of study 4.2 individuals in the day series study were genotyped for the 9 single nucleotide polymorphisms investigated earlier. The results may be seen in Table 27. Minor allele frequencies are shown for the L-L phenotype and for the other PBAD phenotypes combined. Allele frequencies have been compared between these 2 groups using Fisher’s Exact Test.

The FGFR4 SNP rs376618 shows significantly higher proportion of the C allele in L-L phenotype than in the other phenotypes with a MAF of 37.5 vs 9.1 %. MAF in study 4.2 for the whole cohort for ID and PBAD was 28.5% and 22.1% respectively. When the L-L and other phenotypes are compared with the entire cohort from study 4.2 there is no significant difference in proportions of the minor allele.

The ASBT SNP rs188096 has a higher proportion of the A allele in the L-L phenotype compared with the entire cohort and compared with the other phenotypes. The difference does not reach significance. The FGF19 SNP rs948992 has a lower proportion of the G allele in the L-L group than the rest (18.8% vs 45.5%), again this does not reach significance. In study 4.2 the MAF for ID and PBAD was 36.6% and 33.1% respectively.
4.5 Ageing study

27 individuals’ samples were made available for analysis. Age ranged from 23 to 92 years evenly spaced out across the decades (7 aged 20 – 39, 7 aged 40 – 59, 7 aged 60 – 79 and 6 aged 80 and over). There were no gastroenterological symptoms or comorbidities. 7 individuals took medication including anti-hypertensives, statins, warfarin and a bisphosphonate. Body mass index ranged from 20.4 to 26.8 kg/m$^2$ with a median of 23.4 kg/m$^2$.

Fasting serum FGF19 levels were surprisingly low in this cohort of healthy individuals, possibly reflecting differences in separation and storage. Levels ranged from 13.8 to 246.6 pg/mL. Median FGF19 was 71.0 pg/mL. There was no difference in FGF19 for men and women (72.8 and 80.1 pg/mL, p = 0.97). There was a significant correlation with age. FGF19 increases with age ($r = 0.48$, $p = 0.01$), see figure 47a.

![Figure 47(a) FGF19 against age. (b) Median ages with min and max shown](image-url)
The 20 year age ranges were compared (Figure 47b). There was a significant difference in median between the 20 – 39 and the 80 + age groups. Other differences if FGF19 between the groups were non-significant.

Table 28 Subject characteristics and FGF19 in ageing study

<table>
<thead>
<tr>
<th>ID</th>
<th>Age</th>
<th>BMI</th>
<th>Gender</th>
<th>FGF19</th>
<th>ID</th>
<th>Age</th>
<th>BMI</th>
<th>Gender</th>
<th>FGF19</th>
</tr>
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<tbody>
<tr>
<td>F</td>
<td>27</td>
<td>21</td>
<td>M</td>
<td>45.5</td>
<td>D</td>
<td>72</td>
<td>26.8</td>
<td>M</td>
<td>130.4</td>
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<tr>
<td>N</td>
<td>34</td>
<td>22.9</td>
<td>F</td>
<td>27.7</td>
<td>P</td>
<td>79</td>
<td>24.1</td>
<td>M</td>
<td>40.3</td>
</tr>
<tr>
<td>L</td>
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<td>22.2</td>
<td>F</td>
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<td>AA</td>
<td>67</td>
<td>25.9</td>
<td>M</td>
<td>32.1</td>
</tr>
<tr>
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<td>20.6</td>
<td>F</td>
<td>52</td>
<td>AD</td>
<td>70</td>
<td>26</td>
<td>M</td>
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<td>M</td>
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<td>AG</td>
<td>75</td>
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<td>F</td>
<td>102.3</td>
</tr>
<tr>
<td>AL</td>
<td>29</td>
<td>24</td>
<td>F</td>
<td>39.1</td>
<td>AM</td>
<td>72</td>
<td>20.4</td>
<td>F</td>
<td>176.4</td>
</tr>
<tr>
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<td>20.5</td>
<td>F</td>
<td>80.1</td>
<td>AQ</td>
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<td>24.4</td>
<td>F</td>
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</tr>
<tr>
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<td>54</td>
<td>24.2</td>
<td>M</td>
<td>52.4</td>
<td>M</td>
<td>81</td>
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<td>F</td>
<td>204.5</td>
</tr>
<tr>
<td>I</td>
<td>43</td>
<td>22.2</td>
<td>F</td>
<td>13.8</td>
<td>BB</td>
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<td>F</td>
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<tr>
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<td>22.6</td>
<td>F</td>
<td>28.5</td>
<td>BK</td>
<td>86</td>
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<td>F</td>
<td>95.9</td>
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<td>BM</td>
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<td>21.3</td>
<td>F</td>
<td>134.3</td>
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<tr>
<td>AT</td>
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<td>24.5</td>
<td>M</td>
<td>102.6</td>
<td>BN</td>
<td>92</td>
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<td>M</td>
<td>117.5</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

FGF19 levels from this group of healthy individuals were much lower as a whole than in the larger group recruited for study 1. A comparison has been made between the FGF19 levels in this ageing study and subjects from study 1 within the same age and BMI ranges, with negative SeHCAT studies (Figure 48).

Figure 48 Comparison within age groups of FGF19 levels between the different study populations. Study 1 comparison group only includes those within same BMI range as the ageing study with negative SeHCATs (medians, min and max)
As can be seen at each age range median fasting FGF19 was between 2.5 and 4.7 times higher in the study 1 groups (p = 0.001 or less for each group).
4.6 OBADIAH
4.6.1 General Characteristics

35 subjects were recruited and entered the study. 7 withdrew prior to taking obeticholic acid and are therefore excluded from any analysis. There remained 28 subjects, 10 in the primary BAD arm, 10 in the secondary BAD arm and 8 idiopathic diarrhoea controls. Only PBAD and ID subjects are considered in this thesis as the SBAD patients were recruited by my colleague Jonathan Nolan and are included in his thesis.

Primary BAD: There were 7 males and 3 females with median age of 47, range 24 – 74 years. All had SeHCAT retention of under 10%, median 4.8%. 5 of the 10 used bile acid sequestrants prior to the study.

<table>
<thead>
<tr>
<th></th>
<th>PBAD</th>
<th>SBAD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>10</td>
<td>8</td>
</tr>
<tr>
<td>Sex (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>7 (70)</td>
<td>3 (37)</td>
</tr>
<tr>
<td>Female</td>
<td>3 (30)</td>
<td>5 (63)</td>
</tr>
<tr>
<td>Age (y)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>47</td>
<td>39</td>
</tr>
<tr>
<td>Range</td>
<td>24 – 74</td>
<td>25 – 68</td>
</tr>
<tr>
<td>SeHCAT (7d retention %)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number</td>
<td>10</td>
<td>8</td>
</tr>
<tr>
<td>Median</td>
<td>4.8</td>
<td>25.7</td>
</tr>
<tr>
<td>Range</td>
<td>0.8 – 9.3</td>
<td>16.0 – 40.0</td>
</tr>
<tr>
<td>Previous BA sequestrant use (n)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>No</td>
<td>5</td>
<td>8</td>
</tr>
</tbody>
</table>

Idiopathic Diarrhoea: There were 8 subjects, 5 male and 3 female. The median age was 39 years, range 25 – 61 years. SeHCAT retention ranged from 16 to 40%, median 25.7%.
4.6.2 FGF19

**Fasting levels, pre OCA:** In primary BAD the median fasting FGF19 level was 132pg/ml, with a range of 102 – 168. This is similar to our previously reported levels(71). Idiopathic diarrhoea subjects had a median fasting FGF19 on D0 of 116pg/mL; lower than in PBAD but the difference is not significant (p = 0.6).

**Comparison with Study 1:** Median D0 fasting FGF19 for PBAD is not significantly different for the prospectively recruited cohort in Study 1 (medians 133 pg/mL vs 148 pg/mL p = 0.48). Fasting FGF19 was significantly lower in the ID group in Obadiah than in study 1 (Medians 116.5 pg/mL vs 236 pg/mL p < 0.005).

