Metabolomic Variability & Differential Risk in Primary Biliary Cholangitis

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ABSTRACT:

Primary Biliary Cholangitis (PBC) is a progressive cholestatic autoimmune condition that can lead to end-stage liver disease (ESLD). The hallmark, anti-mitochondrial antibodies (AMA) are present in 95% of PBC patients. 10% of patients have AMA positivity but no symptoms and normal LFTs (AMANL). The symptoms associated with PBC can significantly impact patients' physical and psychological well-being. Unfortunately, there are only a limited number of treatment options available.

This study aimed to identify metabolic signatures in serum, urine, and faeces that could differentiate PBC from AMANL and PBC patients with symptoms from asymptomatic patients. Healthy Volunteer controls and Primar Sclerosing Cholangitis (PSC) as cholestatic controls were included. We used high-throughput metabolomic techniques of Nuclear Magnetic Resonance (NMR) and Mass Spectrometry (MS). Ethical approval was sought (REC: 15/EE/0455). Principal component analysis (PCA) followed by Orthogonal Partial Least Squares-Discriminant Analysis (OPLS-DA) gave robust differentiating models, which were cross-validated with multivariate CV-ANOVA analysis.

51 PBC patients and 15 AMANL patients (median age: 64 years for PBC vs. 57 years for AMANL, p=0.12) participated in the study. Ninety percent of the PBC patients were receiving Ursodeoxycholic acid (UDCA) therapy. We found that total bile acids (BAs) were significantly higher in PBC patients compared to those with AMANL (p=0.001), with secondary BA levels also elevated (p=0.0001). The increase in serum BAs among PBC patients was linked to metabolites of UDCA and Lithocholic acid (LCA). Notably, LCA levels were also elevated in the faeces of the PBC group.

We compared the profiles of PBC patients with pruritus to those without pruritus. Hyocholic acid (HCA) levels, a secondary BA produced by gut microbiota, were significantly higher in the serum of PBC patients with pruritus (median: 2036 vs. 395, p=0.0003). HCA levels were raised in both cholestatic groups PBC (561.5) and PSC (1080) when compared to HVs (300.6), p=0.003 and p=0.0003 respectively. There was no difference comparing PBC with PSC (p=0.25) and PSC with PBC pruritus (p=0.12). Additionally, no differences were observed when evaluating profiles of PBC patients experiencing fatigue or cognitive impairment compared to asymptomatic patients.

The notably high levels of HCA in the PBC pruritus group raise questions about whether HCA is a direct cause of pruritus or a result of more complex metabolic alterations. It is important to note that due to the cross-sectional design of this study, we could not establish a "cause and effect" relationship. The study also lacks microbiome data, which may influence bile acid composition.

DEDICATION

My thesis is dedicated to my loving parents, sister and wife.

I would have been lost without their support!

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LIST OF ABBREVIATIONS

A2BP1	Ataxin-2-Binding Protein 1
AAPC	Average Annual Percentage Change
AAA-IgA	Anti-F-actin IgA
AC	Adenyl Cyclase
AGA-IgA	Anti-gliadin IgA
AIH	Autoimmune hepatitis
ALF	Acute Liver Failure
ALFF	Amplitude of Low Frequency Fluctuation
ALP	Alkaline Phosphatase
ALT	Alanine transaminase
AMA	Anti-Mitochondrial antibody
AMANL	AMA-positive normal LFTs
ANA	Anti-nuclear antibodies
ANIT	Alphanaphthyl isothiocyanate
ANOVA	Analysis of Variance
APASL	Asian Pacific Association for the Study of the Liver
APAP	Acetaminophen
AST	Aspartate transaminase
AT	Anaerobic threshold
ATP	Adenosine Triphosphate
ATX	Autotaxin
AU	Arbitrary Unit
AUROC	Area under the receiver operating curve
AVLT	Auditory verbal learning test
BAs	Bile Acids
BEC	Biliary epithelial cell
BILISA	Bruker IVDr Lipoprotein Subclass Analysis
BSEP	Bile salt export pump
BZF	Bezafibrate
CA	Cholic Acid
CAR	Constitutive Androstane Receptor
CAMCOG	Cambridge cognition examination
CCA	Cholangiocarcinoma
CD	Coeliac disease
CDCA	Chenodeoxycholic acid
CFTR	Cystic fibrosis transmembrane conductance regulator
CHB	Chronic Hepatitis B
CI	Cognitive impairment
CK	Creatine Kinase
CLD	Chronic Liver disease
CNS	Central nervous system
CMV	Cytomegalovirus
CPET	Cardiopulmonary Exercise Test
CR	Concordance rate
CRC	Colorectal cancer

CRF	Case Report Form
CSPH	Clinically significant portal hypertension
CT	Computerised tomography
CTLA	Cytotoxic T-lymphocyte antigen
DCA	Deoxycholic acid
DDC	3,5-diethoxycarbonyl-1,4-dihydrocollidine
DNA	Deoxyribonucleic acid
DS	Dominant stricture
EASL	European Association for the Study of Liver
EC50	Effective concentration 50
EMV	Electromagnetic wave
ERCP	Endoscopic retrograde cholangiopancreatography
ERK	Extracellular signal-regulated kinases
ESLD	End-stage liver disease
ESS	Epworth Sleeping Scale
FA	Fatty Acid
FC	Fold change
FDR	First degree relative
FGF	Fibroblast growth factor
FIS	Fatigue Impact Scale
FoxP3	Forkhead box protein
FXR	Farnesoid X receptor
GC	Gas Chromatography
GCA	Glycocholic acid
GCDCA	Glycochenodeoxycholic acid
GCP	Good Clinical Practise
GLCA	Glycolithocholic acid
GLP	Glucagon-like peptide
GPCR	G-protein coupled receptor
GWAS	Genome-wide association studies
H&E	Haematoxylin and Eosin
HADS	Hospital Anxiety and Depression Scale
HC	Healthy controls
HCA	Hyocholic acid
HCC	Hepatocellular carcinoma
HDCA	Hyodeoxycholic acid
HDL	High-density lipoprotein
HE	Hepatic encephalopathy
HIBEC	Human Intrahepatic biliary epithelial cells
HLA	Human Leukocyte antigen
HOMA IR	Homeostatic model assessment of insulin resistance
HR	Hazard ratio
aHR	Adjusted hazard ratio
HRT	Hormone replacement therapy
HSC	Hepatic sinusoidal cells

HTA	Human tissue act
HV	Healthy volunteers
IBAT	Ileal Bile acid transporter
IBD	Inflammatory Bowel Disease
ICP	Intrahepatic cholestasis of pregnancy
I-FABP	Intestinal fatty acid binding protein
IFN	Interferon
IL	Interleukin
IQR	Interquartile range
ITT	Intention-to-treat
IVDr	Invitro diagnostic platform
LBD	Ligand binding domain
LCA	Lithocholic acid
LDL	Low-density lipoprotein
LFTs	Liver function tests
LPC	Lysophosphatidylcholine
LT	Liver transplant
MAIT	Mucosal invariant T cell
MARS	Molecular Adsorbent Recirculating System
MELD	Model of end-stage liver stage
MHC	Major histocompatibility complex
MRCP	Magnetic resonance cholangiopancreatography
MRI	Magnetic Resonance Imaging
MS	Mass spectrometry
MTR	Magnetisation transfer ratio
MWDI	Mean worst daily itch
NASH	Non-alcoholic steatohepatitis
NDS	Non-dominant stricture
NHS	National health services
NMR	Nuclear magnetic resonance
NK	Natural killer
NR	Nuclear Receptor
NRS	Numeric rating scale
NTCP	Sodium-dependent taurocholate cotransport peptide
OCA	Obeticholic Acid
OGS	Orthostatic Grading Scaling
OPLS-DA	Orthogonal Projections to Latent Structures Discriminant Analysis
OPTN	Organ procurement and transplant network
OR	Odds ratio
PAMP	Pathogen associated molecular pattern
PBC	Primary Biliary Cholangitis (Cirrhosis)
PBMC	Peripheral blood mononuclear cells
PC	Phosphatidylcholine
PCA	Principal component analysis
PCr	Phosphocreatine response

PCR	Polymerase chain reaction
PDC	Pyruvate dehydrogenase complex
PDH	Pyruvate dehydrogenase
PDFG	Platelet-derived growth factor
PFIC	Progressive familial intrahepatic cholestasis
PIS	Patient information sheet
PLS-DA	Partial Least-Squares Discriminant Analysis
PPAR	Peroxisome Proliferator-Activated Receptor
PPG	Portal pressure gradient
PSC	Primary Sclerosing Cholangitis
PXR	Pregnane X Receptor
QoL	Quality of life
QSM	Quantitative susceptibility mapping
RCT	Randomised control trial
RLS	Restless leg syndrome
RPC	Reverse phase chromatography
rPBC	Recurrent Primary Biliary Cholangitis
rsFC	resting state- Functional connectivity
RMST	Restricted mean survival time
RR	Relative risk
SIP	Sphingosine-1-phosphate
SLE	Systemic Lupus Erythematous
SHP	Small heterodimer partner
SMA	Smooth muscle antibody
SNP	Single nucleotide polymorphism
SPECT	Single photon emission computed tomography
SREBP	Steroid response element binding protein
SSRI	Selective serotonin reuptake inhibitor
TCA	Taurocholic acid
TCDCA	Taurochenodeoxycholic acid
Tfh	Follicular helper T cell
TGR	Takeda G protein-coupled receptor
TLR	Toll-like receptor
TNF	Tumour necrosis factor
Treg	Regulatory T cell
UDCA	Ursodeoxycholic Acid
US	Ultrasound
ULN	Upper limit of normal
UPLC	Ultra Performance Liquid Chromatography
UTIs	Urinary tract infections
VAS	Visual analogue scale
VDR	Vitamin D receptor
VIP	Variables of Importance
VLDL	Very low-density lipoprotein
WT	Wild type

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Outline of thesis

Aims and Hypothesis:

This study hypothesizes that environmental factors modify disease development, which can explain why at-risk individuals may or may not develop overt disease. Individuals are at risk of developing PBC either due to an autoimmune response or genetic predisposition.

Three population groups particularly at increased risk of developing PBC may serve as important settings for exploring environmental triggers, prevention, or modification of cholestatic liver disease. These groups include:

1) Individuals who produce the characteristic PBC autoantibody (AMA) but do not exhibit liver injury, as indicated by normal liver biochemistry (normal alkaline phosphatase levels in most studies).

2) Daughters of mothers with PBC, face a 30-fold increased risk of developing the disease.

3) Patients who have undergone liver transplantation for PBC, have a 1 in 5 chance of developing recurrent PBC within 10 years of receiving their graft.

We propose that PBC is a multi-step process (see Figure 1). In this model, genetically susceptible individuals or those exposed to environmental triggers may develop an autoreactive immune response that causes early bile duct damage. This autoreactive response, along with the resultant early bile duct damage, can be benign and might not lead to significant liver injury. Thus, we suggest that a second step is necessary for the clinical manifestation of the disease.

The effectiveness of first-line and second-line bile acid therapies in treating PBC, combined with extensive experimental data indicating a critical role for hydrophobic bile acids (which appear to be enriched in the bile pool in PBC) in bile duct injury, implies that the composition of the bile acid pool serves as the second factor, transforming initial immune injury into progressive bile duct damage.

Researchers hypothesize that individual variations in the gut microbiome can influence a person's risk of developing a pathogenic hydrophobic bile acid pool. This variation may stem from differences in the inherited maternal microbiome or changes to the microbiome throughout life, potentially through the use of antibiotics or immunosuppressive drugs, affecting bile acid modification during the gut phase of the enterohepatic circulation.

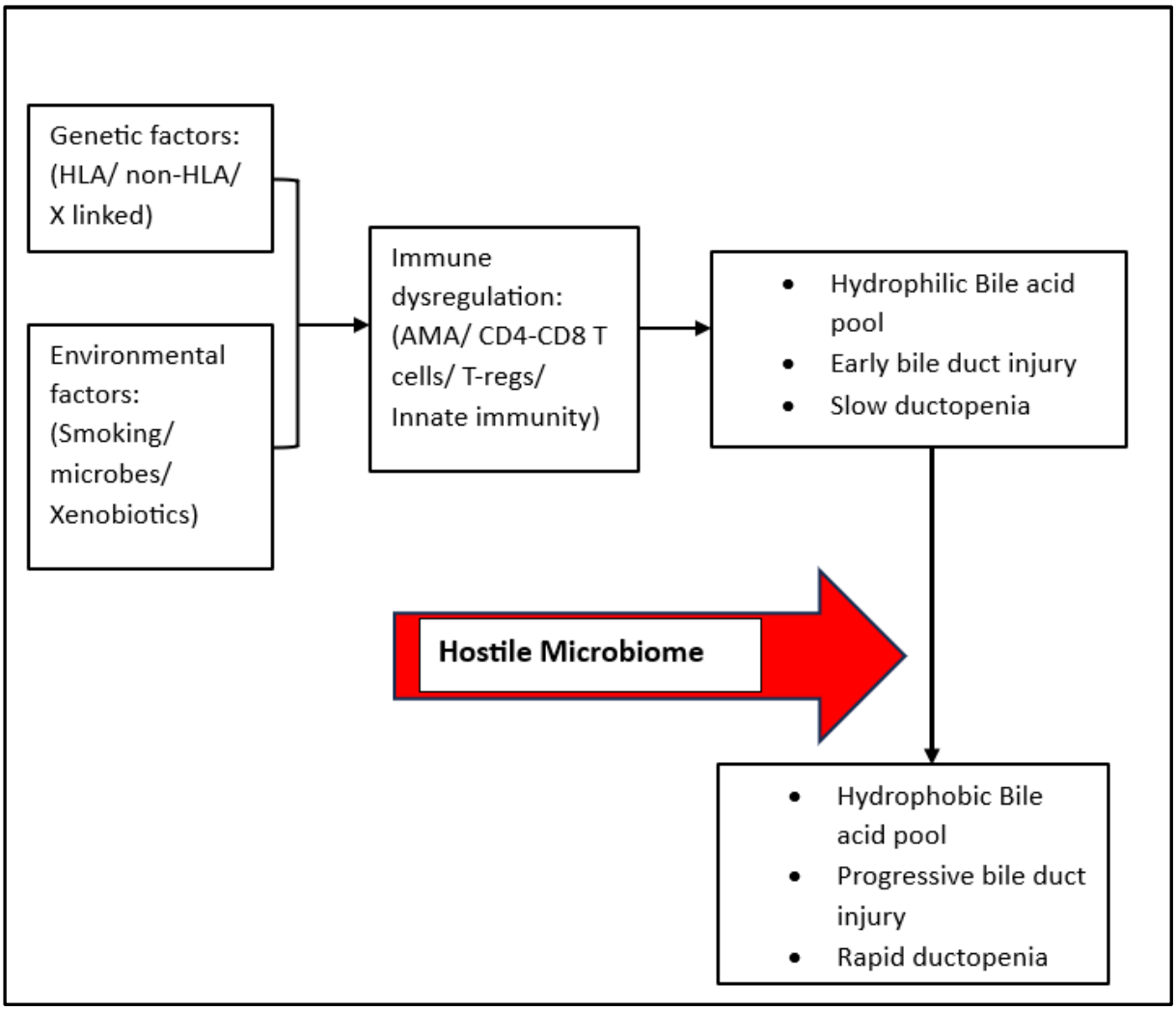


Figure 1: PBC disease progression

The development of an autoreactive immune response to mitochondrial antigens, arising because of the combination of genetic and environmental risk factors, is necessary for the development of PBC. The existence, however, of individuals in both the normal and post-PBC liver transplant population with the autoreactive response but no overt PBC means that the development of such responses is not, in isolation, sufficient for disease development. We postulate that the immune injury results in early bile duct damage which is, in the absence of other modifying factors, only slowly progressive giving disease progression only over many years. We postulate that in these individuals the bile acid pool is skewed towards a hydrophilic profile resulting from the presence of a benign gut microbiome meaning that although impaired bile flow results in increased biliary epithelial exposure to bile acid the non-toxic nature of hydrophilic bile means that no exacerbation of the immune injury is seen. The presence of a hostile microbiome drives bile acid modification within the gut towards a hydrophobic profile which results in enhanced bile duct injury and a vicious cycle of further cholestasis, exposure to hydrophobic bile acids, and bile duct injury.

Aims:

In this proposed observational study, we aim to conduct metabolomic analyses on blood (serum), urine, and stool (faeces) samples collected from the following groups:

- Non-transplanted PBC patients
- Patients with positive AMA without overt PBC and normal ALP (AMANL)
- Patients with PSC as cholestatic controls
- Healthy volunteers (HV)

The thesis aims to investigate the following questions:

1. Is there a molecular signature indicating a 'second hit' in the pathogenesis of PBC when compared to patients with AMA but without overt PBC (AMANL)? Is there a difference in the metabiome?

2. Is there a molecular signature that predicts symptoms in PBC patients? Additionally, is there a signature that can predict the response to Ursodeoxycholic Acid (UDCA) treatment in these patients?

3. Is there a molecular signature that differentiates the underlying cholestatic processes in PBC patients compared to those with PSC?

Hypothesis:

This study will test the following hypotheses:

1. AMA-positive patients show a differential metabolic profile in the urine, serum, and faeces of those who develop liver disease (PBC) compared to those who do not currently have liver disease (AMANL).
2. Symptoms in PBC are associated with distinct metabolic profiles.
3. PBC has a distinct biofluid metabolic profile when compared to cholestatic controls (PSC).

Scope of thesis:

My thesis investigates the metabolic signatures found in the serum, urine, and faeces of patients with PBC. The findings from this study may aid in designing future research aimed at identifying biomarkers for disease progression and potential therapeutic targets. Current treatment options for PBC are limited to UDCA, which is the first-line agent and is effective in only 60-80% of patients. Second-line treatments, such as Obeticholic Acid (OCA) and fibrates, have limited use due to potential side effects. Additionally, at least half of the patients experience debilitating symptoms such as fatigue, pruritus, and cognitive impairment, either individually or in combination, and they have inadequate resources for managing these symptoms. To date, only a few metabolomic studies have been conducted on PBC patients.

A subset of patients exhibits positive antibodies for PBC yet maintains normal ALP levels, with a one-in-six risk of progressing to PBC. **Chapter 3** examines significant metabolic differences in the serum, urine, and faeces of PBC patients compared to AMANL.

The symptoms of PBC can be severely debilitating, yet there is limited understanding of the underlying disease mechanisms and treatment options. **Chapter 4** will investigate potential metabolic markers that differ in the serum, urine, and faeces of PBC patients who experience fatigue, pruritus, or cognitive impairment, in contrast to those without these symptoms. As of now, only a small number of metabolomic studies have explored the effects of UDCA therapy. This chapter will also examine metabolic signatures distinguishing patients who respond to UDCA therapy (based on the Toronto criteria) from those who do not.

Another unique group includes patients with PSC, which, like PBC, is a rare cholestatic autoimmune liver disease associated with high morbidity and mortality. Currently, there are no effective treatments for PSC aside from liver transplantation in cases of end-stage liver failure. Cholestasis arises from the underlying pathological processes in both disease groups; however, significant differences exist in their natural history, prognosis, and symptom burden. **Chapter 5** will explore potential metabolic markers that differentiate patients with PBC from PSC patients.

Chapter 1. Introduction

1.1 Primary Biliary Cholangitis:

1.1.1 Introduction and Epidemiology:

Definition:

PBC is a chronic cholestatic autoimmune liver disease. PBC was formerly known as Primary Biliary Cirrhosis, but as all the patients do not have cirrhosis, the terminology has been changed to cholangitis (Beuers et al., 2015, Lleo et al., 2017). PBC is characterised by progressive inflammatory lymphocytic destruction of the bile ducts (Talwalkar and Lindor, 2003). PBC mainly affects the small and medium bile ducts compared to PSC which affects large bile ducts predominantly (Lleo et al., 2017). Left untreated, PBC can progress to liver fibrosis and cirrhotic ESLD needing a liver transplant (LT) (Talwalkar and Lindor, 2003). PBC predominantly affects female patients (female: male ratio of 9:1) (Talwalkar and Lindor, 2003, Ponsioen, 2015, Lleo et al., 2016).

History:

In 1761, Giovanni Battista Morgagni, an Italian anatomist and pathologist first described a long-standing obstructed liver as hard, green, and scirrhous. Historically this is regarded as the first ever description of secondary biliary cirrhosis. Almost a decade later in 1851, Addison and Gull, in what was called Addison-gull syndrome first presented a clinical report of nonobstructive biliary cirrhosis (Addison, 1851). Another decade later, the term Primary Biliary Cirrhosis was coined after work from American lipidologist E. H. Ahrens, linking PBC to lipid derangements (Xanthomatosis) (Ahrens Jr et al., 1950). The first series of PBC patients followed for 10 years, was presented in 1959 by Sheila Sherlock (Sherlock, 1959).

Epidemiology:

The incidence and prevalence of PBC exhibit significant regional variability (Kim et al., 2000a, Ray-Chadhuri et al., 2001). A latitudinal geoepidemiological pattern has been hypothesized due to the disease's highest frequency in North Europe (Great Britain, Scandinavia) and North America (North Midwest region) (Abu-Mouch et al., 2003, Invernizzi, 2010, Selmi et al., 2005). There are trends toward an overall rise in the incidence and prevalence of PBC.

An earlier epidemiological study from Rochester over 20 years (1975-1995) reported 46 cases of PBC (Kim et al., 2000b). The overall age-adjusted incidence of PBC (per 100,000 person-years) was 2.7 (95%CI 1.9 –3.5). The incidence for men and women was 0.7 (95% CI, 0.1–1.3) and 4.5 (95%CI, 3.1–5.9) respectively. When studied in blocks of 5 years incidence remained unchanged 2.5 (between 1975-79), 2.5 (1980-84), 3.0 (1985-89), and 2.8 (1990-95), p=0.6. The overall prevalence of PBC (per 100,000 persons) reported in this study was 40 (95% CI 27–53). The prevalence in men and women is 12 (95% CI 1.1–23) and 65 (95% CI 43 – 88) respectively.

A Chinese study of 1016 PBC patients between 2000-2015 reported an overall average annual incidence of 8.4 per million population (Cheung et al., 2017). The trend was an increase from an incidence of 6.1 per million in 2000 to 8.1 per million in 2015 (p=0.002). The overall average prevalence was 56.4 per million population, with an increase from 31.1 in 2000 to 82 in 2015 (p<0.0001).

Similarly, a study from Victoria (Australia) in 2002 with 249 PBC cases reported an overall prevalence of 51 cases per million. There was a significant rise in prevalence compared to 19 cases per million reported in 1991 (p<0.00001) (Sood et al., 2004). The prevalence was reported highest in the migrant population from Greece (p=0.03), Italy (p=0.03) and Britain (p=0.01).

A population-based study from the U.K. showed a rising trend in the prevalence of PBC in the early 1990s (James et al., 1999). 770 cases were included in the study (between 1987 and 1994) from the North England region with a population of 2.05 million. The overall incidence (per million) varied between 23-66. The mean annual incidence between 1987-90 and 1991-94 was 26 and 31 per million respectively. The prevalence rate had risen from 149 per million in 1987 to 251 per million in 1994, (p<0.00001).

A Dutch study of PBC patients (N=992) with a population of 5.8 million, between 2000-2008, reported a mean overall annual incidence (per 100,000) of 1.1 (Boonstra et al., 2014b). The incidence in women was higher 1.9 compared to men 0.3. Point prevalence in 2008 was 13 per 100,000 population. As shown by the Cochrane–Armitage test for trend, there was an overall increase in incidence and prevalence trends over the study period (p<0.001). This was attributable to a net increase in the number of PBC cases rather than a decrease due to death (p=0.007).

A meta-analysis of 24 international studies conducted between 1972-2007, reported a wide variation in the incidence and prevalence. The incidence ranged from 0.3-5.8 per 100,000 population. The prevalence ranged from 1.9-40 per 100,000 population(Boonstra et al., 2012a).

Another metanalysis by Zeng et al included 18 cross-sectional and cohort studies conducted till 2019 from the Asia-pacific region (Zeng et al., 2019). The overall pooled incidence was estimated at 8.6 per million per year. The lowest incidence was reported in New Zealand 8.1 and the highest in South Korea 8.6. The overall pooled prevalence (cases per million) was found to be 118 (95%CI 49-187). The prevalence was higher in females (104, 95%CI 42-165) compared to males (19, 95%CI 4.9-32). The highest prevalence was reported from Japan (221 cases per million) and the lowest in Australia (35 cases per million).

A recent systematic review and metanalysis included 47 population-based studies that reported geographical variation and trends in PBC incidence and prevalence (Lv et al., 2021). The pooled incidence was reported at 1.76 (range 0.23-5.3) per 100,000 population. The lowest reported incidence was from Estonia (0.23) and the highest was from Italy (5.3). Based on geographical location highest incidence was in North America (2.75 per 100,000), then Europe (1.86), and the lowest in Asia-Pacific (0.84), ($p<0.05$). The pooled global prevalence was reported at 14.6 (range 1.9-40.2) per 100,000 population with the highest prevalence in the United States and lowest in Australia. Based on geographical location highest prevalence was in North America (21.8 per 100,000), followed by Europe (14.5), and lowest in Asia-Pacific (9.8), ($p<0.05$). The pooled incidence has risen in America and Europe in the past 50 years. For example, in America, incidence rose from 1.48 per 100,000 in the 1980s to 3.6 per 100,000 in 2000. There were no reported studies from Asia before 2000, to report trends. Similarly, prevalence is also on the rise worldwide. In comparison between 1971-80 and 2011-20, the prevalence (per 100,00 population) rise was 3.7 to 29.2 in North America and 3.23 to 17.3 in Europe. In Asia-Pacific, the reported rise is 2.02 to 15.3 between 2000-2020.

Associations:

- ***Familial risk:***

Several correlations between PBC risk and other factors have been identified. There is a 6% chance that a patient's family member will get PBC. Research by an American epidemiology group found that familial PBC was most prevalent in sisters (4.3 percent) and mothers (1.7 percent) (Gershwin et al., 2005a). Compared to dizygotic twins, monozygotic twins exhibit a concordance rate (CR) of 65%. A study by Carlo et al included 16 pairs of twins (8 identical) with the diagnosis of PBC. Of the 31, 21 participants had confirmed diagnosis of PBC, and 11 did not have PBC. Within 8 pairs of monozygotic twins PBC was diagnosed in both in 5 pairs, resulting in CR= 0.63. The CR in dizygotic twins in this study was zero (Selmi et al., 2004).

Familial prevalence varies worldwide. An earlier study in the UK involving 736 PBC patients found a familial prevalence of 1.1%, with 10 patients diagnosed with PBC from eight families (Brind et al., 1995). A study from North America (N=405) with PBC in at least one family member of 26 patients' reported familial prevalence of 4.3% (Bach and Schaffner, 1994). Jones et al, (N=160), reported PBC prevalence in first-degree relatives (FDRs) of 0.72% and offsprings (daughters) of 1.2%. The relative risk of PBC in siblings was 10.6 (Jones et al., 1999). A French study of PBC (N=222, 1155 FDRs) and matched controls (N=509, 3037 FDRs), reported a frequency of PBC in FDRS of 4% compared to 0% in controls (OR-6.4, $p<0.002$) (Corpechot et al., 2010). A more recent study from Iceland (N=222), reported a high relative risk of PBC in first-degree relatives (RR=9.2, $p<0.0001$), second-degree relatives (RR=3.6, $p=0.01$) and third-degree relatives (RR=2.6, $p=0.01$)(Örnolfsson et al., 2018). From Japan familial frequency of 5.8% was reported in one study (N=171 PBC, 10 familial PBC in 5 families) (Yanagisawa et al., 2010).

- ***Genetics:***

Microsatellites are short segments of repeated DNA at a particular locus in a chromosome. These DNA segments usually made of 2-10 base pairs are repeated several times in succession at a specific location in a genome (Wright and Bentzen, 1995). These vary in numbers in different individuals and, therefore can be used for genetic fingerprinting (Vieira et al., 2016). A single nucleotide polymorphism (SNP) is a variation in DNA that results in a difference of a single nucleotide compared to the reference sequence (Komar, 2009). SNPs that occur within or near the regulatory regions of genes can impact gene function and contribute directly to disease pathogenesis. Polymorphism refers to the genomic variability between two randomly selected individuals. SNP is the simplest form of polymorphism, that exists between individuals and is estimated to occur at a frequency of one in 1000 base pairs (Brookes, 1999, Abecasis et al., 2010). SNPs can either function independently to cause Mendelian diseases or work in combination to influence more complex diseases.

Several studies have described an association between microsatellites or SNPs and PBC susceptibility. A Japanese study of case-control susceptibility analysis included 126 PBC patients and 95 healthy controls. A total genome scan was done using 400 microsatellite markers. D16S423- microsatellite marker on chromosome 16p showed the strongest association with PBC (59.5% vs 33.7%, OR =2.9, $p_c = 0.002$), and protein-coding gene *ataxin 2-binding protein 1* (*A2BP1*) was targeted as a candidate susceptible gene. Seven SNPs in A2BP1gene were genotyped. Of these genotype frequency of the major C allele at rs6500742 was found to

be significantly associated with PBC (62.3% vs 51.6%, OR=1.55, 95% CI 1.05–2.27, p = 0.02)(Joshita et al., 2010). Another study from Japan (73 PBC patients and 186 healthy controls), using fluorescence-labelled PCR analysed genetic polymorphisms at six microsatellite markers (D6S1568, DQ.CAR, D6S273, TNF-d, C1-2-A, and C3-2-11). Linkage disequilibrium mapping is a genetic method that studies the relationship between genetic polymorphisms and phenotypic variation. Using this technique, they demonstrated that the potential PBC susceptibility gene lies in or around the TNF gene (Allelic association test-allele125 of TNF-d (P = 0.0007, Pc = 0.005)(Yahagi et al., 2007).

Cytotoxic T-lymphocyte antigen 4 (CTLA4), in one study (402 PBC patients and 279 controls) was suggested to have a significant impact on the risk of developing PBC. This was further suggested to play a possible role in the development of AMA antibodies and disease progression to liver transplantation. CTLA4 encodes a self-tolerance regulatory immunoreceptor that is a key regulator. It is known to have genetic associations with various autoimmune conditions (Juran et al., 2008).

The Human Leukocyte Antigen (HLA) complex is a cluster of genes (Class I, II, III) located at the short arm of chromosome six. These genes encode proteins involved in immunogensis and antigen presentation. Italian genome-wide association study (GWAS) in PBC (N=676) and controls (N=1440), reported PBC association genes with HLA class II. The strongest association was observed with DRB*08 ($p=1.6 \times 10^{-11}$), *DRB1*11* ($p=1.4 \times 10^{-10}$), *DRB1*14* ($p=6.9\times 10^{-7}$) *DQB1*05:03* ($p=6.2 \times 10^{-7}$) and *DPB1*03:01* ($p=9.2 \times 10^{-7}$). HLA-DRB1 genes accounted for most of the signals and *DRB1*08* was the strongest predisposing allele ($p= =1.59 \times 10^{-11}$). *DRB1*14* ($p=9.2 \times 10^{-7}$) was risk predisposing and *DRB1*11* ($p=1.4 \times 10^{-10}$) protective allele (Invernizzi et al., 2012). UK PBC Consortium GWAS study (N=2861 PBC) and matched controls (N=8514), reported most significant associations with the *HLA-DQA1*0401* all (OR = 3.1, $p= 6 \times 10^{-45}$), *HLA-DQB1*0602* (OR = 0.6, $p= 2.3 \times 10^{-15}$), *HLA-DQB1*0301* (OR = 0.7, $p = 6.5 \times 10^{-14}$) and *HLA-DRB1*0404* (OR = 1.6, $p= 1.22 \times 10^{-9}$) (Liu et al., 2012).

In contrast to the European population, a GWAS study in an Asian population from Japan, reported a distinct PBC-associated allele profile DRB1*08:03-DQB1*06:01 and DRB1*04:05-DQB1*04:01, indicating ethnic difference. The study reported two distinct HLA regions in the Japanese population, not seen in the European population- *TNFSF15* (rs4979462), [OR= 1.6, $p = 2.9 \times 10^{-14}$] and *POU2AF1* (rs4938534) [OR = 1.4, $p = 2.4 \times 10^{-8}$] (Nakamura et al., 2012). It is important to understand the ethical variations to inform a better understanding of disease modelling.

- ***Other associations:***

A history of urinary or vaginal infections has been associated with an increased risk of PBC (Burroughs et al., 1984). Patients with PBC have a higher frequency of recurrent urinary tract infections (UTIs). Additionally, a specific mutant strain of E. coli, known as the rough type, is found more frequently in stool samples from PBC patients. AMA which targets mitochondrial 2-oxo-acid multi-enzyme complexes—specifically the pyruvate, oxo-glutarate, and oxo-acid dehydrogenase complexes, collectively referred to as M2—are believed to be induced by recurrent UTIs (Van de Water et al., 2001). Research indicates that this subgroup of patients experiences a phenomenon known as molecular mimicry, where there is cross-reactivity between the M2 antigens found in PBC patient sera and similar antigens in bacteria. This suggests that M2 antibodies may be produced in response to urinary organisms in females with recurrent UTIs (Butler et al., 1993, Butler et al., 1995). Card et al conducted a case-control study from the General Practice Research Database. The study included PBC patients (N=800), general population matched controls (N=7991), and unmatched chronic liver disease patients (N=12137) (Varyani et al., 2011). The study found that 29% of PBC patients had experienced a UTI at least 12 months prior to diagnosis, compared to 22% among the control group (OR = 1.5, 95% CI 1.3-1.8). For a history of pyelonephritis, the rates were 1.75% in PBC patients compared to 0.99% in controls, (OR= 1.8). While the study demonstrates a strong association between PBC and UTI, it lacked data regarding the timing of AMA positivity and any abnormalities in liver function tests.

Like many autoimmune illnesses, the presence of other autoimmune diseases raises the likelihood of developing PBC. Rheumatoid arthritis (10%), Systemic lupus erythematosus (SLE) (3%), autoimmune thyroid disease (9%), Raynaud's syndrome (12%), Sjogren's syndrome (10%), and scleroderma (2%) are frequently linked autoimmune disorders (Uddenfeldt et al., 1991, Selmi et al., 2011). A history of past or present smoking (Parikh-Patel et al., 2001), use of Hormonal Replacement Therapy (HRT) (Guattery and Faloon, 1987), regular use of hair dye or nail polish (Padgett et al., 2005), younger age at first pregnancy, and nulliparity are also proposed to be related to the development of PBC (Gershwin et al., 2005a).