![Figure 49 Comparison of fasting FGF19 pre OCA Obadiah vs Study 1 fasting FGF19. Boxes show IQR, 10 – 90 centiles and outliers.](image)

**Primary BAD:** The fasting FGF19 level increased from 132pg/ml to 237pg/ml after 2 weeks treatment with OCA (p = 0.007). 9 out of 10 subject’s FGF19 increased on OCA with a median increase of 71%, IQR 9 – 102%.
Idiopathic diarrhoea: Of the 8 individuals, 6 had an increase in fasting FGF19 after 2 weeks treatment with OCA. (See Figure 50) Median FGF19 level increased by 130%, IQR 14 – 304%. This did not reach significance (p = 0.12)

Figure 50 PBAD: Fasting FGF19, C4, BA AUC before and after 2 weeks OCA. Medians and IQR fig A - C. Individual values D - F

Figure 51 ID: Individual fasting FGF19 before and after 2 weeks OCA
Figure 52 ID group before and after 2 weeks OCA. IQR and 10 – 90 centile

The 3 groups overall have a significant increase in FGF19 from a median of 74 pg/ml to 152 pg/ml (p = 0.0004)
6 hour response to OCA: FGF19 increased significantly in the 6 hours after OCA in all groups. The groups differed in D0 AUC (Kruskal-Wallis P=0.004) and peak FGF19 values (P=0.0003) with the responses being significantly highest in primary BAD, followed by the idiopathic controls. There were no significant differences between the responses on D0 compared to D14 in FGF19 AUC or 6h peak in any of the groups. In PBAD the median peak value on D0 was 1278 pg/ml, which was not significantly different from that on D14 1216 pg/ml. Median peak FGF19 levels found in the idiopathic group were 496 pg/ml and 560 pg/ml on D0 and D14.

Figure 53 FGF19 and bile acid changes during serial sampling over six hours. Individual patient results are shown on the day of the first dose (D0) and after 2 weeks of OCA treatment (D14). Fasting samples were obtained and then OCA 25mg was taken orally
**FGF19, C4 and Bile Acids: fasting, 6h AUC and peak values on D0 and D14**

<table>
<thead>
<tr>
<th></th>
<th>Primary BAD (n=10)</th>
<th>Secondary BAD (n=10)</th>
<th>Idiopathic controls (n=8)</th>
<th>Overall (n=28)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>D0</td>
<td>D14</td>
<td>P</td>
<td>D0</td>
</tr>
<tr>
<td><strong>FGF19 (pg/ml)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fasting</td>
<td>132</td>
<td>237</td>
<td>0.007</td>
<td>32</td>
</tr>
<tr>
<td><strong>6h response AUC</strong></td>
<td>3945</td>
<td>3825</td>
<td>0.72</td>
<td>401</td>
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<tr>
<td><strong>6h peak</strong></td>
<td>1278</td>
<td>1216</td>
<td>0.51</td>
<td>102</td>
</tr>
<tr>
<td><strong>C4 (µg/L)</strong></td>
<td>16(^a)</td>
<td>3(^b)</td>
<td>0.03</td>
<td>104</td>
</tr>
<tr>
<td></td>
<td>1.0–4.0</td>
<td>0.9–3.0</td>
<td></td>
<td>2.0–6.0</td>
</tr>
<tr>
<td><strong>Bile Acids (µmol/L)</strong></td>
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<td>0.9</td>
<td>0.13</td>
<td>2.5</td>
</tr>
<tr>
<td>Fasting</td>
<td>1.0–4.0</td>
<td>0.9–3.0</td>
<td></td>
<td>2.0–6.0</td>
</tr>
<tr>
<td><strong>6h response AUC</strong></td>
<td>34.5</td>
<td>20.9</td>
<td>0.02</td>
<td>32.0</td>
</tr>
<tr>
<td></td>
<td>22.5–64.8</td>
<td>12.9–30.3</td>
<td></td>
<td>20.5–39.0</td>
</tr>
<tr>
<td><strong>6h peak</strong></td>
<td>7.5</td>
<td>4.0</td>
<td>0.02</td>
<td>6.5</td>
</tr>
<tr>
<td></td>
<td>4.0–15.0</td>
<td>2.8–7.5</td>
<td></td>
<td>3.8–9.3</td>
</tr>
</tbody>
</table>

Values are medians and IQR. AUC = area under the curve. D0 and D14 values were compared by Wilcoxon paired rank tests. Significant values (P< 0.05) are in bold.

\(^a\) Data were incomplete in two patients
4.6.3 7α-hydroxy-4-cholesten-3-one (C4)
C4 decreased between D0 and D14 in response to OCA significantly in primary BAD (16 – 3 μg/L, p = 0.03) and in the idiopathic diarrhoea group (9 – 1 μg/L, p = 0.02).

4.6.4 Bile acids
There was no significant change in fasting bile acids within each group between D0 and D14. When the data is combined however there is a significant reduction in median fasting serum BA level from 2.0 μmol/L to 1.0 μmol/L (p = 0.04). Bile acids were measured for 6 hours post OCA on day D0 and D14 and the AUC was calculated. There were significant falls in AUC over the OCA treatment period in the groups. In the primary BAD group the AUC fell in response to OCA from 34.5 to 20.9 μmol/LHr (p = 0.02). In the idiopathic group values fell from 29.0 μmol/L to 20.0 μmol/L (p = 0.02).

4.6.5 Clinical symptoms
Primary BAD: Clinical symptoms were compared between weeks 2 and 4. There were significant improvements in total numbers of stool, Bristol stool form and the stool index. Median weekly stool fell from 23 to 14 (p = 0.03) with a change in mean BSFS from 5.2 to 4.3 (p = 0.05). Median stool index improved from 113 to 76 a reduction of 34% (p = 0.005). Loperamide was used by 2 individuals however its use was reduced during the OCA treatment period. Symptoms of diarrhoea worsened in week 6 one full week after having stopped the OCA. BA sequestrants were resumed at this stage in 2 patients. Urgency was significantly reduced by 49% (p = 0.03).

Idiopathic diarrhoea: There was very little change in frequency, form or stool index in this group. There was an increase in the median number of stool and no change in stool form. The index decreased from 96 to 83 but this was non-significant (p= 0.61). Other symptoms such as urgency and pain did not change in this group.
4.6.6 Association between symptom improvement and biochemical parameters
In the primary BAD group, the percentage change in stool number was associated with the percentage change in fasting FGF19 and BA AUC. This BA response was inversely correlated with the change in fasting FGF19. Additionally, the percentage change in fasting C4 was significantly associated with reduced urgency score ($r=0.65$, $P=0.04$) and with the change in FGF19 six-hour response ($r=-0.62$, $P=0.05$), indicating that increases in FGF19 resulted in reduction of C4 and new BA synthesis.

4.6.7 Tolerability, liver enzymes and lipid analyses
OCA was well tolerated and only a few minor adverse effects were reported of limited clinical significance. Three patients (two in the secondary BAD group and one in the idiopathic group) reported mild headaches during OCA treatment in week three or four. In the absence of a placebo-treated group, it is uncertain whether these were possibly related to OCA treatment. Another patient with Crohn’s disease experienced worsening of abdominal pain after starting OCA and did not have a bowel movement for 24h. Her symptoms settled on omitting OCA for 1d and then continuing with OCA 25mg on alternate days.