An earlier American epidemiological study identified risk factors associated with PBC. The study enrolled 1,023 PBC patients from 24 centers and compared them to 1,041 controls selected through random-digit dialing.(Gershwin et al., 2005b). Participants completed a standardized questionnaire based on the US National Health and Nutrition Examination Survey (NHANES III), which included 180 questions and 300 sub-questions administered through

telephone interviews. PBC compared to controls had higher rates of SLE (3% vs 0.5%, p=<0.001), autoimmune thyroid disease (9% vs 1%, p=<0.001), Raynaud's syndrome (12% vs 2%, p=<0.001), Sjogren syndrome (10% vs 0.5%, p=<0.001) and history of UTI (59% vs 52%, p=0.0003). The study also found a higher prevalence of PBC among first-degree relatives of PBC patients compared to controls (6% vs. 0.5%, $p < 0.001$), with specific rates of 4.3% in sisters and 1.7% in mothers ($p < 0.01$). Additionally, a history of cigarette smoking was significantly more common in the PBC group (60% vs. 54%, $p = 0.003$). Moreover, greater use of nail polish (29% vs. 22%, $p < 0.001$) and hair dye (38% vs. 35%, $p = 0.04$) was observed among PBC patients. In multivariate analysis, the risk of developing PBC was significantly associated with the following factors: having first-degree relatives with PBC (OR = 10.7), a history of urinary tract infections (OR = 1.6), smoking (OR = 1.6), and hormone replacement therapy (HRT) use (OR = 1.5).

A systematic review and metanalysis (N=21577 from 9 case-control studies), reported a higher risk of PBC in smokers (both current and past), (pooled OR= 1.3, 95%CI 1.03-1.67) (Wijarnpreecha et al., 2019). Another meta-analysis of 5 studies (PBC= 1913 and HC=4697), showed a strong association with a family history of PBC (OR=7.5, 95%CI 1.9-13.2), history of urinary tract infection (OR=2.0, 95%CI 1.4-2.7) and smoking (OR=1.7, 95%CI 1.4-1.9) (Liang et al., 2011).

A multicenter study from Japan with PBC (N=548) and matched HC (N=548) presented similar findings (Matsumoto et al., 2022). Participants completed a comprehensive 121-item questionnaire that covered demographics, anthropometrics, socioeconomic status, lifestyle factors, past histories of autoimmune and non-autoimmune diseases, surgeries, vaccinations, autoimmune diseases in first-degree relatives, and the reproductive history of female participants. The risk factors associated with PBC were a history of smoking (OR=1.7), hair dye use (OR=1.6), history of autoimmune disease (OR=8.9), and first-degree relative with PBC (OR=21).

In another study in PBC (N=97) participants found a significant association between smoking and the degree of liver fibrosis, as determined by Ludwig staging based on liver biopsy (Zein et al., 2006). Among the participants, 49% were classified as having advanced fibrosis (Ludwig's stage 3-4). The advanced fibrosis group had a significantly higher history of smoking (71% vs 33%, p=0.0008), longer duration [>10 pack-years] (71% vs 19%, p<0.001), and greater average pack years (30 vs 17, p=0.04). On multivariate analysis >10 pack years of smoking was significantly associated with advanced fibrosis at presentation (OR=14, 95%CI 4.3-49,

p<0.0001). The results were cross-validated in an independent cohort of 172 PBC patients with 51% having advanced fibrosis (stage 3-4) at presentation.

Similar findings were reported in a French study of 223 PBC patients of which 164 (74%) had liver biopsy at diagnosis (Corpechot et al., 2012). Among them, 11% were active smokers, 23% had a significant smoking history (over 5 pack-years) at the time of diagnosis, and 41% reported passive exposure to smoking. Smokers had a higher proportion of advanced fibrosis (F3-4) compared to non-smokers (33% vs 16%, p=0.02). The smoking intensity, measured in pack-years, was also greater in those with advanced fibrosis (8.1 vs 3.0, p=0.01). In multivariate analysis, a smoking history of over 10 pack-years was linked to an increased risk of advanced fibrosis at presentation (OR= 13).

1.1.2 Clinical presentation and PBC-impact on Quality of life:

PBC Serology:

Serological findings typical of PBC include elevated serum ALP levels and the presence of AMA (Invernizzi et al., 2007). Serological positivity for AMA is the gold standard for diagnosing PBC. AMA is an antibody targeting the E2-subunit of pyruvate dehydrogenase complex (PDH) and is present in more than 90% of PBC patients. A recent metanalysis of 28 studies, reported pooled specificity and sensitivity for AMA of 98% and 84% respectively. The pooled specificity and sensitivity for the AMA-M2 subtype were 96% and 89% respectively. Using hierarchical summary receiver operating characteristic (HSROC), both AMA and AMA-M2 demonstrated high accuracy for diagnosis of PBC (AUC 0.98, 95%CI 0.96-0.99) (Liang et al., 2023).

It is important to note that some patients who are AMA-positive may have normal liver function tests (LFTs), and approximately one in six of these individuals may eventually develop PBC (Vergani et al., 2004, Dahlqvist et al., 2017, Hirschfield and Heathcote, 2008, Ong et al., 2014). Hence, a diagnosis of PBC requires abnormal LFTs in addition to AMA positivity. Corpechot et al conducted a large prospective study across 63 French immunology laboratories. Over 1 year follow up 1318 patients were identified to have positive AMA. 216 patients had previously established diagnosis of PBC, 275 patients had a new diagnosis of PBC and 229 patients had AMA positive without a current diagnosis of PBC. The incidence and prevalence (per 100,000 population) of AMA positivity were 1.7 and 40.4 respectively. The incidence and prevalence (per 100,000 population) of PBC were 1.0 and 24.3 respectively. In patients without PBC, the prevalence of AMA was 16/ 100,000 population. 75 % of these had normal ALP. ALP was >1.5

ULN in 13% of patients. 9 of the 91 patients over a median follow-up of 4.0 years developed PBC, giving cumulative 1-,3-and 5-year incident rates of 2%, 7% and 16% respectively (Dahlqvist et al., 2017).

Xenobiotics-induced oxidative stress has been suggested to trigger the production of AMA. Research conducted by the Acute Liver Failure Study Group in the United States, which involved serum samples from patients with Acute Liver Failure (ALF), has shown that severe oxidative stress-related liver injury can lead to AMA production. The study included 217 serum samples from patients, collected at the time of index admission, and then again at 12 and 24 months. Serum from patients with PBC and healthy volunteers was used as positive and negative controls, respectively, for comparison. The serum samples were analyzed for titer and reactivity with 2OADC-E2. Forty percent of the ALF patients were found to be AMA positive, with reactivity to all major mitochondrial autoantigens. The group further demonstrated that among the acetaminophen (APAP)-related ALF cases, 35% had AMA with similar antigen and epitope specificity as PBC patients. This finding indicates that the lipoic domain of the Pyruvate Dehydrogenase Complex-E2 (PDC-E2) is a potential target for reactive oxygen radicals induced by APAP. The majority of APAP (85%) is metabolized in the liver to non-toxic glucuronide and sulfate conjugates. However, the remaining 15% is converted to N-acetyl-p-benzoquinoneimine (NAPQI) by the microsomal cytochrome P450 isoenzyme. NAPQI is a highly electrophilic metabolite that is primarily detoxified by combining with reduced glutathione (GSH). (Harvison et al., 1988, Athersuch et al., 2018, McGill and Jaeschke, 2013). In cases of APAP toxicity, there is a depletion of hepatic GSH, which leads to an increased accumulation of NAPQI (Larson, 2007, Ramachandran and Jaeschke, 2018). In a PDC-E2 molecule, lipoic acid is attached to lysine via an amide group on the outer surface of the complex. Due to its flexible structure, lipoic acid can rotate, allowing accessibility of the PDC-E2 dithiolane ring for reductive acylation. During APAP toxicity, the depletion of glutathione potentially results in the formation of neo-antigens due to NAPQI-related oxidative modifications of the native PDC-E2. Studies have suggested that glutathiolation of PDC-E2 masks its immunogenicity during apoptosis, thereby blocking its recognition by AMA. In PBC, biliary epithelial cells (BECs) do not glutathionylate PDC-E2 during apoptosis (Howard et al., 1998, Mao et al., 2004, Odin et al., 2001).

According to European guidelines, an elevated ALP and AMA at a titer≥1:40 in adults, in the absence of other systemic disease are sufficient for a diagnosis of PBC (2017) (European Association for the Study of the, 2017). Approximately 30% of patients with PBC also test positive for anti-nuclear antibodies (ANA). Although these antibodies are less sensitive, some

exhibit a high level of specificity for PBC, reaching up to 95%. In cases of AMA-negative PBC, immunofluorescent staining can aid in diagnosis by highlighting perinuclear rims (using anti-gp210) and nuclear dots (using anti-sp100). Generally, a liver biopsy is not required for diagnosing PBC due to the high specificity of serological markers. However, a biopsy may still be necessary if PBC-specific antibodies are absent or if there is a concurrent presence of autoimmune hepatitis (AIH), PSC, or non-alcoholic steatohepatitis (NASH).

Clinical presentation and symptoms of PBC:

The clinical presentation can vary among patients. Most individuals are asymptomatic at the time of diagnosis, and abnormal liver chemistry is often discovered incidentally during routine blood tests for unrelated issues. (Parés and Rodés, 2003, Selmi et al., 2011). In rare cases, individuals who have progressed to cirrhosis may present late for treatment, showing complications like jaundice, variceal hemorrhage, and hepatic encephalopathy (HE) (Ali et al., 2011a).

Pruritus is the most common symptom associated with PBC. Pruritus can affect up to 75% of patients at some point during the disease (Hegade et al., 2017). A prevalence of 50-70% of pruritus has been reported in PBC in various studies(Heathcote, 1997, Koulentaki et al., 2006, Hegade et al., 2019a). The natural history of pruritus in PBC can be quite varied. Intractable pruritus is an independent indication for a liver transplant, in the absence of liver failure (Kuiper et al., 2010). Only limited data exists on the risk factors of pruritus in PBC. Pruritus can develop at any stage of the disease and may even predate the date of diagnosis. A study from America (PBCers organization) reported that symptoms of pruritus were experienced by 75% of the patients, preceding the diagnosis of PBC (n=238), on average for 2-5 years. The survey found that the itch was mainly worse at night, affecting the quality of sleep. The itch was reported to worsen by heat, meals, or premenstrually (Rishe et al., 2008). Another study from the U.K. in a large cohort of PBC patients (n=770) and follow-up of up to 28 years, reported the development of pruritus' in 45% and 60% of asymptomatic patients over 5 and 10 years respectively (Prince et al., 2002a). Talwalkar etal, in their review of outcomes of pruritus in UDCA clinical trials, reported that serum ALP level and Mayo risk score were independent risk factors for pruritus ($P < 0.0001$) (Talwalkar et al., 2003). In untreated PBC patients' the annual risk of development of pruritus was reported to be 27% (n=91) and improvement/ resolution without treatment was 23% (n=91). A study (N=1753) from the UK-PBC cohort found young age at diagnosis and a higher level of ALP at 12 months to be associated with persistent high pruritus'(Hegade et al., 2019a). Similarly in another study (n=2353) younger age (< 30 years)

at presentation was found to be associated with more severe pruritus' using a visual analogue scale (VAS)(Carbone et al., 2013b). The same study also reported a higher severity of pruritus in those who were UDCA nonresponsive ($p < 0.005$), biochemically associated with raised ALP.

Another frequently seen and debilitating symptom of PBC is fatigue. Forty to eighty percent of patients report experiencing weariness (Jones and Newton, 2007b, Khanna et al., 2018, Khanna et al., 2019). Up to thirty percent of the patients are unable to work, and more than twenty percent of the patients suffer from extreme fatigue (Dyson et al., 2016). Cognitive impairment (CI), while also connected with fatigue, was identified, and validated as a key symptom influencing PBC patients (Jacoby et al., 2005). 80% of PBC patients report CI, with 50% claiming moderate to severe concentration and/or memory problems (Khanna et al., 2018, Newton et al., 2008b). This is irrespective of disease stage and severity (as judged by liver function or histology) and is not reversed by LT (Pells et al., 2013). A study of 2002 patients recruited to the UK-PBC Research Cohort with a completed PBC-40 questionnaire reported significant fatigue and cognitive impairment in 60% and 36% respectively. The combination of severe cognitive impairment and fatigue was observed in 8% of the patients. Among patients with fatigue, 55% also had cognitive impairment, a group termed "fatigue with cognitive symptoms" in the study. For this group fatigue severity significantly correlated with cognitive impairment severity ($r^2=0.22$, $p<0.001$). Patients in this group were more likely to have severe fatigue compared to the no-cognitive impairment fatigue group (OR=3.8, $p<0.0001$). Furthermore, the Perceived Quality of Life (QoL) Impairment score, PBC-40 (social and emotional domain), Hospital Anxiety and Depression Scale (HADS-D, HADS-A), and Epworth Sleeping Scale (ESS) were all higher in this group ($p<0.0001$ for all). The group was associated with a higher risk of UDCA suboptimal response rate (OR=1.5, $p<0.05$) (Phaw et al., 2021)

Up to 73% of PBC patients exhibit sicca symptoms, which are characterised by dry mucosae such as the eyes and mouth. Subjective xerostomia is present in approximately 45% of patients, and concomitant dysphagia is more prevalent in this population (Mang et al., 1997, Kruszka and O'Brian, 2009).

Metabolic bone disease in PBC is a well-recognized complication (Hirschfield et al., 2018). Hepatic osteodystrophy is a term used to define bone disease in patients with chronic liver disease; and can present with bone pain and pathological fractures. Osteoporosis characterised by diminished bone volume and osteomalacia due to lack of Vitamin D3 characterised by diminished bone mineralization and the presence of osteoids is at the core of the metabolic bone disease. Several causes of bone disease in PBC have been defined. Dysregulation of vitamin D metabolism in PBC patients includes poor absorption, impaired conversion to its activated form

25-OH vitamin D, and increased urinary excretion, leading to malabsorption of calcium and osteomalacia (poorly mineralised bone)(Compston and Thompson, 1977, Cuthbert et al., 1984, Menon et al., 2001). Bone pain in PBC patients is believed to be associated with osteoporosis, osteomalacia, and rarely periostitis. BSG recommends osteoporosis risk assessment in all PBC patients and treatment in line with national guidelines. Similarly, EASL recommends assessment for osteoporosis with a DEXA bone scan at presentation and follow-up as indicated(Hirschfield et al., 2017).

Up to 30% of PBC patients suffer from restless legs syndrome, a substantially greater frequency than in the general population. Abdominal discomfort is a possible presenting sign of PBC (Anderson et al., 2013). It is frequently found in the right upper quadrant of the abdomen, with approximately 20% of PBC patients suffering from chronic right upper quadrant discomfort (Khanna et al., 2018).

Portal pressure is a gradient (PPG) between the portal and hepatic vein pressure. Normal portal pressure is up to 6mm of Hg. Portal hypertension is defined by PPG >6mm Hg and there is a significantly increased risk of variceal bleeding when PPG is >12mm Hg. A study in PBC patients (N=132), where PPG was measured through a percutaneous approach, reported portal hypertension in 35% and significant portal hypertension in 19% of the patients. Significant correlations were seen between PPG, and Mayo risk score ($r^2 = 0.26$, $p < .001$) and the liver biopsy Ludwigs stage ($r_s = 0.41$, $p < .001$). There was a significant difference in transplant-free survival in no (PPG <6 mm Hg, N=86), moderated (PPG 6-12mm Hg, N=20), and severe (PPG >12 mm Hg, N=26) portal hypertension ($p<0.0003$) (Huet et al., 2008). A retrospective study from Vienna (N=333 PBC patients) with a median follow-up of 5.8yeras, reported clinically significant portal hypertension (CSPH) in 38% of patients. The 10-year risk of decompensated CLD (dCLD) was 12% in those who were compensated. The risk in compensated CLD (cCLD) without CSPH and with CSPH was 22% and 14% respectively. The overall liver-related mortality was 7-fold higher in those with CSPH. PBC with the CSPH group had significantly lower 10-year survival rates than those without CSPH (57% vs 78%, $p<0.02$) (Burghart et al., 2022).

Portal hypertension can occur in the early stages of PBC. A study conducted in the United Kingdom with 86 participants found that portal hypertension (defined as a PPG >5 mm Hg) was present in 86% of the patients. Of this cohort, 45% had clinically significant portal hypertension. Furthermore, 72% of the patients had stage 1 or 2 PBC according to Ludwig's classification on histology, and about half of them had normal bilirubin levels (Warnes et al., 2021). Similar findings were reported in a study from China involving 180 participants, where 47% had CSPH

upon presentation. Liver biopsy results were available for 58% of participants (n=104), and within this group, 65% (68 out of 104) were classified as having Scheuer stage 1 or 2 PBC. Notably, 20% of the patients with stage 1 or 2 PBC also had CSPH. In this subset of early-stage patients, splenomegaly was identified as a strong predictor of poorer outcomes (HR = 29, $p < 0.0001$). Moreover, the 5-year transplant-free survival rate was significantly lower in patients with CSPH compared to those without (59% vs. 95%, HR = 6.8, $p < 0.001$) (Zhang et al., 2025).

Oesophageal varices can be present in 10% of patients at the time of presentation. A study involving 256 PBC patients from Japan reported that varices were observed in 8.5% of participants. Among these patients, 85% were classified as having Ludwig's stage 1 or 2 PBC, and 5% had varices at presentation, indicating clinically significant portal hypertension. Both low platelet counts and high alkaline phosphatase (ALP) levels were significantly associated with the risk of developing varices ($p < 0.001$ for both) (Ikeda et al., 2012). Similar results were reported in a study from Mayo Clinic (N=325). In their cohort, 6% of the patients with early-stage PBC had oesophageal varices (Ali et al., 2011a). Transplant-free survival rates were significantly better in patients without varices compared to those with varices. The 10-year survival rate was 83% for those without varices, compared to just 26% for those with them ($p < 0.001$), as reported in a retrospective study from a transplant center in the U.K. involving 330 patients (Patanwala et al., 2013).

An international study (N=4565) reported an incidence of HCC in PBC of 3.4/ 1000 patient years. Risk factors associated with HCC development in PBC were hepatic decompensation (HR 9.8, $p<0.0001$), UDCA non-response (based on Paris-I criteria) (HR 4.5, $p<0.0001$), advanced disease (HR 2.7, $p=0.02$), male sex (HR 2.9, $p<0.0001$), low platelets (HR 1.7, $p<0.0001$) and raised AST (HR 1.24, $p<0.0001$) (Trivedi et al., 2016).

PBC- Impact on Quality of Life (QoL):

The symptoms of PBC not only have a physical impact on patients but also affect their social, psychological, and sexual lives. This can lead to relationship breakdowns, loss of employment, depression, and in extreme cases, complete social isolation (Hale et al., 2012). A landmark national study in the UK conducted by Jones et al. examined the impact of PBC symptoms on perceived QoL in 2,353 subjects. They used several assessment tools: the PBC-40 questionnaire, the Epworth Sleepiness Scale, which measures daytime drowsiness; the Orthostatic Grading Scale (OGS), which assesses vasomotor autonomic dysfunction; and the Hospital Anxiety & Depression Scale, which evaluates levels of anxiety and depression. The

study revealed a complex pattern of significant impacts on patients. Autonomic dysfunction is a well-recognised symptom prevalent in the early and late stages of PBC. OGS (score of >/= 4 [range 0-20]) is a validated measure of vasomotor autonomic dysfunction (Newton et al., 2007). A significant number of PBC patients report increased daytime somnolence independent of associated obstructive sleep apnoea contributing in part to their feeling fatigued. An ESS score of 10 or more (range of 0-24) indicates clinically significant daytime somnolence (Newton et al., 2006). Depression and anxiety are common in PBC patients and exhibit a complex interplay with other PBC symptoms, especially fatigue, one leading to another and eventually forming a vicious cycle (Zigmond and Snaith, 1983). A significant correlation was observed between the severity of fatigue and HADS-D scores ($p < 0.0001$). However, depression as a standalone symptom was uncommon, even in patients experiencing severe fatigue. Instead, combinations of depression with sleep disturbances or autonomic symptoms were more frequently seen in those with severe fatigue. This highlights the complex relationship between depression, sleep disturbance, and fatigue in these patients, suggesting that depression may be more a consequence of fatigue rather than its primary cause. Approximately 35% of patients reported impaired perceived quality of life, compared to only 6% among healthy controls ($p < 0.0001$), and only 20% felt that their perceived health status was good. The severity of symptoms in PBC patients was significantly greater across all domains of the PBC-40 compared to healthy controls, with fatigue having the most substantial negative impact on quality of life. Furthermore, autonomic dysfunction showed significant correlations with fatigue, cognitive impairment, and sleep disturbance (Mells et al., 2013).

Another important finding from a study of a cohort of 1,990 patients indicated that a younger age at presentation is associated with a greater negative impact on perceived quality of life ($p < 0.01$), with social dysfunction being the most significant contributing factor. (Dyson et al., 2016).

1.1.3 Pathogenesis of PBC:

Pathogenesis of PBC is poorly understood, especially the relationship between immune and cholestatic processes. Chronic cholestasis may be caused by or result from autoimmune reactivity, according to competing theories (Liaskou et al., 2014). An intricate cycle of damage to the epithelial cell lining of the small bile ducts is described in one proposed mechanism. Cholestasis and bile duct atrophy result from neoantigen exposure and the subsequent breakdown of immune tolerance to the E2 component of the PDH complex (Jones, 2008,

Hirschfield and Gershwin, 2013). However, a decrease in bile flow in the biliary ducts and a shift toward a more hydrophobic bile acid pool are at the heart of the disease process.

The biliary tree is lined by biliary epithelial cells (BEC). Through apical and basolateral transmembrane channels and exchangers, BECs lead to bile formation. In PBC there is targeted damage of BECs, resulting from loss of immunotolerance to the E2 subunit of mitochondrial PDC-E2 complex. PDC-E2 which is located on the inner membrane of mitochondria is an immunodominant antigen. PDC-E2 contains a lipoic acid-lysine bond which is crucial for antigen recognition and immune activation. Exposure to a putative environmental stimulus causes apoptosis of BECs. Normally apoptotic cells modify and clear the mitochondrial PDC-E2 by binding to glutathione (Ravichandran and Lorenz, 2007). However, in PBC this regulation is impaired (Odin et al., 2001). As a result within the apoptotic bleb, the lysine-lipoyl epitope remains intact. In a study using cultured human intrahepatic BECs (HIBECs), HeLa, CaCo-2, non-transformed human keratinocytes, and bronchial epithelial cells, apoptosis was induced using UV radiation. Pre- and post-induction of apoptosis all cell lines were tested from sera of PBC (N=30), HC (N=20), and autoimmune liver disease (N=20). PDC-E2 staining was only observed in HIBECs (mean no of cells PDC-E2 stain positive= 88) compared to other cell lines (mean=0), $p<0.0001$. Further when a mouse monoclonal AMA antibody was added to the cell line, PDC-E2 on apoptotic BEC blebs reacted without the need for a permeabilization agent (Lleo et al., 2009). The lysine-lipoyl epitope immunocomplex is recognised by circulation AMA. The resultant antigen-antibody complex offsets a complex immune response resulting in further apoptosis of surrounding BECs. Lleol etal, in their invitro study using HIBECs and sera from PBC patients and healthy controls, demonstrated that when apoptotic bodies from HIBECs are cultures with PBC sera macrophages, they produce proinflammatory cytokines only in the presence of AMA. Compared to controls there was a two-fold increase in the secretion of IL-6, IL-10, IL-12, and MIP-1b; and a ten-fold increase in TNF-alfa ($p<0.001$) (Lleo et al., 2010).

Dysregulation of innate and adaptive immunity in response to loss of tolerance to PDC-E2 further propagates injury of BECs. The presence of highly specific AMA and heavy infiltration of portal tracts with CD4+ and CD8+ T cells in PBC emphasize the role of immunity. PBC patient portal tracts are rich in chemokines CX3CL1, CXCL9, and CXCL10. These chemokines recruit CD4 and CD8+ T cells leading to BEC apoptosis. BECs express Toll-like receptors (TLRs). Microbial pathogen-associated molecular patterns (PAMPs) like membrane LPS bind to TLRs leading to the release of chemokines (CX3CL1, IL-8) and activation of NK- kB proinflammatory pathways. CX3CL1 is produced by vascular endothelial cells of the portal vein in response to TLR ligand (TLR3 and 4) stimulation. CX3CL1 upregulation happens in

BECs in response to TLR3 stimulation. TLR4 activates CX3CR1+ monocytes which invade the liver cells and upregulate CX3CL1 by BECs-mediated TNF-alfa secretion. The result is an inflammatory response leading to cholangitis (Shimoda et al., 2010). NK cells exhibit both inhibitory and activating receptors, the net effect of which is key to the regulation of innate immunity. This net effect results in target cell killing whilst maintaining self-tolerance at the same time. Shimoda etal in their in vitro study demonstrated that in a high NK/BEC ratio, and the presence of APC, NK cells activate CD4+ autoreactive T cells, which are cytotoxic to autologous BECs (Shimoda et al., 2015). At low NK/BEC ratio, a cytoprotective effect is seen by IFN-γ mediated expression of Class I and II MHC molecules (Shimoda et al., 2015). Mucosal invariant T cells (MAIT) are a novel subset of innate-like T cells. Their expression is restricted to MHC class I (Treiner et al., 2003). They can be activated in response to microbial antigens or can self-activate and this results in the production of pro-inflammatory cytokines including IFN-g, TNF-a, and IL-17 (Ussher et al., 2014). In a study of 25 PBC patients and 19 chronic viral hepatitis controls, MAIT cells were reduced in both peripheral blood (0.66% vs 1.89%, $p=0.04$) and liver tissue (2.23% vs 13.0%, $p=0.002$)(Setsu et al., 2018). IL-7R (83% vs 92%, $p<0.001$) and IL-18R (73% vs 82%, $p=0.03$) levels were also significantly reduced in serum of PBC patients. Further analysis in pre- and post-6-month UDCA treated patients (N=7), showed a significant increase in MAIT cells after UDCA treatment (0.42% vs 0.27%, $p=0.02$). However, expression levels of IL-7 and IL-18 did not recover after UDCA treatment. Findings may indicate persistent activation and depletion of MAIT cells in PBC. Low levels of MAIT despite biochemical response with UDCA, indicate persistent inflammation.

Other T cells implicated in immunopathogenesis include follicular helper T cells (Tfh), regulatory T cells (Treg), and TH17 CD4+ helper T cells. Tfh is located in the germline centers in lymphoid follicles. They facilitate B cell differentiation, production of antigen-specific antibodies, and B cell memory. An Invitro study using sera from PBC patients (N=69), autoimmune hepatitis (N=16), and HC (N=20), reported several interesting findings (Wang et al., 2015). PBC patients had higher levels of circulating Tfh cells (17.8%) compared to AIH (13.7%, $p<0.05$) and HCs (9.8%, $p<0.01$). Higher levels of IL21-producing IL21+Tfh were higher in PBC compared to HCs (12% vs 2.8%, $p<0.05$). Levels of Tfh were significantly associated with B cells in PBC serum ($r=0.52$, $P<0.04$). Compared to UDCA non-responders Tfh cells were significantly lower in UDCA responders ($p=0.02$). Tfh cell levels were higher in AMA + patients (18.4%) compared to AMA -ve (13.9%), $p<0.05$). This may imply that Tfh cells may be specifically involved in AMA production.

Tregs are classified into two types depending on their developmental pathway. Natural Tregs (nTregs) are derived from the thymus and induced Tregs (iTregs) are peripherally derived. Tregs have suppressive function and control excessive immune response through modulation of antigen-presenting cells, killing of target cells, suppression of inhibitory cytokines and metabolic pathways (Liberal et al., 2015). A study of PBC patients (N=36), Chronic Hepatitis B (CHB) (N=28), and HC (N=28), demonstrated that peripheral concentration of Tregs was lower in PBC (3.9%) compared to CHB (7.4%, $p<0.01$) and HC (5.2%, $p<0.05$) (Rong et al., 2009). Another study of PBC patients (N=91), PBC relatives (N=28), and HC (N=41) reported similar findings(Lan et al., 2006). Lower levels of Tregs were seen in PBC (4.3%) compared to HCs (5.67%), $p<0.0002$. Development of Tregs depends upon forkhead box protein P3 (FoxP3). Reduced levels of FoxP3-Treg were seen in PBC compared to HC (4.3% vs 5.9%, $p<0.04$). A disease stage-dependent difference in FoxP3 expression was observed in the study. FoxP3 mean fluorescence intensity was not different in early (Stage 1 and 2) PBC (mean 17.3) compared to HCs (17.3). FoxP3 mean fluorescence intensity was higher in late-stage (3 and 4) PBC compared with early-stage PBC (19.7 vs 17.3, $p<0.03$) and HC (19.7 vs 17.3, $p<0.002$). Sisters and daughters of PBC patients also showed lower levels of CD4+CD25+Tregs compared with HCs (4.0% vs 5.3% $p< .0005$).

The liver and biliary tree have a high concentration of bile acids (BAs), which are by-products of cholesterol metabolism. Their role in regulating cellular bioenergetics is gaining increasing recognition. The human body has a robust enterohepatic circulation mechanism that maintains the levels of bile acid pools in the liver and intestine. Particularly when the profile is hydrophobic, an overabundance of BAs in the liver (biliary tract) is toxic. It can seriously affect the bile ducts (Russell, 2003b, Hofmann and Hagey, 2014, Kuipers et al., 2014, de Aguiar Vallim et al., 2013). Cholangiocytes and hepatocytes are protected from toxic bile acids by a "biliary HCO^3 umbrella," as described by De Vries and colleagues. For human cholangiocytes to maintain this biliary HCO^3 umbrella, they need the Cl^-/HCO^3 exchanger AE2, the HCO^3 exporter, and a fully functional biliary glycocalyx. Defective AE2 expression has been observed in patients with PBC (Prieto et al., 1999, Medina et al., 1997, Banales et al., 2012). Defective AE2 leads to sensing of intracellular alkaline environment by intracellular adenylyl cyclase (sAC). sAC causes BA acidification making them hydrophobic and cell permeable, which ultimately leads to BEC apoptosis. An in vitro study in H69 cholangiocytes demonstrated that bile salt-induced apoptosis is regulated by sAC. They found that compared to controls in AE2 knockdown H69 cholangiocytes there was a 2-fold increase in expression of sAC and related protein. Further AE2 knockdown, sensitised H69 cholangiocytes to etoposide-induced

apoptosis as determined by Caspase 3/7 activity ($p<0.001$). They further demonstrated that sAC inhibition by KH7 or sAC knockdown by tetracycline-induced short hairpin RNA prevented bile salt-induced apoptosis (Chang et al., 2016). MicroRNAs (miRNA) are non-coding RNAs that can modulate post post-transcription expression of genes. miRNA-506 inhibits translation and reduces AE2 activity by binding to the 3-untranslated region (3-UTR) of AE2 mRNA. In an in-vitro study using PBC (N=6) and normal liver (N=6) biopsies, miRNA-506 was found to be overexpressed (FC=3.4, p=0.001) in PBC liver cholangiocytes (Banales et al., 2012). Using real-time PCR, they demonstrated that pre-miRNA-506 transinfected H69 cholangiocytes overexpressed mature miRNA-506 resulting in a marked reduction in AE2 protein. In the cultured PBC cholangiocytes, miRNA-506 inhibition resulted in increased AE2 activity. AE2 expressed by BECs is the major Cl-/HCO3 exchanger which forms a bicarbonate-rich umbrella on the apical surface of BECs by regulating intracellular pH and biliary bicarbonate secretion. AE2 downregulation results in an alkaline intracellular environment. This leads to sAC-mediated acidification of BAs, increased BA hydrophobicity, and increased cell membrane permeability. All these changes sensitise BECs to apoptosis and lead to the formation of PDC-E2-containing apoptotic blebs. Accumulation of apoptotic blebs causes cytokine and chemokine secretion, upregulating immune activity and leading to progressive cholestasis and fibrosis.

Nuclear receptors (NRs) like farnesoid X receptor (FXR), pregnane X receptor (PXR), peroxisome proliferator-activated receptor gamma (PPAR-α), and constitutive androstane receptor (CAR) have been better understood, thanks to the decades of work that have gone into elucidating the mechanisms of BA metabolic pathways (Wittenburg et al., 2003, Sinal et al., 2000, Uppal et al., 2005, Kovacs et al., 2008). As transcription factors, NRs are essential for BA biosynthesis, transport, and metabolism. **The key functions of these nuclear receptors are discussed in Chapter 1.4.**

1.1.4 Pathogenesis of Pruritus In PBC:

Although the precise pathophysiology of cholestatic pruritus remains unknown, the available evidence points to a mixed cause. The neurological basis of general itching is distinct from that of pain, with neurons and neurotransmitters that are specific to itching (Beuers et al., 2014). Local deposition of excess bile salts has been postulated as the tissue-level mechanism for itch in cholestatic disease (Aretaeus, 1865). Bile salts, opioids, lysophosphatidic acid modified by autotoxin (autotaxin-lysophosphatidic acid axis), and serotonin are cholestatic pruritogens that have been suggested (Hegade et al., 2019b, Imam et al., 2012, Beuers et al., 2014). The best available evidence suggests a complex interaction between these agents (Hegade et al., 2016b).

- **Autotaxins:**

Autotaxin (ATX) is a human motility-stimulating protein. ATX hydrolyses choline from lysophosphatidylcholine (LPC) to produce a bioactive phospholipid lysophosphatidic acid (LPA)(Perrakis and Moolenaar, 2014). ATX is present in the circulating blood and effects of ATX are mediated mainly by the enzymatic formation of LPA. A study by a Dutch group proposed LPA (an ATX) as a potential pathogen in cholestatic pruritus. They measured Cytosolic free calcium levels, ATX activity, LPA (quantitative), bile salts, histamine, tryptase, μ-Opioid Activity, and Substance-P levels in sera from patients with cholestatic disorders (subjects- PBC and ICP) and Pregnant women and health volunteers (controls)(Kremer et al., 2010). Neuronal Ca^{+2} was higher in cholestatic (PBC pruritus) and ICP sera compared to controls. LPA was raised and identified as a major Ca^{+2} agonist in cholestatic patients with pruritus. Serum ATX was increased in cholestatic patients with pruritus compared to those without pruritus ($p<0.001$) and patients with ICP compared to pregnant controls ($p<0.0001$). The group also demonstrated that intradermal LPD injection to mice induced itch response, a finding which was previously demonstrated by Hashimoto etal(Hashimoto et al., 2004). In the same study, temporary nasobiliary drainage significantly reduced serum ATX levels ($n=4$, $p<0.05$) and associated relief in pruritus.