Lipid measurements showed higher values for fasting total and LDL-cholesterol (median increases of 10.4% and 19.7% respectively, both $P<0.001$ overall) and a reduction in triglycerides (median decrease of -11.8%, $P=0.06$) after two weeks OCA treatment. HDL-cholesterol did not change significantly. Similar magnitudes of changes were found in all three groups and are comparable to those published previously.(109). There were no significant changes in median values of liver biochemistry parameters, including alkaline phosphatase, alanine transaminase, bilirubin and albumin, which all remained in the normal range.
5. Discussion

5.1 Study 1
This study has successfully recruited 95 subjects, mostly from the Department of Nuclear Medicine at the Hammersmith Hospital. Those with other GI disease were excluded and analysis focussed on PBAD and ID. When the data were combined with the cohort recruited identically by my predecessor there were 162 subjects, 93 with ID and 69 with PBAD. This has allowed greater statistical power and may enable more subtle interactions and weaker associations to be discovered.

A cut off of 15% 7 day SeHCAT retention has been used in this study to define BAD. It has been found in a systematic review that using this cut off the proportion of individuals with chronic diarrhoea who have a positive SeHCAT scan is 26%. The prevalence rate in these studies ranged from 18% to 53%(60). In this study 69 of 162 subjects have positive SeHCAT scans: 42.6 %. 18 individuals had severe BAD with 7 day SeHCAT retention of < 5%, a percentage of 11.1%, similar to the proportion of 10% found in the systematic review.

General characteristics and symptoms
General characteristics were similar between ID and PBAD. Although this is not a case control study similarities in age, ethnicity and symptoms makes the prospective study more powerful, allowing more meaningful comparison of other factors such as biochemistry. Neither age, sex nor ethnic make-up were significantly different between ID and PBAD. The ratio of F : M was higher in ID than PBAD but not significantly so. Those with severe PBAD have had the condition significantly longer than those with moderate and mild PBAD, and they tended to have had it longer than those with ID. It is not clear why individuals with a SeHCAT of < 5% have had symptoms longer. It may be that the underlying aetiology of PBAD worsens with time, and that those with severe PBAD have gone through a period of time with milder disease during which their SeHCAT retention may have been higher. In a long-term study of PBAD (mean follow up 99.2 months) of 23 diagnosed with the condition, 3 had died at follow up, 3 lost to follow up and 3 diagnosed with IBD. Of the remaining 14, 7 showed improvement and 7 were still symptomatic. With repeat SeHCAT scanning, 5 of the 7 symptomatic had positive tests (< 5% in this study) and all of the asymptomatic subjects tests had become negative (110). In a similar study 20 subjects were followed up of whom 16 had a repeat SeHCAT test. In 13 of 16 symptoms persisted and SeHCAT remained low and almost identical to previous values (median follow up time 88 months)(111). 3 subjects showed considerable increase in SeHCAT retention. There is mixed evidence regarding stability of SeHCAT over time, although there is no evidence of worsening SeHCAT retention in the literature.

The only symptom to be significantly different in proportions between ID and PBAD was reported steatorrhoea (increased proportion in the severe PBAD group). This has not been quantified in our patients and depends on observation by the patient. This is well described in PBAD and in the past has been ascribed to ‘true’ bile acid malabsorption leading to
reduced BA pool and therefore reduced emulsification of fats by BA(59). In the presence of
a normal or enlarged BA pool size, steatorrhoea may be caused by uncoordinated BA
secretion into the small bowel as in post-cholecystectomy subjects or it may have a cause
unrelated to BA such as bacterial overgrowth.

Body mass index
As has been demonstrated in our previous studies, BMI of individuals with PBAD is
significantly higher than those with ID (86)(82). Again, BMI correlates negatively with
SeHCAT retention, and there is a weaker association between BMI and FGF19 when taking
all ID and PBAD subjects together. Within the PBAD diagnosis BMI has previously been
shown to correlate with FGF19 but this significant association has not been demonstrated in
this study (86). Further correlations have been shown between BMI and age as well as BMI
and serum triglycerides. Higher BMI with increased age is well described in the normal
population, and associations between BMI and triglycerides are also recognised (112)(113).

Different mechanisms may explain the finding that PBAD subjects have higher BMIs. The
weak association of FGF19 with BMI implicates the hormone. Previous studies have
revealed that FGF19 plays a role in glucose disposal, lipid regulation and insulin sensitivity
(114)(115)(116). FGF19 has also been found to be associated with the development of
NAFLD and the metabolic syndrome in obese adolescents and lower FGF19 levels have been
found in obese subjects compared with a healthy control group (117)(118). Despite the
multiple metabolic effects of FGF19 its actions may not account for the difference in BMI
found in this study. The association with SeHCAT is stronger and FGF19 independent
mechanisms may explain BMI differences. BAs have been shown to act as metabolic
signalling molecules through both the cell surface receptor TGR5 and the nuclear receptor
LXR. Changes in serum BA levels and BA pool size can affect glycaemic control, BMI and
insulin sensitivity (119)(120)(121). No association has been found in our study between BMI
and fasting BA however. A limitation of this part of the study is the sole measurement of
fasting biochemical parameters. Changes or peaks values of FGF19 and/or BAs may have
displayed greater associations with BMI; these factors are explored in the day series study.
Finally, factors other than the metabolic effects of FGF19 and BAs may be responsible for
differences in BMI between ID and PBAD. Diet is a confounding variable which is impossible
to exclude as the study was not designed to record this information. Other factors such as
intraluminal BA concentration and differential lipid absorption may account for differences
and are not possible to detect with the methodology used. Differences in the gut
microbiome can lead to differences in nutrient extraction through fermentation of
carbohydrates to absorbable short chain fatty acids (122). The BA profile and metabolism is
highly dependent on microbial activities and therefore differences in the microbiome may
account for differences in BA metabolism and BMI.

FGF19 and Lipids
This study has again confirmed the finding that median fasting FGF19 is lower in
PBAD than in diarrhoea controls, with those subjects with lowest SeHCAT retention (< 5%)
having lowest, followed by moderate (5-10%) followed by mild (10 – 15%) PBAD (4)(87). The positive correlation with SeHCAT has also been shown again.

It is clear that PBAD is a condition characterised by low fasting FGF19, with the most severe forms having the lowest values. Associations with other variables are providing insights into possible mechanisms. Strong associations are shown between SeHCAT retention and lipids, in particular triglycerides. Abnormalities in lipid composition have been demonstrated in subjects with aberrant bile acid metabolism previously (123), triglycerides have been shown to correlate with C4, a marker of bile acid synthesis(124). C4 has not been measured in this study but this group has previously shown a strong negative correlation between 7 day SeHCAT retention and fasting serum C4 (87). Therefore, findings of elevated triglycerides in elevated bile acid synthesis support findings from elsewhere. The higher FGF19 values found in the patients with fasting hypertriglyceridemia suggest these patients constitute a different subgroup and are in keeping with previous work. Gälman and colleagues studied a large group of over 400 normal subjects without clinical diarrhoea, with measurements including FGF19, C4 and triglycerides(124). Overall they found a negative correlation between FGF19 and corrected C4, and between C4 and triglycerides. In their patients with high C4 above the 95th percentile, 35% had raised triglycerides >2.4mmol/L. This is a similar proportion to that found in our PBAD patients who would also be expected to have raised C4. Patients with familial hypertriglyceridemia have been shown to have an increased rate of bile acid synthesis and abnormal absorption has been suggested(123). They have been shown to have reduced expression of ASBT mRNA and protein to about half of control values 21 although no increase in ASBT sequence variants has been found(125). We have not shown whether these patients in our cohort with diarrhoea have reduced ASBT, and it is unclear why they should have essentially normal fasting FGF19 values, but they do appear to constitute a subgroup of about 30% with different characteristics meriting further study.

FGF19 and BA
Total serum BAs are influenced by factors other than new synthesis, as measured by C4, such as ileal BA reabsorption and hepatic uptake and may not always reflect BA pool size (126). The positive correlation we have shown between FGF19 and total BA in the entire cohort presumably reflects varying ileal BA absorption and the stimulation of FGF19 synthesis that occurs during this process. The relationship between total BA and FGF19 is maintained in the PBAD group suggesting appropriate FGF19 synthesis in response of enterocyte exposure to BAs. The absence of a significantly lower BA level in PBAD does not support any notion of a true BA malabsorption taking place in PBAD, however nor does it support the theory of an enlarged BA pool size previously proposed (4). The lack of correlation between BA and SeHCAT likely reflects the fact that SeHCAT retention is a measure of BA turnover and in PBAD and ID a steady state of BA metabolism is attained with more than one factor influencing the measured BA level. As noted previously numerous factors influence fasting total serum BAs other than the BA pool size.
Variation of FGF19 and Reproducibility
The results from previous work by our group has shown that FGF19 remains stable over 2 days at room temperature. It has also been shown that freeze – thaw cycles affect values minimally.

Repeated measurement of fasting FGF19 within the same individual was made possible as some subjects from study 1 also participated in Study 4 where a 9am fasting sample was taken. Most of the subjects showed little variation in FGF19 however in five of the 20 the variation was much greater at more than 200 pg/ML. This variation may be due to different sampling times - from study 1 the sampling time was at any time 9am – 5 pm, although subjects has always been instructed to fast. The study 4 samples were all taken at 9am. It is possible that in either study subjects did not in fact fast and that one of the samples was a post prandial sample. In future studies admitting subjects to the research ward the night before and ensuring compliance with fasting would exclude this confounding factor.

Vitamins A and D
In this study no significant differences has been demonstrated in serum vitamin A or vitamin D between diarrhoea control subjects and those with primary BAD. This is an important finding as there is strong evidence that abnormalities in the FGF19 – FGFR4 signalling axis are the primary abnormality underlying primary bile acid diarrhoea and that vitamins A and D are essential to FGF15/19 synthesis. Prospective studies by our group involving over 150 subjects have shown reduced median levels of fasting FGF19 in PBAD versus controls (147 vs 225pg/ml , p < 0.001) (87). Diarrhoea controls are included in this prospective study showing that diarrhoea per se is not the cause of reduced levels of FGF19 previously reported. Duration and severity of symptoms was similar between the 2 groups.

An unexpected link was previously found between vitamins A and D and bile acid biosynthesis in knockout mouse models (41). Bile acid pool size was found to be bigger in VDR-/- mice. Quantitative PCR on liver showed that CYP7A1 was up-regulated as was ileal FGF15. Physiological concentrations of vitamin D were then studied in an ileal explant culture for 6 hours which revealed a dose-dependent relationship with FGF15 expression. Chromatin immunoprecipitation (CHIP) analysis was performed which demonstrated that VDR binds directly to the FGF15 promoter, directly regulating its expression.

Lack of relationship between FGF19 and vitamin D in our subjects makes a causative role in primary BAD less likely. Further studies are required in humans to explore the role of vitamin D in FGF19 expression, for instance in an explant culture.

In further work by Schmidt et al it was found that mice fed vitamin A had greatly increased ileal FGF15 expression and reduced CYP7A1 expression. Through the use of selective RAR and RXR ligands in wild type mice it was determined that FGF15 induction occurred only through RXR. Analysis of liver gene expression revealed that repression of CYP7A1 also occurs separate to the FGF15 mechanism, through SHP induction. In another study retinoic
Acid was found to suppress CYP7A1 and lead to increased expression of FGF19 and SHP in HepG2 cells and human hepatocytes (127).

In this study serum retinol has been measured whereas in the studies mentioned retinoic acid or retinyl palmitate has been used. Furthermore it is acknowledged that serum retinol does not provide an accurate assessment of whole body vitamin A status (128). These are potential flaws in our study.

It may be speculated that a deficiency in vitamins A or D may cause a deficiency of FGF19 in a subset of PBAD subjects. It is also possible that in another subset of subjects with low FGF19 but normal or high dietary vitamin A & D consumption, that excess BAs in the bowel lumen lead to increased emulsification and increased fat soluble vitamin absorption, offsetting those subjects with low serum levels. There is no evidence of a biphasic pattern to vitamin A and D levels to support this.

It remains a possibility that although serum levels of the vitamins do not play a role in impaired FGF19 production, there may be a defect in the vitamin sensing proteins. Abnormalities in VDR, FXR or RXR may not lead to abnormal levels of vitamins A and D however they may cause aberrant signalling and impaired expression of FGF19.

This sub-study has failed to demonstrate any association between the fat soluble vitamins A and D and FGF19 or SeHCAT retention in a prospectively recruited cohort of patients with chronic diarrhoea or bile acid malabsorption. It is unlikely dietary vitamin A or D or serum levels play a causative role in the pathogenesis of primary bile acid malabsorption. Given the central role of these vitamins to FGF15/19 expression however, further investigation into the roles of their receptors and downstream pathways may be warranted.
**Vitamin B12**

Vitamin B12 (cobalamin) is released from dietary proteins by gastric acid. It is bound initially by R protein in saliva. In the duodenum R protein is hydrolysed by pancreatic enzymes and intrinsic factor (IF) binds to the B12. IF is synthesised by gastric parietal cells. It is resistant to proteolysis and passes to the terminal ileum where IF binds to specific receptors on the enterocyte where B12 absorption takes place. The Schilling test in which radiolabelled B12 and IF are administered was used as a test of terminal ileal function but is no longer available (129). It is known that those with terminal ileal resection or disease are at risk vitamin B12 deficiency however no true impairment of absorption has ever been demonstrated in PBAD (130). Clearly vitamin B12 levels will be affected by many factors but if differences in patient’s B12 levels could be demonstrated in PBAD then this may provide evidence of a true malabsorptive defect in the terminal ileum of those individuals.

B12 levels will be affected by diet, particularly a vegan diet. Absorption is impaired by achlorhydria, protein R release is prevented by lack of proteolysis and gastric pathology can lead to impaired IF synthesis. B12 levels can also be affected by medication and increased by small intestinal bacterial overgrowth. These confounding factors may effect B12 levels in the study population however as most individuals have no GI disease this will remove a number of confounding variables.

Although significance was not reached there is a near correlation between FGF19 and serum B12 levels. Furthermore, there is a nonsignificant trend to lower B12 levels in those with PBAD. It is well described that small intestinal bacterial overgrowth may lead to vitamin B12 deficiency; the finding of lower levels in PBAD may indicate that at least in some individuals there maybe bacterial overgrowth causing lower B12 levels and perhaps impairing BA absorption. BA composition can have biological effects on intraluminal bacteria and this is explored further in the introduction section on the microbiome. There are different possible explanations for the near associations seen. PBAD may be a condition in which there is a lower intraluminal BA concentration with reduced FXR activation and lower FGF19 levels and lower bactericidal properties. This may lead to SIBO and lower B12 levels. Alternatively, SIBO may be the primary event leading to metabolism of BA and B12 resulting in reduced FXR activation, lower FGF19 levels and lower B12. Finally there may be more complex relationships between the variables plus other factors such as the immune system.
5.2 Genetic polymorphisms

Genetic mutations in enzymes involved in BA kinetics have been linked to BA diarrhoea both as familial, dominant, single gene mutations and in more subtle associations discovered in changes in BA metabolism in large cohorts of patients (131). In study 2 we have explored mutations in a number of genes. No clear single gene mutation with a large effect has been demonstrated. However a number of possible smaller effect mutations have been uncovered.

It has been reported that dysfunctional mutations in ASBT can lead to familial forms of PBAD (32). The infantile form of the disease leads to depleted BA pool size and reduced solubilisation of dietary lipid. The previously reported A171S mutation in ASBT has not been shown to significantly more prevalent in our population and no consistent abnormalities in the biochemistry of those individuals has been demonstrated. It is therefore highly unlikely that this polymorphism plays a role in the majority of subjects with PBAD. The finding that 2 of 3 subjects with AA genotype had severe PBAD with unexpectedly normal/high FGF19 certainly warrants further investigation suggesting a possible role for this mutation in a very small subset. An insignificant increase in the proportion of the L-L individuals in the day series had genotype A, again suggesting a possible role for this mutation.