- **Bile Salt Theory:**

BAs have also been implicated as potential pruritogens in cholestatic liver disease using direct and indirect evidence from various studies. Though one suggestion is activation and degranulation of mast cells leading to pruritus, the exact underlying pruritogenic mechanism of

BAs is not fully understood(Quist et al., 1991). One of the earlier studies conducted in healthy individuals where they injected bile salts intradermally, showed that dihydroxy bile salts and their conjugates were more effective pruritogens than trihydroxy bile salts(Kirby et al., 1974). The application of unconjugated DCA and CDCA in a cream vehicle has also been shown to induce pruritus(Varadi, 1974). OCA is a semisynthetic bile acid and a potent FXR agonist. Pruritus is the commonest side effect of OCA therapy (both in the PBC and non-PBC group) and supports the proposal that bile salts potentially modulate pruritus by FXR activation. In a phase II double-blind, placebo-controlled trial of PBC patients (n=165) with OCA given at doses of 10 mg, 25 mg, or 50 mg pruritus incidence was reported at 47% (p=NS), 87% (p<.0003), and 80% (p<.006), respectively vs 50% in the placebo group(Hirschfield et al., 2015b). Similarly in the phase 3 trial of OCA (n= 217) in patients with PBC, randomized to receive OCA at a dose of 10 mg and a dose of 5 mg with adjustment to 10 mg if applicable or placebo over 12 months, reported a higher incidence of pruritus in the OCA group (56% in the 5–10 mg group and 68% in the 10 mg group) compared to 38% in the placebo group(Nevens et al., 2016). However, the counterargument to bile salt theory is that no association between serum bile acid levels or bilirubin and hepatic itch intensity has been seen. For example, women with intrahepatic cholestasis of pregnancy (ICP) do not necessarily have significantly increased bile acid levels. Nasobiliary drainage, MARS, and cholestyramine therapy, which can significantly reduce pruritus, do not always significantly reduce serum BA levels(Hegade et al., 2016a, Beuers et al., 2006, Pusl et al., 2006, Geenes and Williamson, 2009). Recent work has identified bile acids as natural ligands for MRGPRX4 expressed in human dorsal root ganglion (hDRG) neurons and co-expressed with histamine itch receptor HRH1. BA stimulation of these receptors leads to neuronal activation and transmission of pruritus signalling(Yu et al., 2019, Yu et al., 2021).

- **Role of IL-31:**

Interleukin-31 (IL-31) is a known pruritogen in skin conditions like atopic dermatitis and prurigo nodularis. Anti-IL-31 receptor A (IL-31RA) antibody [nemolizumab] has been successfully used to treat itch in these patients(Ruzicka et al., 2017, Ständer et al., 2020, Furue et al., 2018). A study in serum samples obtained from five clinical studies of non-steroidal FXR agonist cilofexor evaluated serum IL-31 levels as a potential marker of pruritus(Xu et al., 2023). Samples were taken from healthy volunteers (n = 60) who participated in a phase 1 study and phase 2 placebo-controlled studies of patients with noncirrhotic NASH (n = 140), cirrhotic NASH (n = 20), noncirrhotic PSC (n = 52) and noncirrhotic PBC (n = 71). Baseline IL-31 levels

in PSC and PBC were positively correlated with the Visual Analog Scale for pruritus (ρ = 0.44, p = 0.002 [PSC]and ρ = 0.39, $p < 0.001$ [PBC]) and 5-D itch scores (ρ = 0.42, p = 0.003 [PSC] and ρ = 0.48, $p < 0.001$ (PBC]). Cilofexor did not alter serum IL-31 in patients with PSC and PSC. However, in contrast, in patients with NASH receiving cilofexor 100 mg, cilofexor dose-dependently increased IL-31 from Week (W)1 to W24. In patients with NASH, IL-31 was higher in those with Grade 2–3 pruritus than those with Grade 0–1 pruritus [W1 (5.1 X FC, p = 0.002), W4 (2.9 X FC, p = 0.005), W8 (3.8 X FC, p = 0.014), W12 (4.5 X FC, $p < 0.001$), and W24 (7 X FC, p = 0.01)]. *IL-31* messenger RNA (mRNA) was elevated in hepatocytes from patients with PSC and NASH compared to HVs. The percentage of IL-31 + cells in liver biopsy samples was 0.74% (NASH), 0.85% (PSC) compared to 0.18% (HV). The group also evaluated the effect of FXR agonists on human hepatocytes in chimeric PXB mice. They found increased *IL-31* mRNA expression in human hepatocytes (7.8-fold higher compared to vehicle, $p < 0.001$) and serum levels of human IL-31 (p = 0.005). Further hepatic IL-31 mRNA correlated with serum IL-31 levels (ρ = 0.61, $p < 0.001$). Clinical trials of Seladelpar a potent and selective peroxisome proliferator-activated receptor (PPAR)-delta agonist in PBC, have reported a significant reduction in patient-reported pruritus in those with moderate-to-severe pruritus(Mayo et al., 2024). Further, the evaluation of IL-31 levels in this study group reported the following findings(Kremer et al., 2024). Baseline IL-31 levels closely correlated with pruritus numerical rating scale (NRS, 0–10) ($p <$ 0.0001), total BAs ($p <$ 0.0001), and conjugated BAs ($p <$ 0.0001). Decreases in IL-31 were observed with Seladelpar 5 mg (−30%, p = 0.0003) and 10 mg (−52%, $p < 0.0001$) compared to placebo (+31%). Patients with clinically meaningful improvement in pruritus (NRS ≥ 2 decrease) demonstrated greater dose-dependent reductions in IL-31. The findings add further evidence to the role of IL-31 in pruritus in liver disease and the potential of being a therapeutic target in managing cholestatic itch.

1.1.5 Pathogenesis of Fatigue in PBC:

Although the etiology of fatigue is poorly understood, several bioenergetic abnormalities have been identified. PBC patients exhibit fatigue with both peripheral and central components.

A hand grip protocol, in fatigued PBC patients demonstrated a significantly rapid decline in muscle function with repeated activity (Goldblatt et al., 2001). Intramuscular acidosis contributes to muscle fatigue during prolonged muscle activity (Rico-Sanz, 2003, Allen et al., 1995). In fatigued PBC patients, a novel magnetic resonance spectroscopy revealed a significant mitochondrial dysfunction resulting in excessive acidosis after prolonged exercise, and a delay in recovery. In PBC patients, a ratio between phosphocreatine responses (PCr) by mitochondria

and adenosine triphosphate drive (ATP) was no longer tightly regulated (the ratio being higher in fatigued PBC patients). This was also associated with high anti-PDC levels (Hollingsworth et al., 2008). Using impedance cardiography and magnetic resonance, it is determined that PBC patients have a significantly lower PCr/ATP ratio in their cardiac muscle (Jones et al., 2010). Using cardiopulmonary exercise testing (CPET), it has been demonstrated that PBC patients undergoing LT evaluation have a lower baseline anaerobic threshold (AT) compared to controls(Prentis et al., 2011). Over ninety-five percent of PBC patients have high titers of AMA directed against the E2 and E3BP subunits of PDH (pyruvate dehydrogenase), the key enzyme in anaerobic metabolism regulation. These findings suggest that fatigued PBC patients may exhibit an excessive shift in metabolism from aerobic to anaerobic pathways, resulting in an excessive accumulation of lactic acid (Teoh et al., 1994, Yeaman et al., 2000).

This central component characterized by neurophysiological abnormalities is associated with cognitive symptoms, sleep disturbance, and depressive symptoms (van Os et al., 2007, Cauch-Dudek et al., 1998, Montagnese et al., 2013). A mouse model of inflammatory liver injury suggested a possible immune-mediated inflammatory pathway involving the entry of inflammatory cells into the central nervous system (CNS) and subsequent central neural changes and ensuing behavioural alterations. MCP-1/CCL2 was found in the periventricular and perivascular regions of BDR mice at day 5. Further immunohistochemical staining of brain sections from BDR mice at day 10 revealed the transmigration of CFSE-labelled monocytes into the periventricular and perivascular regions of cerebral parenchyma. The findings suggest that leukocytes may cross the blood-brain barrier into the CNS (D'Mello et al., 2009) 2009). Mosher et al. investigated the resting-state functional connectivity (rsFC) of deep grey matter brain structures, using resting-state functional magnetic resonance imaging (rsfMRI). Patients with PBC had significantly altered rsFC levels compared to healthy controls. Changes in deep grey matter rsFC were associated with symptoms of fatigue, and verbal working memory performance and may reflect chronic immune-mediated signalling between the liver and brain (Mosher et al., 2017b).

1.1.6 Pathogenesis of Cognitive Impairment in PBC:

PBC patients have impaired sensory-emotional neuronal networking, which is connected to symptom burden, according to functional Magnetic Resonance Imaging (MRI) investigations. However, no physiological mechanism has been established to be responsible for this (Mosher et al., 2017b). Additionally, a correlation has been shown to exist between a decline in cognitive

performance and autonomic dysfunction, white matter lesions, and dysregulated cerebral circulation (Frith et al., 2012, Frith et al., 2009, Newton et al., 2008a).

In an earlier study, Goodwin et al found that patients with chronic liver illness had lower regional cerebral blood flow in the right anterior cingulate region using single photon emission computed tomography (SPECT)(O'carroll et al., 1991). The study included 10 patients with established liver cirrhosis and 10 matched healthy controls. Neuropsychological testing was done using trail making test, digit symbol substitution, auditory verbal learning test (AVLT), and Cambridge cognition examination (CAMCOG). Significant impairment in all cognitive tests except Trail A was found among cirrhotic (CAMCOG $p=0.004$, AVLT $p=0.008$, digital symbol $p=0.001$, Trail B $p=0.007$, and Trail A $p=0.3$). Significant differences in the regional cerebral blood flow existed between cirrhotic patients and controls ($p=0.02$). Functional abnormalities in the limbic cortex and basal ganglion correlated with cognitive dysfunction Trail B test ($r=0.74$, $p<0.05$) and digit symbol ($r= -0.76$, $p<0.05$). The loop between the anterior cingulate gyrus and basal ganglion regulates the attention span in the brain. Similar findings were seen in a more recent study of PBC patients. A pilot study from a Polish group of 20 prospectively recruited female PBC patients showed a positive correlation between impaired right frontal lobe perfusion and cognitive symptoms (Raszeja-Wyszomirska et al., 2017). Fatigue Impact scale (FIS) was measured at the time of performing a brain perfusion scintigraphy SPECT. FIS is comprised of physical, cognitive, and psycho-social domains. There are 40 items (scoring 0-4) giving a total score of 160. Using Spearman's correlation coefficient, a positive correlation was seen between the FIS cognitive domain and decreased right frontal lobe perfusion ($p =0.02$).

PBC is characterised by the frequent occurrence of dysregulation of blood pressure and autonomic dysfunction, both of which are independently connected to CI and weariness.

One study reported significantly more falls in PBC patients (N=97) compared to PSC (N=15) and non-liver disease controls (N=96)(Frith et al., 2010). A self-reported falls questionnaire comprising of falls in 5 years, falls in 12 months, >1 fall in 12 months and no falls, was used. A significantly greater number of patients had falls in the PBC group (73%) compared to PSC (20%) and non-liver disease controls (49%), $p<0.001$ for both. Postural hypotension as measured by OGS was significantly worse in PBC fallers ($p<0.001$).

According to the findings of a study conducted by Jones et al on 164 patients diagnosed with PBC, at least one symptom of orthostatic hypotension was present in 60% of patients, with 46% of patients experiencing moderate to severe symptoms. The study recruited 164 PBC patients, 31 PSCs, and 50 HCs. Patients completed the validated OGS questionnaire (score >9 diagnostic

of orthostatic hypotension). In addition, ESS, HADs, and FIS scores were calculated. Formal autonomics assessment was performed on 20 patients with orthostatic dizziness using Lying-standing blood pressure and head-up tilt test. Using OGS, they were able to determine that the symptoms of cardiovascular autonomic dysfunction were more severe and more frequently reported in PBC patients than in matched normal and PSC controls (mean 3.2 +/- 3.4 versus 1.3 +/- 1.9, p= .005). Neurally mediated hypotension in PBC was higher (65%) compared to the non-PBC population from the syncope clinic (34%), p<0.005)(Newton et al., 2007).

In another study conducted on 198 PBC patients with CI by Jones et al., the decline in cognitive function was associated with autonomic abnormalities (Newton et al., 2008a). The study was conducted in 3 phases. In phase 1 (N=198) PBC patients' prevalence of cognitive symptoms was determined using the PBC-40 questionnaire. Mild, moderate, and severe cognitive impairment was reported in 20%, 34%, and 19% respectively, and this was unrelated to abnormalities in LFTs or histological staging. In phase 2 (N=28) PBC patients and matched controls were subjected to trail-making and full-scale IQ test. Significant correlations were seen between the PBC-40 cognitive domain and verbal scale of IQ score (r= -0.4, p<0.05), performance scale of IQ score (r= -0.5, p<0.001), and visual constructive ability (r= -0.6, p=0.0005). In terms of autonomic function, a significant correlation (positive) was seen between IQ score and baseline systolic blood pressure (p=0.01). In phase-3 (N=11) underwent an MRI brain to identify white matter lesions. White matter lesion was seen in all patients with the greatest load in the basal ganglion and frontal lobe. Total lesion load correlated (negatively) with the cognitive function (p=0.01).

An MRI study found changes in the interoceptive regions of the brain in PBC patients (N=15) compared to healthy controls (N=17)(Mosher et al., 2019). Thalamic and insular volume, neuronal activity, and iron deposition were measured using resting-state functional MRI (rsfMRI) and quantitative susceptibility mapping (QSM). Thalamic volume was significantly reduced bilaterally in PBC patients (p=0.006). There was no difference in susceptibility mapping (p=0.7) indicating the absence of inflammation. Neuronal activity was measured using the amplitude of low-frequency fluctuations (ALFF) with rsfMRI. UDCA non-responders were found to have significantly lower ALFF compared to responders (p<0.05). Similarly, ALFF of the anterior insula was significantly reduced in the PBC group (p=0.01). No correlation of these changes was found with the duration of the disease, indicating changes may be present since the early stages of PBC.

A study from the same group evaluated changes in the deep grey matter region of the brain responsible for learning, memory, and emotional signalling (Mosher et al., 2017a). Resting state

functional connectivity (rsFC) and its association to symptoms was evaluated in PBC female patients (N=20) compared to HC (N=21). The Fatigue Severity Scale, PBC-40 questionnaire, Hamilton Depression Rating Scale (HAM-D), Trail Making Test (A and B), Digit Span Test, Wechsler adult intelligence scale, and the Corsi Block-Tapping Test were all used to assess cognition. Reduced rsFC of the thalamus and increased rsFC of the putamen were observed in fatigued patients (N=8, $p<0.05$). Digit Span test was significantly lower in PBC patients ($p=0.03$), and this was associated with increased rsFC of the right insula and decreased rsFC of the right inferior lateral occipital cortex.

A U.K.-based study reported changes in the brain in early PBC and correlation with cognitive impairment (Grover et al., 2016). Thirteen patients with biopsy-proven stage 1 or 2 PBC and 17 HC were recruited. MRI brain was performed and Magnetisation transfer ratio (MTR) was calculated for frontal white matter, caudate, putamen, globus pallidus, and thalamus. MTR correlates with the myelin concentration in tissue. In PBC patients MTR was significantly reduced in frontal white matter ($p=0.01$), caudate ($p=0.01$), putamen ($p<0.0001$), globus pallidus ($p<0.05$), and thalamus ($p<0.0001$). Further, a correlation was seen between Putamen MTR and PBC-40 cognitive domain ($r=-0.61$, $p<0.05$).

These studies raise an important question: could changes in the brain contributing to cognitive decline be a result of autonomic dysregulation in PBC? More research is needed for a comprehensive understanding of this process.

1.1.7 Management of PBC and symptoms:

Management of cholestasis in PBC

1. <u>First-line therapy:</u>

UDCA (Ursodeoxycholic Acid) was the first therapy shown to be beneficial for treating cholestasis in PBC. UDCA was the only therapy licensed up until the year 2016. UDCA is a physiological component of BAs, and makes up about 3% of the chemical composition of bile acids. UDCA is beneficial to patients with PBC through several different mechanisms. These mechanisms include its anti-apoptotic and anti-inflammatory effects on cholangiocytes (Beuers, 2006, Lazaridis et al., 2001, Miura et al., 2001, Takigawa et al., 2013, Tanaka and Makino, 1992, Tanaka et al., 1996). UDCA protects cholangiocytes from the harmful effects of BAs by

stabilizing the "biliary HCO^3 umbrella,". The administration of UDCA has been shown to improve liver function tests, resulting in a significant drop in ALP, and GGT, in addition to the reduction in cholesterol, and IgM (Combes et al., 1995, Heathcote et al., 1994, Poupon et al., 1991a). Research has shown that this is associated with significant clinical implications, including a delay in the histological progression of the disease and increased transplant-free survival.