Other potentially significant findings have been made in study 2. Although no strong relationships with any one polymorphism have been shown, a number of associations with a smaller effect have been uncovered by this study. 2 individuals with the FXR SNP rs61755050 had severe PBAD and low serum FGF19. The study is not powered adequately to detect associations in a polymorphism with low minor allele frequencies, however it is very possible that this rare polymorphism may have a very significant physiological effect and may be implicated in pathogenesis in a small subset of individuals. The G allele in klotho-β rs1761824 has been shown by others to be associated with faster gut transit in response to chenodeoxycholic acid and proportionally is higher in our PBAD group (70). With 6 out of 7 AA subjects having ID there is a possibility that homozygosity is protective. A pharmacogenetic study has shown that both the KLB SNP above and the FGFR4 SNP rs376618 influenced the degree of acceleration in constipation predominant IBS in response to chenodeoxycholate (69). The FGFR4 SNP in study 2 revealed significant findings in a significantly increased proportion of ID subjects being heterozygous. The significance of this finding is currently unclear; however further enquiry may be worthwhile.

What has become clear through the genotyping of SNPs in our cohort of subjects divided into PBAD or ID according to 7 day SeHCAT retention is that no clear relationship exists for any single polymorphism. Instead a number of potential future lines of enquiry have been uncovered. A number of SNPs have been shown to possibly confer a minor degree of disease susceptibility and an additive effect may exist with increasing risk burden according to number of SNPs as has been shown in other diseases. This risk may involve interaction with an environmental agent to trigger the condition; for example an infective precipitant as
in post infectious IBS. Others have performed complete exome sequencing in those with IBS-D and controls but without the benefit of further subdividing according to SeHCAT retention (105). Further studies are warranted including exome sequencing on this existing cohort of patients.

It is important to emphasise the hypothesis generating nature of these genetic studies. The sample size is only able to pick up strong genetic associations such as in a single gene disorders. Most diseases are not single gene disorders and this is likely to be the case in PBAD. If there is a genetic contribution it is likely to be a much more subtle contribution from multiple genes as is the case in other diseases such as coeliac and Crohn’s. There are multiple levels at which genetic variation may play a role in the disorder from BA absorption at the enterocyte, to FGF19 sensing at FXR, to FGF19 production and so on through the hormonal axis. The individual’s response to excess BAs in the colon may also have a genetic basis. Minor contributions may be made by polymorphisms at multiple levels. This study has highlighted possible weaker associations discussed above that may be further explored in future studies.
5.3 Bile Acid Species Characterisation

In study 1 significant associations were discovered with regard to chemically measured total fasting bile acids in the prospectively recruited cohort. Total BA correlated strongly with fasting FGF19; however BA did not correlate with 7 day SeHCAT retention. Comparison of median BA between PBAD and ID also did not reveal significant differences.

In study 3 10 subjects each from the ID and PBAD groups were selected. They were intentionally selected to have either strongly positive or negative SeHCAT scans; otherwise the 2 groups were similar in terms of age, sex and clinical characteristics. The BA species have their results expressed in arbitrary units which allows comparison between the 2 groups and allows for potential correlation with other factors. The sum of BA species in each individual correlates very strongly with the previously chemically measured total BA. This is an expected but important finding as it validates the results. The total bile acids were not significantly different between PBAD and ID. This mirrors the findings in the larger cohort with chemically measured BA.

The 17 BA were compared between PBAD and ID and only one species was found to be significantly different. αTauro-muricholic acid was significantly lower in PBAD. With a p value of 0.0043 this is highly significant, uncorrected. Using a Bonferroni correction, a value of 0.003 would have to be attained.

Bile acid characterisation using UPLC-MS has been performed by others in conventionally raised and in germ free mice (132). It is well known that the gut microbiota affects bile acid composition through deconjugation, dehydroxylation and dehydrogenation of primary BAs in the colon. The gut microbiota have been shown to reduce the BA pool size in rats compared with germ free animals(133). Sayin et al have shown significantly reduced levels of muricholic acid but not cholic acid in conventionally raised compared with germ free mice. They also found that tauro conjugated alpha and beta muricholic acids functioned as FXR antagonists, resulting in reduced expression of FXR dependent genes including FGF15. In mice therefore it has been shown that the gut microbiota regulated BA metabolism through increased levels of the FXR antagonistic BAs alpha and beta tauromuricholic acids. We have found lower levels of alpha-muricholic acid in PBAD. If this BA functions as a human FXR antagonist one would expect to see higher levels of serum FGF19 in PBAD, however as has been shown, lower levels exist. It must therefore be assumed that either alpha-tauromuricholic acid levels are not involved in the aetiology of PBAD, or potentially that this BA is profoundly agonistic. The latter is unlikely as the amino acids in the ligand binding domain are greatly conserved between species(134).

The BA pool size consists of those BA in the systemic circulation, those in the portal circulation, BA in the biliary system including the gall bladder and those BA in the small bowel. Therefore this study does not measure the BA pool size directly and will be affected by a number of factors including BA absorption from the small bowel. It has been shown that systemic BA levels correlate strongly with total BA pool size and although accurate
estimation of BA pool size is not possible the systemic BA levels does allow comparison of pool size levels between patients (135). It has been shown that the BA pool size may be elevated in PBAD (84) but this has not been found to be the case in this study. No significant difference in total BA was found, nor were there any significant differences in the individual major BAs. Different BAs have differing affinity for FXR and it is possible that FGF19 differences are accounted for through more subtle interactions between different BAs and FXR(35).
5.4 Meal stimulated response
Elsewhere in this study and published previously is clear evidence of abnormalities in fasting FGF19 in PBAD (4)(87). Post prandial FGF19 has also been explored briefly (4). This study has aimed to further describe abnormalities in FGF19 over a period of time including the post-prandial period. This aimed to allow descriptions of different patterns of FGF19 levels and allow analysis of FGF19 at different time points, exploring associations with other parameters.

The main finding has been the description of the different phenotypes of FGF19 response. Almost half the PBAD patients had the L – L pattern, where no sample was above 300pg/mL, which differs from the typical meal-stimulated normal response shown previously(40). The lack of significant FGF19 response resembles a fasting pattern but is also similar to that which has recently been shown in ileal Crohn’s disease (136). Those subjects with the L - L response have higher BA turnover as measured by 7 day SeHCAT retention than those with the other described phenotypes. The L – H phenotype is characterised by low ( < 200pg/mL) fasting FGF19 followed by levels that rise to over 400pg/mL, which is similar in post prandial response to the pattern in healthy volunteers(40). It is not clear what would cause low fasting but normal post prandial FGF19 response. It may be presumed that low fasting levels do not reflect any defect in synthesis but may be low in response to reduced FXR activation by luminal BAs.

The H – H pattern of persistently elevated FGF19 has also been described. This could be due to prolonged stimulation overnight, lack of fasting or perhaps a stress response. An impaired hepatic response to FGF19 due to receptor differences in FGFR4 or KLB-β (or further downstream) could result in excessive bile acid synthesis and high FGF19 levels. Variants in the FGFR4 and KLB genes described by Camilleri and colleagues are particularly interesting in this regard(69)(105). They have been shown to be associated with colonic transit and to interact with each other. Although we found no overall significant differences in any allele frequency between the PBAD and ID groups, there were borderline differences in genotype frequencies for the FGFR4 and KLB variants in the small subgroups, and higher FGF19 levels, suggesting these genes should undergo appropriately powered further studies in patients with well-defined PBAD.

This study only examined responses for a 6 hour time period and it would be useful to measure FGF19 over a 24 hour time period. This would be most helpful to further examine the H - H pattern to investigate FGF19 levels in the hours preceding breakfast. The description of FGF19 patterns has helped define subtypes of PBAD and has been shown to reflect different severity of SeHCAT retention. What it has not done, however, is demonstrate any differences in clinical parameters such as symptoms, duration of the condition or co-morbidity. The finding of a significant association between the 3pm FGF19 and bowel movements per 24 hours may give insight into what time point is the most
clinically significant for measurement of FGF19. This could be further investigated as in the Obadiah trial with formal diary carding on larger numbers (137).