The earliest evidence for UDCA in PBC comes from a study that began in 1982 and was reported in 1987. In this study, fifteen consecutive patients were treated with UDCA at a dosage of 13-15 mg/kg for 24 months. Significant improvements were observed at 24 months compared to baseline values in ALP levels (mean of 213 vs. 612 IU/l, $p<0.001$), gamma-glutamyl transferase (GGT) levels (122 vs. 554 IU/l, $p<0.001$), and bilirubin levels (22 vs. 36 µmol/l, $p<0.001$) (Poupon et al., 1987).

A multicentre double-blind placebo-control trial followed this. Biopsy-proven PBC patients were randomized to receive UDCA 13-15mg/kg/d (n=73) or placebo (N=73). In the UDCA group, there was a significant improvement in bilirubin, ALP, AST, ALT, GGT, cholesterol, and IgM ($p<0.001$, for all). Treatment failure as defined by a rise in bilirubin >70 µmol/L was observed in 6 patients in the UDCA group compared to 13 in the placebo ($p<0.01$) (Poupon et al., 1991b). Overall, 95 liver biopsies were available during the follow up which showed improvement in the UDCA group of the mean histologic score ($p<0.002$).

UDCA has also been shown to delay histological progression to cirrhosis. A study from the Mayo clinic compared non-cirrhotic PBC patients on UDCA (mean duration 6.6 years, N=16) to non-cirrhotic PBC patients on D-penicillamine or placebo (mean duration 5.6 years, N=51). The overall rate of progression to cirrhosis was less in the UDCA group (13% vs 49%, $p=0.01$). The pattern was maintained when UDCA was compared individually to placebo (13% vs 43%, $p=0.05$) and with D-penicillamine (13% vs 53%, $p=.007$) (Angulo et al., 1999a). Similar results were observed in a French study of 103 PBC patients with 162 paired liver biopsies. Using the Markov model, they found a lower rate of progression from early fibrosis to advanced fibrosis or cirrhosis in UDCA-treated patients compared to placebo (&5 vs 34% per year, FC=-5, $p<0.002$) (Corpechot et al., 2000). Another study combined data from four clinical trials, where paired liver biopsies were available at time points 36 months apart (UDCA treated, N=200 and Placebo, N=167). There was significantly lower progression in the UDCA group in baseline stage 1/2 PBC compared to placebo (51% vs 63%, $p<0.03$) (Poupon et al., 2003).

UDCA treatment has also been shown to slow the progression of developing varices. A multicenter, randomized, double-blind, placebo-controlled trial (n=180) with four years of follow-up performed gastroscopy every two years. The risk of developing varices was lower in the UDCA group compared to the placebo at 4 years (16% vs 58%, p=0.001) (Lindor et al., 1997).

There are mixed reports in terms of progression to hepatic decompensation or liver-related events (OLT/ death). In a French randomized control trial biopsy proven PBC patients received UDCA (13-15mg/kg/d, N=72) or placebo (N=73) for 2 years. After two years of open-label extension, all the patients received UDCA. The response probability was higher in the UDCA-treated group compared to placebo followed by the UDCA group (RR=0.28, p<0.002). The likelihood of OLT alone, and OLT and death combined were lower in the UDCA group (RR=0.21, p=0.003 and RR=0.32, p=0.005 respectively) (Poupon et al., 1994). Combined data from 3 studies compared UDCA-treated patients (N=273) and placebo (N=275). Transplant-free survival at 4 years was higher in the UDCA group (RR=1.9, p<0.001). The study also reported improved survival in stage 4 disease in the UDCA group (N=68) compared to placebo (N=65), p<0.01 (Poupon et al., 1997).Another randomized placebo-control trial of UDCA (N43) compared to placebo (N=43) from Greece with a mean follow-up of 8 years did not find any difference in decompensation (51% vs 45%) or liver-related events (OLT or death) (44% vs 30%), p=NS (Papatheodoridis et al., 2002).

A meta-analysis of (N=1447 PBC patients) comparing UDCA with placebo or no intervention reported the results from 16 RCTs. The median percentage of advanced cirrhosis was 51% and follow-up was 24 months. There was no difference between the two groups in all-cause mortality (6.4% vs 6.6%, RR=0.97), all-cause mortality, or liver transplantation (12.1% vs 12.6%, RR=0.96), pruritus (52% vs 53%, RR=0.96) and fatigue (65% vs 72%, RR=0.90). UDCA did slow the progression of the histological stage (23% vs 38%, RR=0.62) (Rudic et al., 2012). Further, a multicenter retrospective cohort study (N=1047) of PBC patients with response to UDCA therapy based on the Paris-2 criterion using adjusted restricted mean survival time (RMST) analysis reported complication-free survival gain (Corpechot et al., 2024). They included patients who were on UDCA therapy for at least 12 months and at least one documented adequate response (ALP < 1.5 X ULN). The primary endpoint was defined as survival without serious clinical events (complications of cirrhosis, OLT, and death). The incidence of serious clinical events (N=81) was found to be 17 per 1000 person-years. After adjusting for the duration of UDCA therapy, normal ALP levels were associated with complication-free survival (aHR= 0.57, 95% CI 0.27-0.99, p=0.04). The 10-year complication-

free survival rate in those with normal ALP compared to those without at the time of entry to study was 85.7% vs 73.2% ($p < 0.001$). This translates to an absolute complication survival of 7.6 months at 10 years. The gain was much higher in young patients who had raised MLS with an absolute gain of 52.8 months at 10 years.

Corpechot etal, in a retrospective cohort study (N=780) of PBC transplanted patients, reported the effect of preventative UDCA therapy (long term and started within 2 weeks post-transplant)(Corpechot et al., 2020). 24% of patients in the study were on preventive UDCA therapy. In a median follow-up period of 10.7 years, 30% of patients developed recurrent PBC. Preventative UDCA therapy was associated with lesser rates of rPBC (HR=0.41, 95% CI 0.28–0.61, $p < 0.0001$), graft loss (HR=0.33, 95% CI 0.13–0.82, $p = 0.01$), liver-related death (HR=0.46, 95% CI 0.22–0.98, $p = 0.04$) and all-cause mortality (HR=0.69, 95% CI 0.49–0.96, $p = 0.02$)

Despite its efficacy, approximately forty percent of PBC patients do not respond well to UDCA or their response is suboptimal (Corpechot et al., 2011, Kuiper et al., 2009). Risk factors for UDCA non-response include male gender and women diagnosed with the condition who are younger than 55 years of age (Carbone et al., 2013b). GLOBE and UK-PBC scores are two relatively new continuous risk assessment models that have recently been validated in a larger population originating from a more varied geographical location, which includes Europe (Carbone et al., 2016). The response to UDCA therapy has been shown to improve survival comparable to that of matched healthy populations when these prognostic models were used (Lammers et al., 2015, Carbone et al., 2016). UDCA is a drug that is safe to use and is generally well-tolerated by patients. The most common adverse reactions to UDCA include mild gastrointestinal distress, which may manifest as diarrhea. Allergic reactions to UDCA are rare.

Over several decades, various models have been developed to define and quantify the response to UDCA. This list is presented in (Table 1).

	Criterion	Definition of UDCA Non-Response at defined duration of therapy	Study participants	Reference
1	Rochester Criterion	ALP≥ 3X ULN or Mayo score ≥4.5 at 6 months	180	(Angulo et al., 1999b)
2	Barcelona Criterion	ALP drop of ≤40% and ALP >ULN at 12 months	192	(Parés et al., 2006)
3	Paris-I Criterion	One of the parameters: ALP >3 ULN, AST >2 ULN, or bilirubin >1 mg/dL at 12 months	292	(Corpechot et al., 2008)
4	Toronto Criterion	ALP >1.67 ULN at 24 months	69	(Kumagi et al., 2010)
5	Rotterdam Criterion	Bilirubin ≥1 X ULN and/or Albumin < 1X ULN at 12 months	375	(Kuiper et al., 2009)
6	Ehime Criterion	Decline in GGT≤70% and GGT≥1 X ULN	83	(Azemoto et al., 2009)
7	Paris-2 Criterion	One of the parameters: ALP ≥1.5 ULN, AST≥1.5 ULN, or bilirubin >1 mg/dL at 12 months	165	(Corpechot et al., 2011)

Table 1: PBC prognostic models

Summary of prognostic PBC models defining UDCA non-response at a specified duration of therapy. (ALP: Alkaline Phosphatase, GGT: Gamma-glutamyl transferase, AST: Aspartate aminotransferase, ULN: Upper limit of normal).

2. <u>Add-on therapies for managing Cholestasis in PBC:</u>

- **Obeticholic acid (OCA)**:

OCA is a semisynthetic derivative developed from the naturally occurring primary human BA, chenodeoxycholic acid (CDCA). OCA is an FXR ligand and is one hundred times more potent than CDCA (Fiorucci et al., 2005a, Fiorucci et al., 2005b). By inhibiting the synthesis of BAs from cholesterol and increasing their clearance from hepatocytes, by activating FXR, OCA has the ultimate effect of lowering the overall quantity of BAs that are toxic to cholangiocytes

(Ananthanarayanan et al., 2001). Through its FXR-mediated mechanism, OCA has also been demonstrated to have anti-cholestatic, anti-fibrotic, and anti-inflammatory effects. Phase 2 and phase 3 clinical trials of OCA in patients with PBC who did not respond to UDCA demonstrated a significant improvement in liver biochemistry but did not show any significant effect on PBC symptoms in their clinical trials(Gerken and Nitschmann, 2017, Hirschfield et al., 2015a, Jones et al., 2011).

A randomized double-blind placebo control trial of OCA randomized 165 patients with PBC and ALP levels between 1.5-10 X ULN, to receive OCA 10mg, 25mg, 50mg, or placebo for 32 months. There was a statistically significant change in mean ALP levels in all OCA groups (24% in 10mg OCA, 25% in 25mg OCA, and 21% in 50 mg OCA) compared to placebo (3%), ($p<0.0001$ for all). Response as assessed by different criteria was superior in the OCA group- Paris I ($p=0.02$ in 25m OCA), Paris II $p=0.04$ in 10mg OCA, 0.02 in 25mg & 50mg OCA), Toronto I (0.01 in 10mg OCA, 0.005 in 25mg OCA and 0.006 in 50mg OCA) and Toronto II (0.02 in 25mg OCA and 0.01 in 50mg OCA group). There were significant increases from baseline (%changes) in FGF-19 levels in OCA 10mg ($p=0.0007$), 25mg ($p<0.001$) and 50mg ($p=0.02$). For the OCA group, there was a significant decrease in C4 levels ($p=0.02$, $p<0.001$ and $p<0.003$) and BAs ($p=0.01$, $p<0.001$ and $p<0.001$) for 10mg, 25mg, and 50mg respectively. The most common side effect was pruritus (Hirschfield et al., 2015b).

POISE: Phase 3 randomized, double-blind, placebo-controlled, parallel-group trial of OCA randomized 217 PBC patients with UDCA non-response (defined by ALP >1.67 ULN) or intolerance to receive 5-10mg of OCA (N=71), 10mg OCA (N=73) or placebo (N=73). The primary endpoint was an ALP< 1.67 ULN, with at least 15% reduction from baseline, and normal or < 1 X ULN bilirubin. This was achieved in 46% in 5-10mg, and 47% in 10mg OCA compared to 10% in the placebo group ($p<0.001$ for all). A 15% reduction from baseline was achieved in 77% in both the 5-10mg and 10mg groups compared to 29% in placebo ($p<0.001$). Using the PBC-40 questionnaire, itch domain scores were significantly worse in the 10mg OCA group at 3 months ($p<0.001$). 9% of participants withdrew from the study. 4% withdrew due to pruritus with the majority in the 10mg OCA group (Nevens et al., 2016).

COBALT: Phase 3b/4 confirmatory trial patients on 5-10mg OCA were compared to placebo or external control (Komodo database- US healthcare claims). The study could not address primary endpoints and was terminated early due to functional unbinding and cross-over to commercial OCA. The primary endpoint was time to uncontrolled ascites, hospitalization for hepatic decompensation, MELD score ≥15, OLT, or death. In ITT analysis (N=166) occurrence of the primary endpoint was 28% in OCA and 295 in the placebo group (HR=1.0). In EC

analysis (N=1051)) the occurrence of the primary endpoint was 10% and 21% in OCA and non-OCA groups respectively (HR=0.4, p=0.001) (Kowdley et al., 2022).

Italian group reported outcomes from a multicentre real-world cohort of OCA-treated PBC patients (RECAPITULATE). The study compared OCA-treated PBC patients (N=437) with GLOBAL PBC controls (N=831). The study showed better liver-related event-free (HR=0.3) and transplant-free survival (HR=0.3) ($p<0.001$ for both) (Terracciani et al., 2024).Using the Italian RECAPITULATE (N = 441) and the IBER-PBC (N = 244) data, the group created an OCA response score that includes ALP, ALT, GGT, bilirubin levels, age, pruritus and presence of cirrhosis. The scores predict OCA response probability at 12 and 24 months for various response criteria- POISE, ALP <1.67 ULN, or (normal ALT, ALP, and bilirubin) (De Vincentis et al., 2024).

OCA is now approved for use as a second-line treatment for patients who have not responded adequately to UDCA or who are intolerant to it. The most common side effects of OCA include dizziness, peripheral edema, pruritus, and fatigue. Additionally, there have been reports of drug-induced liver injury linked to OCA; therefore, caution should be exercised when administering this medication.

- **Fibrates:**

It has been well known that fibrates, particularly **fenofibrate and bezafibrate** (BZF), can normalize ALP. They are agonist agents for the peroxisome proliferator-activated receptor (PPAR). These agents have been used for a long time to treat hyperlipidaemias. Feher et al., in 1993 for the first time reported their effect on lowering serum ALP (25% reduction in serum ALP) after six weeks of BZF therapy for hyperlipidaemia (Day et al., 1993). Since then, numerous studies have reported that fibrates improve liver biochemistry in patients with PBC who have a suboptimal response to UDCA (Ohmoto et al., 2006, Cuperus et al., 2014, Shen et al., 2021). As a result, fibrates have been proposed for use as an adjunct to UDCA in patients with PBC who do not respond to treatment with UDCA. In a meta-analysis of six trials (131), including 84 patients, there was a significant improvement in ALP in patients treated with Fenofibrate and UDCA combination compared to UDCA alone. In addition, a retrospective study conducted by Hegade et al, in 23 patients treated with fenofibrate and UDCA combination showed improvement in ALP. There was no improvement in predicted survival, as shown by the UK-PBC risk score(Hegade et al., 2016c).

Bezafibrate:

An earlier study from Japan evaluated Bezafibrate monotherapy and combination therapy in PBC. In study 1 (N=45) patients were randomised to receive Bezafibrate 400 mg OD (N=20) or UDCA 600mg OD (N=25)(Iwasaki et al., 2008). There was a significant reduction in ALP levels in both arms, with 59% of patients in the BZF group and 38% in the UDCA group achieving normalization of ALP at 24 weeks. However, the difference was not significant when the two groups were compared (p=0.2). In study 2 (N=22) patients were randomised to receive a combination of BZF+UDCA (N=12) or UDCA alone (N=10). In this group patients were UDCA non-responders (defined by ALP >1.5 ULN after 26 or more weeks of UDCA treatment). There was a significant reduction in ALP and GGT levels in the combination therapy group at 52 weeks (p<0.01), with normalisation of ALP in 60%. Self-limiting myalgia (N=1) and raised CK (N=3) were reported in combination therapy in study 2. Another Japanese retrospective study (N=118) enrolled PBC patients who received at least 12 months of UDCA therapy followed by at least 12 months of combined UDCA+BZF therapy (Honda et al., 2019). There was a significant reduction in the mean GLOBE score from 0.5 to 0.12 after 12 months of combination therapy (p<0.0001). The 3- and 5-year transplant-free survival rate was significantly higher with combination therapy (p<0.0001). Similarly using the UK-PBC score, the risk of a 5-year liver-related transplant or death was significantly reduced with combination therapy (p<0.0001).

More recently double-blind, placebo-controlled phase 3 trial (BEZURSO) recruited 100 PBC patients with UDCA nonresponse defined by Paris 2 criterion at 6 months (ALP/ ALT >1.5 ULN or raised bilirubin) (Corpechot et al., 2018). Patients were randomised to receive BZF 400mg OD (N=50) or placebo (N=50) in addition to UDCA. The primary endpoint was defined as the complete normalisation of biochemistry (ALP, AST, ALT, bilirubin, and albumin). This was achieved in 31% of BZF compared to 0% in the placebo group (p<0.001). Itch intensity score (0-10 VAS) difference of change -95% (95%CI -240% to 50%). The difference of relative increase in fatigue (measured by a 3-stage fatigue scale- absent, intermittent, and continuous) at 24 months between BZF and placebo group was -28% (95%CI -47% to -8%). Quality of life score (measured using the Nottingham Health Profile (NHP) questionnaire) did not show any difference between the two groups. The difference of relative change in 10-year risk of liver-related death or transplant at 24 months between the BZF and placebo group as predicted by GLOBE score was -58% (95%CI -73% to -44%) and UK-PBC score was -51% (95%CI -71% to -322%). 20% of patients in the BZF group and 10% in the placebo group reported myalgia.