An important finding from the study is the discovery that FGF19 correlates with serum BA levels at the previous time point (90 minutes earlier). It is clear from examining the individual FGF19 and BA traces that they exhibit a similar topography, albeit with FGF19 changes following the changes in BA. Following the description of different FGF19 patterns this finding goes someway to potentially explain abnormalities in FGF19. The fact that BA changes are followed by FGF19 clearly suggests that abnormalities in BA may be an underlying abnormality.

This study has allowed clearer definition of the abnormalities in FGF19 in PBAD and has uncovered possible underlying causes. Limitations are that there were too few diarrhoea controls or healthy controls to allow meaningful comparison. A lack of formal diary carding meant that patient memories were relied upon for symptom data which may have introduced bias. Finally, the time was limited to 6 hours which although providing much greater information than the fasting only sample ‘snap shot’, still does not allow a description of nocturnal BA metabolism that a 24 hour study would.
5.5 Ageing Study

Reviewing the literature there is no clear relationship between age and fasting serum FGF19. Our group has previously demonstrated a significant positive association of FGF19 with age (87). A study of BA and lipid metabolism in 435 healthy and 23 cholecystectomised individuals showed no relationship between age and FGF19(124). In a further study of 81 healthy subjects with normal glucose tolerance and 91 with impaired fasting glucose tolerance a significant negative association was found between fasting serum FGF19 and age(138).

There are 2 highly significant findings from study 5, the ageing study. Firstly it has been found that fasting levels of FGF19 are between 2 and 4.7 times lower than median levels in the prospectively recruited cohort in study 1. Secondly, a statistically highly significant positive association between age and FGF19 has been found.

The median fasting FGF19 levels are unexpectedly low in this ageing study. The serum values are much lower than both the diarrhoea control subjects from study 1 and lower than published values in healthy individuals in the literature. Median fasting FGF19 levels from study 1 in diarrhoea controls is 235 pg/mL, similar to values found in healthy individuals by other groups(139)(118). The cause of this discrepancy is not obvious. Confounding in recruitment is unlikely as the ageing cohort was carefully recruited to have no significant co-morbidities and was carefully sex and BMI matched. Behaviour of subjects with regard to fasting may confound the results, however both study groups were both asked to fast before the sample was taken. Other possibilities relate to sample preparation, storage and processing. Venesection and centrifuge protocols were the same between the 2 studies. In the ageing study samples were kept for over 6 months at –20°C, considerably longer than the samples from study 1 which were kept for 3 months at most at -80°C. Thawing and ELISA was performed by the same individual by the same protocol. Furthermore, a standard mid-pool sample was use as a comparator in different ELISA batches and this was not found to be significantly different between the relevant batches. Sample storage therefore is the likeliest cause for the discrepancy in FGF19 values.

Despite the possibility of degradation of FGF19 during storage the association with age is nevertheless strong and it might be assumed that it remains valid as storage related degradation would affect all samples equally. That the subjects were BMI matched is important as BMI increases with age and this would have been a strong confounding variable. Unfortunately other serum parameters were not available for the study group including lipids and serum BAs. In future studies on the effect of age it will be important to analyse other variables to determine if the age association of FGF19 is independent of these factors.
5.6 OBADIAH Study

In this study it has been demonstrated that Bile Acid Diarrhoea may be treated with a potent FXR agonist which in certain subgroups leads to significant clinical improvement and significant alterations in bile acid homeostasis. A significant increase in the primary end point, fasting FGF19 was shown in PBAD. No significant change in fasting FGF19 in ID was seen.

It has been shown in previous work that subjects with primary and secondary BAD have lower fasting FGF19 compared with diarrhoea controls. Furthermore it has been shown in serial measurements of FGF19 over the course of 6 hours different patterns of FGF19 secretion between different individuals with BAD and compared with healthy individuals. It has been shown in Study 4 that some individuals have lower fasting levels of FGF19 while others have levels which are higher pre-prandially and stay high post prandially, suggestive of a possible ‘upstream’ defect in the FXR – CYP7A1 axis (40)(140). Others have demonstrated differences in klotho β genotype in some subjects with IBS-D (69). With OBADIAH it was hypothesised that OCA would lead to changes in fasting FGF19 and would also lead to higher post prandial FGF19 levels, through activation of FXR.

Evidence of FXR activation

Changes in BA homeostasis have been demonstrated in this study through the activation of FXR. Activation of FXR was shown through significant increases in fasting FGF19 in PBAD. It was also demonstrated through high levels of circulating FGF19 after OCA administration, up to 1723pg/mL with a median peak level of 1278pg/mL in PBAD. These levels are consistent with work that has been done in other studies and significantly higher than in previously done 6 hour post prandial studies (without OCA) on those with PBAD where the peak FGF19 level was 866pg/mL (91). Changes in bile acid metabolism were reflected in significantly reduced total BAs in the entire cohort as well as reductions in C4 level in the groups. These changes indicate that in PBAD OCA was able to enter the ileal enterocyte and activate the nuclear BA receptor. Within the idiopathic diarrhoea cohort there was an increase in fasting FGF19 which did not reach significance. This may in part be due to smaller cohort size. Previously published work using an in vitro ileal explant system has shown that incubation with OCA leads to increased FGF19 transcript expression. FGF19 protein secretion was also seen to be potently induced through incubation with OCA (141).

PBAD

Significant clinical improvement was seen in this group. All 10 subjects had an improvement in the composite score, the Diarrhoea Index. A limitation of this study is the lack of placebo control groups so clinical improvement may be due to the ‘placebo effect’. Of note, however, is the correlation between % change in fasting FGF19 and % change in stool frequency and the correlation between % change in BA AUC also with % change in stool frequency. Therefore, those with greater increase in FGF19 had the greatest improvement in stool frequency suggesting that clinical improvement is related to altered BA physiology and its effects on GI function, rather than placebo or psychological causes. Elevation by OCA
of FGF19 to levels found in normal individuals leads to reduction in BA synthesis as shown by the significant reduction in C4. This reduction in biosynthesis leads a reduction in BA secretion into the small bowel which is then reflected in a significant reduction in peak BA and BA AUC. This reduction in BA in the TI may lead to reduced concentrations in the colon where their effects on water and electrolyte secretion plus motility effects is not realised. Other potential mechanisms for action of OCA may be a direct effect on colonic mucosa, it has been shown to attenuate colonic secretory function and prevent diarrhoea in an experimental model by others (142).

**Idiopathic diarrhoea**

Changes in serum FGF19 were less marked in the ID group. Fasting FGF19 significantly lower in OBADIAH than in Study 1, the prospective cohort study (116 vs 236 p=0.06). Indeed, fasting FGF19 was lower in the ID group than in PBAD in this study although not significantly so. The median FGF19 was also lower in the ID group in OBADIAH compared with the PBAD group in Study 1, but again not significantly so (116 vs 148 p = 0.192. The increase in fasting FGF19 after 2 weeks of OCA was also non-significant although there were significant changes in C4 and peak BA and BA AUC. Although overall symptoms did not improve in the ID group overall, symptoms change did correlate with % change in BA AUC (R=-0.83 p = 0.021). The ID group with their normal SeHCAT retention would not have been expected to respond clinically to OCA as they have no defect in BA metabolism. The fact that they have significantly different fasting FGF19 to Study 1 ID subjects does suggest that either the 8 subjects recruited to OBADIAH are in some way atypical or different to those recruited to previous studies, or that there may have been problem with sample storage or the FGF19 assay.

**Tolerance and safety**

In this study OCA was well tolerated with minimal potential side effects and has been noted without a placebo arm it is not possible to say whether side effects such as headache are due to OCA. The most significant finding was a significant increase in LDL and HDL cholesterol, due to significantly decreased bile acid synthesis as reflected by an 80% decrease in C4 overall. BA synthesis is the primary route for cholesterol excretion and therefore this increase in cholesterol is unsurprising. The findings in OBADIAH with regard cholesterol levels mirror those found in previous study in subjects with non-alcoholic fatty liver disease. It is not known what effect these changes in serum cholesterol would have in the long-term but clearly this would need to be explored further before using OCA for longer periods in bile acid diarrhoea.
6. Summary

This research has, through the recruitment of a large number of individuals allowed the exploration of a number of different lines of enquiry into the pathogenesis and diagnosis of primary bile acid diarrhoea. It has attempted to further describe the condition and factors that differentiate it from diarrhoea predominant IBS. Significant clinical characteristics include longevity of symptoms, presence of steatorrhoea and elevated body mass index.

Importantly, it has again been shown the central role FGF19 plays in the aetiology of this condition with low fasting median levels found in PBAD. Different patterns of FGF19 secretion have been described in individuals with PBAD, potentially allowing further investigation into different underlying aetiologies relating to the different FGF19 patterns or phenotypes. Associations between FGF19 and other factors have been investigated including bile acids, lipids, B12 and the fat soluble vitamins A and D.

For the first time a possible group of individuals with severe PBAD, high triglycerides and high/normal FGF19 has been identified likely to represent a subset with a different aetiology to their disease, potentially an ileal transporter defect.

Among the strongest correlations demonstrated in this research has been that between FGF19 and total bile acids. This applies both to the fasting levels shown in study 1 as well as postprandial levels explored in the day series in study 4. There is evidence from study 4 that changes in BA level may precede changes in FGF19 and may therefore be the factor determining abnormalities in serum FGF19. There are many factors determining systemic serum BA level including TI and hepatic BA transport, spill-over rate between portal and systemic circulations as well as other factors including the role of the microbiome in degradation and conversion of BAs. There is evidence from work on ileal tissue that FGF19 expression is lower in those with lower SeHCAT retention (143). This may suggest a role of reduced BA transport in enterocytes however it has also been shown that FGF19 response to incubated chenodeoxycholate acid (unconjugated and therefore transmembrane transport is not transporter dependent) is also proportional to SeHCAT retention suggesting that reduced ileal transporter expression or activity is not the primary underlying abnormality. Other possibilities include abnormalities in transcellular transport but differences in these transporters has not been demonstrated(80). Inflammation has been shown to reduce FGF19 in Crohn’s disease(143) and inflammatory cytokines can inhibit FXR(144), however there is no histological evidence of inflammation in PBAD. Bile acids have been further explored in this research through the characterisation of BA sub species through UPLC-MS. This revealed a significant reduction of the FXR antagonistic BA α tauro-muricholic acid in PBAD subjects, the significance of which remains unclear.

In study 6 the OBADIAH study this research has shown that PBAD may be treated with the FXR agonist obeticholic acid leading to significant clinical improvement which correlate with significant alteration in BA homeostasis. This study showed that a significant increase in
fasting FGF19 subjects is possible through the use of OCA to levels comparable with healthy volunteers. The significance of this finding is firstly that it suggests that lower FGF19 in PBAD is not due to a defect in production but possibly an abnormality in the pathway that leads to FXR exposure to BA. Secondly OBADIAH has shown clear potential therapeutic benefit which should be explored further.
7. Weaknesses

**Study 1.** This part of the study relies on self-reported symptoms to define diarrhoea. It is defined as 3 or more Bristol Stool Form stools of type 6 or 7 for more than 3 months. This inherent subjectivity makes analysis and comparison of symptom severity challenging to interpret. The nature of this investigation is to explore underlying physiological mechanisms and reliance on self-reported symptoms introduces undesirable bias. Although much more time consuming a study which examines stool weight and form objectively would allow more meaningful analysis.

FGF19 increases very significantly post-prandially and therefore it is of great importance that the samples taken were genuine fasting samples. All patients were asked to fast before the sampling period but it was not possible to check that this was actually done. The sampling period was early afternoon for most patients so it is quite likely that some will have eaten in the preceding few hours. This could only be negated by admitting patients to a research ward in the hours before or by using a reliable serum marker of the post prandial phase.

**Study 2.** This part of the project investigated polymorphisms in candidate genes involved in BA homeostasis. It explored specific polymorphisms that have been linked to other diseases involved with BA metabolism or diarrhoeal conditions. By only exploring 9 SNPs it was a very narrow search and has not investigated other polymorphism or genetic factors. Due to the sample size it was only powered to pick up very strong genetic effects. It is possible that PBAD has a partly genetic basis due to weaker contribution from a large number of polymorphisms which this study is not powered to detect. Exome sequencing would provide a much greater yield of information. Genome Wide Association Study (GWAS) would involve much greater subject numbers and expense but would explore weaker associations.

**Study 3.** This study aimed to measure BA species in 10 individuals with PBAD and 10 with ID. For a study with this sample size measuring biochemical factors with extreme accuracy it would have been beneficial to ensure subjects had exactly the same diet for the preceding few days and to measure other factors such as stool form and weight. Measurement of BA species in stool the same time would have provided further information for analysis and allowed comparison with systemic circulation BA species.

**Study 4.** This meal stimulated study corrected a number of factors that were missing in the above studies by examining subjects under the same conditions. The main weakness was not being 100% certain that the 9am FGF19 sample was a true fasting sample. Another weakness is that the study was limited to 6 hours. Both of these issues could be solved by admitting patients to research ward at 6pm staying NBM until 9am with sampling starting at midnight and continuing for 24 hours. Stool form and weight could also be measured.

19 of the 21 subjects had PBAD. There is effectively no diarrhoea or healthy control group. These are important to have to define normal FGF19 patterns post-prandially.
Study 5. This study examined fasting FGF19 levels in a healthy group of volunteers of normal BMI. This study has shown fasting FGF19 significantly lower than in other studies in this project or by others. The results are consistently lower throughout different ages and individuals. Compared with the other studies the difference is that these samples were not taken or immediately processed or stored by our immediate research group. The measurement of FGF19 was done in exactly the same way by our group. Possibilities for lower FGF19 include:

- Different storage temperature. These were stored at -20°C instead of -80°C. It is not known if this would lead to significantly greater degradation of FGF19 protein.
- Extra freeze – thaw cycles. As not stored by our group may have undergone thawing by others.
- Other factors – eg unrecorded dilution at some point by original research group
- Genuine lower FGF19 levels for physiological reasons.

A weakness of this study is that the samples were taken and stored by another group and it is not possible to completely certain of these conditions.

Study 6. The main limitation of this study is the lack of blinding or placebo control. Recording of symptoms in a diary is subjective and could be influenced by the placebo effect. There were however objective components to this study including the primary outcome measure serum FGF19 as well as secondary outcomes including C4 and BAs. Future studies must be double blinded placebo controlled to avoid potential biases. They may also include comparison with current standard therapies including cholestyramine and loperamide.
8. Future Work
The identification in study 4 of 3 distinct variants of dynamic FGF19 levels would benefit from being explored further both to validate the findings and to investigate these phenotypic differences over a longer time period of 24 hours. This study should include a determination of normal FGF19 pattern through the recruitment of normal healthy individuals and diarrhoea controls. Changes in BA should be explored further and determination of total BA pool size through different methodology will help determine abnormalities in overall BA homeostasis. The increased steatorrhoea found in subjects with severe PBAD raises the possibility of genuine BA malabsorption and reduction in BA pool size. Terminal ileal biopsy and explant culture from the different phenotypes, including individuals with high triglycerides will allow further investigation of a possible transporter defect in this subset.

Extracted DNA from our large cohort of PBAD subjects and controls can now undergo whole exome sequencing in an attempt to seek underlying genetic differences that confer susceptibility to the condition. Identification of candidate genes could be explored further for abnormalities in expression and function through an explant system and quantitative real-time RT-PCR.