3. Experimental therapies:

Several immunosuppressive agents have been tried (examples shown below), without any significant impact on clinical outcome. A significant number of patients do not respond adequately to UDCA, OCA, or fibrates, highlighting an unmet need for effective treatments. As a result, there is an ongoing effort to identify additional therapeutic agents that can provide benefits while maintaining a favorable side-effect profile. Examples of these potential agents include FXR agonists (such as LJN452 and GS-9674), PPAR agonists (such as MBX 8025 and Elafibranor), FGF19 mimetics (NGM-282), and a human monoclonal anti-CD40 IgG4 antibody (FFP104).(Tully et al., 2017, Badman et al., 2020, Trauner et al., 2019, Jones et al., 2017, Schattenberg et al., 2021, Mayo et al., 2018, Floreani, 2020)

- **Budesonide**

A prospective placebo-control, double-blind compared UDCA (10-15 mg/kg/d) (N=20) + Budesonide (3mg TDS) to UDCA + placebo (N=19). Significant improvements in ALP ($p<0.05$) were seen in the UDCA/budesonide group. Using a point score analysis, they found a 30% improvement in liver histology in the UDCA/budesonide group compared to a 3.5% worsening in the UDCA/placebo group, $p<0.001$. There was no difference in changes in bone mineral density in the two groups ($p=0.43$)(Leuschner et al., 1999). Similar results were reported in a randomized controlled trial which compared UDCA 15mg/kg/d _ Budesonide 6 mg/d (N=41) with UDCA alone 15mg/kg/d (N=36) in stage 1-3 PBC for 3 years. Fibrosis score improved in UDCA/budesonide (22%) and worsened in budesonide alone (20%), $p=0.009$ (Rautiainen et al., 2005).

- **Budesonide and Mycophenolate**

An Open-label study of triple therapy with UDCA (13-15mg/kg/d) + budesonide (6mg/d) + Mycophenolate (1500mg/d) in PBC patients with inadequate response to UDCA showed significant improvement at 3 years in median AST (92 vs 39 IU/l), ALT (118 vs 44 IU/l) and ALP (380 vs 168 IU/l), ($p<0.0001$ for all). There was a significant improvement in histological staging (METAVIR, A2-3) (100% vs 30%, $p=0.002$) (Rabahi et al., 2010).

- **Prednisolone and Azathioprine**

A retrospective study compared UDCA 13-15mg/kg/d alone (N=31) to a combination of UDCA + prednisolone + Azathioprine (N=29) [UDCA 13-15mg/kg, prednisolone 30-

50mg/d at induction and 5-10mg/d maintenance, and Azathioprine 50mg/d]. The drop in ALP was significantly higher in the combination therapy group (57% vs 29%, p=0.005). There was an increase in mean albumin levels in the combination therapy group of 52% and a decrease in the UDCA alone group of 6%, (p=0.0002). There was an improvement in the combination therapy group in markers of fibrosis. A significant decrease was seen in the S-index (75% vs 25%, p=0.02) and APRI (52% vs 15%, p=0.01) (Fang et al., 2014).

- **Colchicine and Methotrexate**

Colchicine and methotrexate in addition to UDCA did not show a significant impact on transplant-free survival. In this randomized controlled trial UDCA (15mg/kg/d) + colchicine (0.6mg/d) [N=43] compared to UDCA (15mg/kg/d) + Methotrexate (15mg/ week) [N=42] did not show any difference in 10-year survival (0.57 vs 0.44, p=0.12) (Kaplan et al., 2004).

- **Rituximab**

Rituximab, an anti-CD20 monoclonal antibody given as 2 doses of 1000mg 2 weeks apart in PBC patients (N=14) with inadequate response to IUDCA failed to meet the primary endpoint of 25% reduction in ALP. The median decrease in ALP in this study was 16% (Myers et al., 2013).

- **Tropifexor (LJN452)**

A phase II, double-blind, placebo-controlled study randomized PBC patients (N=61) to receive non-bile acid farnesoid X receptor agonist tropifexor (30 μg [N=11], 60 μg [N=9], 90 μg [N=12}, 150 μg [N=8]) or placebo (N=21) for 4 weeks. Mean ALP levels were decreased in the 60 μg and 90 μg tropifexor groups. A statistically significant reduction in mean ALP was seen in the 60 μg tropifexor group compared to the placebo (p=0.001). However, mean ALP remained above the ULN (104 U/L) in all treatment groups. Pruritus was the most common side effect in the treatment group (53%) compared to placebo (28%) (Schramm et al., 2022).

- **Elafibranor**

In phase 2, a double-blinded, placebo-controlled trial PBC (N=45) with incomplete response to UDCA (ALP >1.67 X ULN) were randomized to receive a dual PPAR-agonist, Elafibranor 80 mg (N=15), Elafibranor 120 mg (N=15) or placebo(N=15) for 12 weeks. The primary endpoint was set as relative reduction (%) in ALP. This was achieved in both the Elafibranor groups compared to the placebo- 80mg group (-48% vs +3.2%), and the 120 mg group (-41% vs 3.2%), ($p<0.001$ for both). Normalisation of ALP was achieved in 13-20% of Elafibranor groups compared to 0% in placebo (Schattenberg et al., 2021).

Management of symptoms of PBC:

1. Management of Pruritus in PBC

1. Cholestyramine:

Cholestyramine is widely used as a first-line agent for treating pruritus in PBC, as well as other causes of cholestatic itch (Lindor et al., 2009, Datta and Sherlock, 1966). It is a non-absorbable quaternary ammonium ion exchange resin that binds to intestinal bile acids, reducing their reabsorption (Trivedi et al., 2017, Hong-Bin et al., 2016). The earliest evidence came from small case-cohort studies. For example, Sheila Sherlock in 1966 in a series of 27 patients who received cholestyramine (6.6-10gm daily) reported complete relief of pruritus in 19 and partial in 4 patients. This was maintained for 6 to 32 months(Datta and Sherlock, 1966). To date, there have only been a couple of small placebo-controlled trials. One single-blind placebo-controlled study of 7 PBC patients showed that pruritus scores were significantly lower during two weeks of treatment with cholestyramine(Duncan et al., 1984). Another double-blind, placebo-controlled, randomized trial (n=10) reported a significant beneficial effect of cholestyramine (9gm daily for 4 weeks) versus a placebo (p=0.01)(Di Padova et al., 1984).

In an earlier study, 10 patients with cholestatic pruritus (PBC=9 and congenital intrahepatic atresia=1) were given flavoured potato powder for one week, followed by cholestyramine. Patients were kept blinded by the change. A complete relief in pruritus was reported in 80% of patients 4-11 days after cholestyramine treatment. Two patients reported nausea and diarrhoea as side effects (Kremer et al., 2011).

The recommended dose of cholestyramine is 4-16 g/day in divided doses. Cholestyramine must be taken 2-4 hours apart from UDCA, as the drug interaction interferes with UDCA absorption(Rust et al., 2000b). Tolerability can be a concern with cholestyramine due to common side effects that, while not severe, can be unpleasant. These side effects include a sour taste, bloating, diarrhea, and constipation.

2. Rifampicin:

Rifampicin is a recommended second-line therapy in cholestatic pruritus (European Association for the Study of the, 2017). It is an antimicrobial agent that inhibits bacterial DNA-dependent RNA synthesis, and its effect in PBC is poorly understood but postulated to be derived from its PXR agonism. Rifampicin compared to placebo has also been shown to significantly decrease serum ATX levels and reduce expression of ATX in HepG2 cells (*in vitro*) in a PXR-dependent manner suggesting an anti-pruritic effect of rifampicin may be via PXR agonism-mediated down-regulation of ATX (Kremer et al., 2012). The earliest evidence came from a report in 6 PBC patients where rifampicin 600mg daily was given for 2 weeks to study its effect in inducing the hepatic metabolism of drugs and bile acids. It was noted that 5 of the 6 patients reported a pronounced decrease in their pruritus(Hoensch et al., 1985). Rifampicin has well-established effectiveness in the treatment of pruritus in PBC in randomised controlled trials and meta-analysis (Bachs et al., 1989, Podesta et al., 1991, Ghent and Carruthers, 1988, Khurana and Singh, 2006).

The recommended maximum dose of rifampicin is 600 mg, usually started at a lower dose of 150 mg and gradually titrated up, while carefully monitoring liver biochemistry (Prince et al., 2002b, Sampaziotis and Griffiths, 2012). Adverse effects of rifampicin include hepatotoxicity, nephrotoxicity, and drug interactions. An earlier study (n=16, follow-up 2-24 months) reported hepatitis in 12.5% (2/16) leading to the discontinuation of rifampicin (Bachs et al., 1992). In contrast another retrospective study (n=105) in PBC or PSC patients treated with rifampicin, reported a low risk of hepatotoxicity (4.8% cases)(Webb et al., 2018).

3. Naltrexone:

Naltrexone and naloxone (Selective mu (μ) opioid receptor antagonist) are recommended third-line agents for cholestatic pruritus(European Association for the Study of the, 2017). It is thought to relieve pruritus in PBC patients via its opioid-antagonistic actions (Ballantyne et al., 1989, Bergasa et al., 1992, Terg et al., 2002). The earliest report of the beneficial effect of naloxone in cholestatic pruritus came from a randomized double-blind placebo-controlled (n=20) of IV naloxone or saline. However, the mean change in itch score induced by naloxone (-0.95) was not different from placebo (-1.33) ($p>0.5$). They demonstrated that naloxone caused a significant reduction in itch score in the placebo non-responder group ($0.05> p > 0.02$)(Summerfield, 1980).

A crossover, double-blind, placebo-controlled study randomized (N=20) PBC pruritus patients, to receive naltrexone 50 mg/d (N=10) or placebo (N-=10) for two weeks. Therapies were crossed over after a one-week washout period. Significant improvement in pruritus as measured by VAS was seen in the naltrexone group ($p<0.0003$). Greater than 50% reduction in 45% of patients and complete resolution of pruritus in 25% was seen with naltrexone (Terg et al., 2002).Another double-blind, placebo-controlled study, treated PBC pruritus patients with naltrexone 50mg/d (N=8) or placebo (N=8). After 4 weeks of treatment, a significant improvement in daytime pruritus (54% vs. 8%, $p<0.001$) and night pruritus (-44% vs. 7%, $p=0.003$) was seen in the naltrexone group (Wolfhagen et al., 1997).

A systematic review including three randomized controlled trials, one controlled clinical trial, one open-label pilot study, seven case reports, and one retrospective notes review, found naltrexone to be effective in relieving pruritus in cholestasis(Murray-Brown, 2021). The recommended maximum daily dose of naltrexone is 50mg/ day; however, it is usually started at a lower dose of 12.5 mg/day and gradually titrated up. The commonest side effects include opioid withdrawal-like reactions during the initial few days of treatment(Jones et al., 2002, Thornton and Losowsky, 1988). Opioid antagonists are contraindicated in patients with acute hepatitis, liver failure, and those receiving opioid medications.

4. <u>Sertraline:</u>

Sertraline, a commonly used antidepressant is a selective serotonin reuptake inhibitor (SSRI). Sertraline can influence the endogenous serotoninergic system which is thought to modulate nociception and perception of pruritus and, therefore, modify the central itch and/or pain signalling. Evidence though small in the form of randomised controlled trials, has shown sertraline to have some beneficial effects in resistant pruritus independent of its antidepressant effect(Browning et al., 2003, Mayo et al., 2007). For example, the randomized, crossover, double-blind, placebo-controlled trial (n=12) of sertraline vs placebo. This followed an open-label, dose escalation to determine the dose with optimal efficacy and tolerability. Participants quantified their pruritus using a 0-10 VAS. Itch scores improved in patients taking sertraline (improved 1.86 raw points) compared to placebo (worsened 0.38 points) (net beneficial effect of 2.24 points, $p > 0.009$)(Mayo et al., 2007). The recommended initial dose is 25mg/day, increased gradually by 25mg every 4-5 days to 75-100mg/day. Side effects include dry mouth, dizziness, nausea, diarrhoea, hallucinations, and insomnia.

5. IBAT inhibitors:

Selective inhibitor of the human ileal bile acid transporter (IBAT), increases excretion of bile acids and reduces bile acid concentrations in the liver and systemic circulation, resulting in reduced pruritus and associated symptoms.

- **Lopixibat/ Maralixibat**

Maralixibat chloride (also known as SHP625, LUM001, or Lopixibat) is a competitive, apical sodium-dependent bile acid transporter competitive inhibitor. Phase 2 study of Maralixibat in PBC pruritus patients randomized 66 patients to receive either Maralixibat (N=42) or placebo (N=24). Following the dose escalation period, patients received Maralixibat 5mg, 10mg, or 20mg in the stable dose treatment phase (a total of 13 weeks of treatment). Change in the Adult ItchRO weekly sum score was the primary endpoint. ItchRO scores significantly reduced from baseline to end of the trial period in the Maralixibat (mean change: –26.5, $p< 0.0001$) and the placebo group (–23.4, $p < 0.0001$) but there was no difference comparing the two groups (mean difference: –3.1, p= 0.48). Compared to placebo, Maralixibat 10 mg (-2.3, p=0.66) and Maralixibat 20mg (-4.0, p=0.44) were no different. However, on a self-reported scale (PIC-Patient Impression of Change, score ≤3 defined treatment response), there was a significant response in the Maralixibat group (90%) compared to 68% in placebo, p=0.03 (Mayo et al., 2019). In another cholestatic condition Progressive familial intrahepatic cholestasis (PFIC), Maralixibat showed improvement in ItchRO in a phase 3 multicentre, randomised, double-blind, placebo-controlled trial. 93 participants were randomised to receive oral Maralixibat (N=47) (initial dose 142·5 μg/kg, escalated to 570 μg/kg twice daily) or placebo (N=46) for 26 weeks. In the bile salt exporter pump (BSEP) deficiency cohort (N=14), there was a significant improvement in the mean ItchRO score compared to controls (–1·1, p=0·006)(Miethke et al., 2024).

- **Linerixibat**

A phase 2b by GLIMMER reported dose-response, efficacy, and safety of an IBAT inhibitor Linerixibat in a multicenter, randomized, parallel-group study (Levy et al., 2023). The study recruited PBC patients with moderate-sever itch (defined as >/=4 on a 0–10 numerical rating scale [NRS]) to receive Linerixibat 20 mg QDS (N=16), 90 mg QDS (N=23), 180 mg QDS (N=27), 40 mg BD (N=23), 90 mg BD (N=22) or placebo (N=36). The primary endpoint was defined as a reduction in mean worst daily itch (MWDI) from baseline. MWDI showed reduction in all groups t week 16 (p=0.03), however when compared to placebo, there was no

significant difference (p=0.12). In their post-hoc analysis, they evaluated the effect of total daily dose using a liner model. A total of 11335 doses of Linerixibat, 8612 of placebo, and 20246 MWDI were included in their final analysis. A significant reduction in MWDI (10%, p=0.5) from baseline was observed. Compared to placebo, monthly itch score also showed a significant change from baseline when compared to Linerixibat 180 mg QDS (LS mean change –0.9, p= 0.04), 40 mg BD (–1.16, p=0.01), and 90 mg BD (–0.95, p=0.04). Diarrhoea was the most common adverse effect (38-68% for Linerixibat compared to 11% in placebo)(Carreño et al., 2024). A 2-part, Phase 3 study of Linerixibat (GLISTEN) evaluating the efficacy, safety, and impact on health-related quality of life (including the PBC-40 questionnaire) is currently ongoing (NCT04950127).

- **Volixibat**

Another IBAT inhibitor Volixibat is being evaluated in a Phase 2 randomized, double-blind, placebo-controlled trial in PBC patients with pruritus (VANTAGE) is recruiting patients and is anticipated to finish in late 2025 (NCT05050136). The primary end point is the mean change in ItchRO scores. Secondary end points include changes in ALP, bilirubin, bile acids, PBC-40 scores, Patient Reported Outcomes Measurement Information System (PROMIS) fatigue, and sleep disturbance questionnaire.

6. <u>Gabapentin:</u>

Gabapentin, due to its proposed effect of increasing the nociception threshold, had been recommended as a potential treatment for cholestatic pruritus(Anand, 2013). A randomized double-blinded placebo-controlled trial of 16 patients with cholestatic pruritus treated with 4 weeks of gabapentin, failed to show any significant therapeutic advantage over a placebo. On the contrary, some patients experienced worsening pruritus as measured by hourly scratching activity (HAS) [mean score 88.5 post-gabapentin vs 53.0 pre-gabapentin] (Bergasa et al., 2006).

7. <u>Phototherapy:</u>

Phototherapy using Ultraviolet B radiations has been suggested as therapy for cholestatic pruritus. Although the mechanism is unknown, results from an observational case series of 13 patients showed significant beneficial effects. There was a statistically significant improvement in pruritus with a decrease in the median visual analogue scale from 8.0 to 2.0 (P <.001) with the average duration of phototherapy being 8 weeks(Decock et al., 2012).