The role of the microbiome in the pathogenesis should be explored further in light of findings of possible reduced B12 in PBAD.

Finally, obeticholic acid has shown clear potential benefit in the treatment of PBAD in the proof of concept study OBADIAH. This should be explored further to determine clinical efficacy in a larger double-blind, placebo-controlled trial. This should include an existing treatment arm. For blinding purposes the use of colesevelam would be preferable to cholestyramine.

Summary of future work

Meal stimulated study
- Study period 24 hours and to include stool form and weight.
- Determination of BA pool size through use of isotope dilution studies.
- Collection of stool for measurement of BAs

Genetics study
- Exome sequencing of DNA of existing cohort of subjects – PBAD and ID controls.
Obeticholic Acid

- Multi-centre double blind randomised control trial Obeticholic Acid in treatment of PBAD
- 3 arms: 1. Placebo 2. OCA 3. Colesevelam
## 8. Appendices

### Appendix 1 Study 1 proforma

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<td>DOB</td>
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<td>Post Code</td>
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<td>Height</td>
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#### Diarrhoea Characteristics

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<tr>
<th>No. bowel movements</th>
<th>/day</th>
<th>/night</th>
<th>&gt;25% of time</th>
<th>Y/N</th>
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<tbody>
<tr>
<td>Duration:</td>
<td></td>
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<tr>
<td>Stool type (Bristol Stool Chart):</td>
<td>Abdo pain:</td>
<td>Urgency:</td>
<td></td>
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<tr>
<td>Blood in stool:</td>
<td>Bloating:</td>
<td>Incontinence:</td>
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<td>Steatorrhoea:</td>
<td>Dietary factors:</td>
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#### Past Medical History

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<th>IBD</th>
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<tr>
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#### Surgical History

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<th>Ileal Resection</th>
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#### Family History

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<td>Other</td>
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#### Medications and Social History

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<th>Alcohol</th>
<th>units/week</th>
<th>Cigarettes</th>
<th>/day and years</th>
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<tr>
<td>Foreign Travel</td>
<td>Y/N</td>
<td>Coffee</td>
<td>/day</td>
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#### Examination

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<th>+ve findings</th>
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#### Investigations

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<td>FIBC</td>
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<td>CRP</td>
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<td>Gluc</td>
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<td>Research</td>
<td>Serum</td>
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<td>Stool</td>
<td>MC&amp;S</td>
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<td>Colonoscopy</td>
<td>TI Bx</td>
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<tr>
<td>Lactose H2 Breath Test</td>
<td>Glucose H2 Breath Test</td>
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#### Imaging

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122
Appendix 2: Study 2 SNP Primers

SNP ID rs1789170 Gene FGF19
Location Chr.11:69509343
GTGCCGTGGGTTCCTCTGGGAA[A/G]TGTCTCTCCCCTTTTAGTCCTAGC
Polymorphism A/G, Transition Substitution

SNP ID rs948992 Gene FGF19
Location Chr.11:69513338
GTGGGCAGAGGGCTGGTGGGCTGGG[A/G]GATGGAAGCAGGTGACACCGGACA
Polymorphism A/G, Transition Substitution

SNP ID rs61755050 Gene NR1H4 FXR
Location Chr.12:100926308
AACGGGGGCAACTGTGTGATGGATA[C/T]GTACATGCGAAGAAAGTGTCAAGAG
Polymorphism C/T, Transition Substitution

SNP ID rs61613822 Gene NR1H4 FXR
Location Chr.12:100887101
AAGTGCATTTCAATTGAAAAATTTG[G/T]ATGGGATCAAAATGAATCTCATTG
Polymorphism G/T, Transversion Substitution
Phenotype MIM: 603826

SNP ID rs376618 Gene FGFR4
Location Chr.5:176517797
GAGGACCCCAAGTCCCATAGGGACC[C/T]CTCGAATAGGCAAGTACACCTAG
Polymorphism C/T, Transition Substitution

SNP ID rs17618244 klotho β
Location Chr.4:39448529
GCCCTGGCCTGGCGCCTCTACGACC[A/G]GCAGTTTCAGGCCCTCACAGCGCGGG
Polymorphism A/G, Transition Substitution

**SNP ID rs188096 ASBT**

Location Chr.13:103705044
CCAATGGAAACAGGAACAACGAGAG[A/C]AACCAGAGATGTACCTAAAGATGA
Polymorphism A/C, Transversion Substitution

**SNP ID rs939885 OSTα**

Location Chr.3:195955762
GCTGACCCTGGGCTTTCTC[A/G]TCCTACCCGACGGCATCTATGACCCAGC
Polymorphism A/G, Transition Substitution

**SNP ID rs7848647 TNFS15**

Location Chr.9:117569046
CATTGACCATTGTTTTAATCGAGTA[C/T]GAGGCCACAGATCGAGGTGACTGTC
Polymorphism C/T, Transition Substitution
Appendix 3: Schedule of events Study 4. Meal stimulated study

Day before sampling day

Nil by mouth from 9pm, except usual medications and small sips of water

Sampling day

08.30 Attend Sir John McMichael Centre

08.45 Interview and assessment

09.00 Peripheral venous cannula inserted. 5ml Blood drawn and processed (described elsewhere)

09.10 Standard breakfast consisting of cornflakes, 2 slices of toast with butter and jam, a banana and tea/coffee provided

10.30 5ml Blood drawn and processed

12.00 5ml Blood drawn and processed

12.10 Standard lunch provided: cheese and lettuce sandwich, crisps, yoghurt and drink.

13.30 5ml Blood drawn and processed

15.00 5ml Blood drawn and processed, cannula removed and participant discharged home.
### Appendix 4 OBADIAH symptom diary

**OBADIAH Daily Symptom and Stool Diary V1.1 1 Nov 2011**

<table>
<thead>
<tr>
<th>Day</th>
<th>Date (e.g. Monday 5 Jan 01)</th>
<th>Hours of Pain? (if no pain, enter 0)</th>
<th>Abdominal Pain Severity?</th>
<th>Urgency?</th>
<th>Bloating?</th>
<th>Treatment</th>
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<tbody>
<tr>
<td>1</td>
<td></td>
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<td></td>
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<td></td>
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</tr>
<tr>
<td>2</td>
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<td>3</td>
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<td>4</td>
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<td>6</td>
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<tr>
<td>7</td>
<td></td>
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</table>

**STOOL FORM AND TIME**

(Form = score 1-7 from 'Stool Form' list below, Time = time of stool)

<table>
<thead>
<tr>
<th>Day</th>
<th>1st</th>
<th>2nd</th>
<th>3rd</th>
<th>4th</th>
<th>5th</th>
<th>6th</th>
<th>7th</th>
<th>8th</th>
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<tbody>
<tr>
<td></td>
<td>Form</td>
<td>Time</td>
<td>Form</td>
<td>Time</td>
<td>Form</td>
<td>Time</td>
<td>Form</td>
<td>Time</td>
</tr>
</tbody>
</table>

**Stool Form**

1 = Separate hard lumps, like nuts
2 = Sausage shaped but lumpy
3 = Like a sausage or snake, smooth and soft
4 = Like a sausage or snake, smooth with cut edges
5 = Soft blobs with clear cut edges
6 = Ruffly pieces with ragged edges, a mushy stool
7 = Watery, no solid pieces

---

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1 Nov 2011 version 1.1
### OBADIAH1 Daily Symptom and Stool Diary

*Only add bowel movements if >8 in any one day. Please add the relevant date.*

<table>
<thead>
<tr>
<th>Day</th>
<th>Date</th>
<th>Stool Form and Time</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Form</td>
</tr>
<tr>
<td>1</td>
<td></td>
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</tr>
<tr>
<td></td>
<td></td>
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</tr>
<tr>
<td>2</td>
<td></td>
<td>Form</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Time</td>
</tr>
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<tr>
<td>4</td>
<td></td>
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<td>5</td>
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