8. Plasmapheresis:

Plasmapheresis has been suggested as a treatment for resistant complications of PBC going as far back as the 1970s (Turnberg et al., 1972, Alallam et al., 2008). However, its use in pruritus related to cholestasis is still in the experimental phase. A recent study of seventeen PBC patients with refractory pruritus who received 129 sessions of plasmapheresis over 40 hospital admissions has been published (Krawczyk et al., 2017). Refractory pruritus was defined as no response to therapy with both cholestyramine and titrated maximum dose of rifampicin (300mg). The itch was quantified using a 10-point numeric rating scale before and after plasmapheresis and at 30 and 90-day time points. The mean pruritus score declined from 8.3+/-1.4 to 3.1+/-2.2 ($p<0.0001$) in all the patients and the anti-pruritic effect was maintained for 90 days ($p<0.0001$). A significant decrease in serum ALT, ALP, AST, GGT ($p<0.001$), and bilirubin ($p<0.002$) was also noted. Plasmapheresis offers a plausible strategy for treating refractory itch in PBC and other cholestatic conditions, however, we need further randomized controlled studies to establish its exact place in the treatment ladder for treating cholestatic itch.

9. Nasobiliary drainage:

Nasobiliary drainage of bile acids helps reduce serum autotoxins and, thereby, exerts an anti-pruritic effect. However, due to the nature of the therapy effects, are not sustainable over longer periods (Beuers et al., 2006, Hofmann and Huet, 2006). A multicentre retrospective study, from Europe of 27 patients undergoing 29 nasobiliary drainage procedures reported improvement in pruritus in 89.6% of patients as measured on a visual analogue scale (score decreasing from 10.0 to 0.3 (P <.0001). A significant improvement in serum bilirubin and alkaline phosphatase was also reported ($p=0.03$ and 0.001 respectively) (Hegade et al., 2016d). As the procedure involves doing an ERCP, the potential of high-risk adverse events of pancreatitis always remains a concern.

10. Albumin dialysis (MARS- molecular adsorbent recirculating system):

Albumin dialysis using an adsorbent recirculating circuit is thought to exert its anti-pruritic effects by removing pruritogens from the circulation(Leckie et al., 2012). In a study of twenty patients with cholestatic pruritus, who underwent albumin dialysis, a significant improvement in pruritus as defined by the visual analogue scale (scores decreased from 70.2 +/- 4.8 to 20.1 +/-4.2 (P <.001)) has been demonstrated. No adverse effects were reported (Parés et al., 2010).

2. Management of Fatigue in PBC

Despite the investigation of several different agents in clinical trials, there are currently no recommended or effective therapies for treating fatigue in patients with PBC. **Rituximab**, a monoclonal anti-CD20 antibody, selectively depletes B cells. In a single-center, double-blind, placebo-controlled trial involving 57 patients, an improvement in the fatigue score (as measured by the PBC-40) was observed in the treatment group. However, this improvement did not reach statistical significance.(Jopson et al., 2015).

Modafinil is a central nervous system stimulant that is effective in treating excessive daytime sleepiness caused by a variety of conditions other than liver disease and has been used as an off-label therapy for PBC-related fatigue(Jones and Newton, 2007b). A randomized, double-blind, placebo-controlled in PBC fatigue who received modafinil (N=20) [100-200mg OD] and placebo (N=20) did not show any significant improvement in fatigue severity as defined by >50% improvement in Fisk Fatigue Impact Scale (FFIS) (p=1.0) (Silveira et al., 2017). An intention-to-treat analysis (N=21) used ESS and PBC-40 questionnaires pre-and, post-treatment with modafinil for 2 months. Patients with ESS >10 (0-24) without OSA were considered suitable for therapy. On ITT analysis, there was a significant improvement in the mean ESS from 15.0 +/- 3.3 pre-treatment to 8.0 +/- 6.0 post-treatment ($p<0.0005$); and mean PBC-40 fatigue domain score (from 46 to 34, $p<0.0001$) (Jones and Newton, 2007a).

Setanaxib is a selective NADPH oxidase isoform 1 and 4 (NOX1/4) inhibitor. In hepatic stellate cells, NOX generates reactive oxygen species promoting liver fibrosis. NOX1/4 inhibitors in animal models have been shown to attenuate liver fibrosis, mRNA expression of the NOX gene, and ROS production (Aoyama et al., 2012). A multicentre, randomised, double-blind, placebo-control trial Phase 2 trial of evaluated efficacy of Setanaxib in PBC patients with persistently elevated ALP (after 6 months of UDCA and at least 3 months of stable dose, before recruitment)(Invernizzi et al., 2023). The study randomised 111 patients to receive Setanaxib 400 mg OD (N = 38), Setanaxib 400 mg BD (N= 36), or placebo (N= 37). At week 24 mean percentage fatigue score change in 400mg BD Setanaxib group was -9.9% (N=30). In comparison minimal change (+0.3%, N=37) in Setanaxib 400mg OD and an increase (+2.4%, N=36) in the placebo group was seen. The mean change difference in fatigue score compared between Setanaxib 400mg BD and placebo was statistically significant (p=0.02). A post hoc analysis reported quality of life outcomes using the PBC-40 questionnaire(Jones et al., 2023). Except for pruritus' (0.1), patients in the Setanaxib 400mg BD group reported mean reductions in all other PBC-40 score domains (fatigue -3.6, emotional -2.0, social -2.2, cognitive -1.4 and symptoms -1.4) at week 24 of treatment. A meaningful response described by 0.5-point

reduction was seen in the emotional domain (44% in BD, 35% OD group compared to 2.8% in placebo), and cognitive domain (27% in BD group compared to 11% in placebo). Absolute change in fatigue score (mean) was greatest in those with moderate-severe fatigue in the BD group (-5.8) compared to the OD group (-3.1) and placebo (1.0).

Other agents, such as antidepressants(Talwalkar et al., 2006b), ondansetron(Theal et al., 2005), and methotrexate (Lindor et al., 1995), have been investigated in clinical trials for the improvement of fatigue with limited success. A double-blinded, placebo-controlled study of **fluoxetine** for PBC fatigue randomized 18 patients to receive Fluoxetine 20 mg OD (N=10) or placebo (N=8) for 8 weeks. The primary study endpoint was a ≥50% reduction Fisk Fatigue Impact Scale (FFIS) score. No statistically significant difference was seen in the FFIS (p=0.4) score and HRQL (secondary outcome) between the two groups. 4 patients discontinued the treatment due to side effects of which 3 reported worsening fatigue (Talwalkar et al., 2006a). In another randomized controlled trial, 17 PBC fatigue patients received **fluvoxamine** 75 mg BD and 16 placebo for 12 weeks. There was no difference in fatigue as measured by VAS fatigue (p=0.5), Fisk Fatigue Severity Scale (physical domain p=0.12, cognitive domain p=0.6 and social domain p=0.9), and Multidimensional Fatigue Inventory (general fatigue p=0.8, physical fatigue p=0.4, reduced activity p=0.13, reduced motivation p=0.7 and mental fatigue p=0.9) (Ter Borg et al., 2004).

Studies have reported conflicting results regarding the improvement of fatigue or the continuation of fatigue in PBC patients after **LT** (147) (Carbone et al., 2013a). A recent meta-analysis of 16 qualifying studies of the treatment of fatigue in PBC found UDCA (7 studies) was not beneficial in treating fatigue in PBC (RR=0.86, p=0.2). They reported some benefits of OLT in PBC fatigue (2 studies, SMD -0.56, p<0.001). A prospective, longitudinal study from the U.K. evaluated the impact of OLT on fatigue in PBC (N=49) using, the PBC-40 questionnaire at listing and 6, 12, and 24 months after OLT and matched non-transplanted PBC patients and community controls. Though there was a significant improvement in fatigue scores in the PBC transplant group from pre-transplant (40.7) to 24 months (26.2), p<0.001, fatigue scores were still significantly higher as compared to control population even after OLT (26 vs 17, p-<0.0001) (Carbone et al., 2013a).

Patients with PBC often suffer from fatigue, which can be difficult to manage. Standard clinical practice suggests investigating and addressing other common causes of fatigue, such as mood disorders, vitamin D deficiency, and sleep disturbances like obstructive sleep apnoea and restless legs syndrome. A comprehensive approach to managing fatigue in PBC patients

includes non-pharmacological interventions, such as pacing strategies, graded exercise therapy, participation in awareness and support group activities. Furthermore, it is essential to have a multidisciplinary team that includes physiotherapists, occupational therapists, and psychologists to provide effective support for these patients. (Jopson and Jones, 2015).

3. Management of Cognitive Impairment in PBC

There are no approved treatments available for CI in PBC, largely due to the lack of complete understanding of the pathophysiology of this complication of PBC.

Postdoc analysis of the double-blind randomized placebo-controlled trial of NADP oxidase inhibitor **Setanaxib** reported changes in quality-of-life measures using the PBC40 questionnaire (Invernizzi et al., 2023). The study recruited 111 patients with PBC and inadequate response to UDCA (defined as ALP and GGT levels ≥ 1.5 times ULN). Patients were randomized to receive Setanaxib 400 mg BD (N=36), Setanaxib 400 mg OD (N=38), and placebo (N=30) for 24 weeks. Based on PBC-40 scores patients were categorized into mild fatigue (N=55) or moderate-severe fatigue (N=56). A meaningful change was described as a 0.5 per item change in each domain. Compared to placebo overall meaningful change was seen in all domains in Setanaxib groups, except the social and cognitive domains. In the cognitive domain, meaningful change was seen in 27% (N=8) in the Setanaxib BD group compared to OD 10.8% (N=4) and placebo 11.1% (N=4). In the fatigue domain, meaningful change was observed in the moderate-severe group with Setanaxib BD (-5.8) and OD (-3.1) compared to placebo (1.0). In the mild fatigue group, there was no meaningful change in Setanaxib BD (-0.6), OD (1.1), and placebo (0.2). The study was not powered for changes in QoL parameters and the number of patients in each sub-group was small.

Management of cognitive impairment focuses on patient and carer engagement, utilizing non-pharmacological interventions such as cognitive stimulation programs and memory enhancement strategies like lists and memory aids.

1.1.8 Transplant-free survival and recurrent PBC after transplant:

Global PBC study group in a study of 785 patients who underwent liver transplantation for PBC, with a median follow-up of 6.9 years (IQR: 6.1–7.9 years) reported PBC recurrence rates of 22% and 36% at 5 and 10 years respectively (Montano-Loza et al., 2019). Recurrence was defined based on abnormal liver chemistry +/- liver biopsy. 67% of all patients had liver biopsy (of these 52% were protocol and 48% abnormal liver chemistry triggered biopsies). The median (IQR) time for recurrence of PBC was s 4.4 (3.4–5.1) years. Risk factors for PBC recurrence were identified as young age at the time of PBC diagnosis (<50 years) at diagnosis (HR=1.79, p < .001), younger age (<60 years) at the time of liver transplantation (HR=1.39, p=0.04), cholestatic liver (bilirubin >100 mmol or alkaline phosphatase >3 X ULN) at 6 months post-OLT (HR=1.79, p=0.008) and use of tacrolimus (HR=2.31, p=<0.001). Ciclosporin use was found to be associated with reduced PBC recurrence (HR=0.62, p=0.001). Based on multivariable Cox-regression analysis PBC recurrence was associated with increased graft loss (HR=2.01, p= .01) and death (HR=1.72, p= .02).

Another study from the Global PBC group reported similar findings. They included UDCA-treated or untreated patients with bilirubin levels ≤1 X ULN at baseline or 12 months (Perez et al., 2020). Subgroup analysis of those with ALP ≤1.67 X ULN at 12 months was done to look for transplant-free survival. 10-year LT survival rates of those with bilirubin ≤0.6 X ULN was 91.3% compared to 79.2% in those with bilirubin >0.6 (p<0.001), [HR=2.12, 95%CI 1.69-2.66, p<0.001]. 10-year transplant-free survival rates in ALP ≤ 1 X ULN and ALP 1.0-1.67 X ULN were 93.2% and 86.1% respectively. Global PBC group in another study evaluated the combination of GLOBE score and ALP in UDCA-treated PBC patients (N=3774) (de Veer et al., 2022). After 12 months of UDCA treatment, 73% had normal and 27% raised GLOBE scores. In patients with normal GLOBE score and age < 50 years, 10-year cumulative transplant-free survival was 97%, 96%, and 94% in those with ALP ≤ 1 X ULN, 1-2 X ULN, and > 2 X ULN respectively. The respective rates for those > 50 years were 91%, 88%, and 82% for those with ALP ≤ 1 X ULN, 1-2 X ULN, and > 2 X ULN. In those with raised GLOBE score ALP at 12 months of UDCA therapy was associated with LT/death (aHR=1.38, p = 0.016). The study again highlights the fact that normal ALP is associated with better transplant-free survival.

1.2 Primary Sclerosing Cholangitis:

Primary Sclerosing Cholangitis (PSC) is a chronic autoimmune cholangiopathy resulting in inflammatory fibrotic damage of the bile duct causing multifocal biliary strictures. In the majority of patients, the strictures will be evident on cholangiogram MRCP or ERCP. However, a small proportion of patients have what is called small duct PSC where cholangiogram is normal and PSC changes are seen on liver histology(Karlsen et al., 2017, Kaplan et al., 2007, Lunder et al., 2016). Two-thirds of PSC patients tend to be males. PSC tends to occur at a younger age (median age of 36-39 years at diagnosis); however, it can occur at any age (Parés Darnaculleta and Group, 2017, Weismüller et al., 2017, Kuo et al., 2019). There is an increased risk of Cholangiocarcinoma (CCA) and colorectal cancer (CRC) in PSC patients (Barner-Rasmussen et al., 2020, Boonstra et al., 2013).

1.2.1 Epidemiology:

There have been few population-based epidemiological studies of PSC and the majority of the data is from North America and Western Europe. The estimated incidence of PSC in the Western world is 1- 1.5 per 100,000 person-years (Karlsen et al., 2017, Boonstra et al., 2012a, Liang et al., 2017a, Card et al., 2008, Lindkvist et al., 2010, Bambha et al., 2003). A population-based epidemiolocal study from the United Kingdom, over 19 years reported annual incidence was 0.91 per 100,000 and the point prevalence 12.7 per 100,000. 53 cases were recorded between 1984 and 2003 (midpoint population 251,000)(Kingham et al., 2004). A U.K.-based study, using the General Practice Research Database, over 5 years (223 cases), reported an overall incidence of 0.41 per 100,000 and a point prevalence of 3.9 per 100,000(Card et al., 2008). In another study on data from UK Clinical Practice Research Datalink, over 16 years (250 cases) reported an overall incidence of 0.68 per 100,000 and point prevalences of 5.5 per 100,000(Liang et al., 2017b). A Dutch study (595 cases), reported an overall incidence of 0.5 per 100,000 and a point prevalence of 6.1 per 100,000 (Boonstra et al., 2013). A Norwegian population-based epidemiolocal study over 10 years (17 cases/ 130,000 population), reported an incidence of 1.3 per 100,000 and point prevalences of 8.5 per 100,000(Boberg et al., 1998). A study from Calgary over 5 years incidence rate of 0.92 cases per 100,000. Small duct PSC incidence was 0.15/100,000, risk ulcerative colitis (RR= 212, 95% CI 116–356) and Crohn's disease (RR= 220, 95% CI 132–343)(Kaplan et al., 2007). A Swedish study (199 cases/1.5 million population) over 14 years, reported an overall incidence of 1.2 per 100,000 and point prevalences of 16.2 per 100,000. They also reported an increase in the incidence of PSC over the study period (average annual percent change [AAPC 3.06, 95%CI

0.01-6.2])(Lindkvist et al., 2010). A Finnish study (632 cases) over 15 years from two districts comprising 29% of Finland's population reported an overall incidence of 1.6 per 100,000 and a point prevalence of 32 per 100,000(Barner-Rasmussen et al., 2020). In other parts of the world, there appears to be a lower prevalence of PSC, however, data remains limited (Isayama et al., 2018, Narciso-Schiavon and Schiavon, 2017).

1.2.2 Aetiology:

The precise etiology of PSC is unknown though a complex interplay of genetic and environmental factors; and gut dysbiosis is suspected. Genetic studies have identified a clear genetic propensity including HLA variations(Jiang and Karlsen, 2017, Ji et al., 2017, Eaton et al., 2015, Srivastava et al., 2012, Kummen et al., 2021). For example, a GWAS study from the Norwegian PSC group in 715 patients and 2962 controls analyse over 2 million SNPs. (Melum et al., 2011). They reported a strong association of PSC-SNPs in the HLA complex at chromosome 6p21, rs3134792 with associated G allele HLA-B*08 and HLA-DRB1*03 ($p = 6.8 \times 10^{-49}$). They also demonstrated non-HLA associations at rs6720394 near *BCL2L11* ($p = 4.1 \times 10^{-8}$) and rs3197999 in *MST1* ($p = 1.1 \times 10^{-16}$). In another study of PSC patients (N=285) compared to healthy controls (N=368) reported association with *HLA-B* at chromosome *6p21*, SNP rs3099844 (OR=4.8, 95%CI 3.6–6.5, $p = 2.6 \times 10^{-26}$) and rs2844559 (OR=4.7, 95%CI 3.5–6.4, $p= 4.2 \times 10^{-26}$). Another UK study of PSC (N=992) and HC (N=5162), reported associations at *MST1* and *IL-2/IL-21* loci. The associated SNPs were rs4147359 (10p15 (*IL2RA*), [$p = 2.6 \times 10^{-4}$], rs3197999 (3p21 (*MST*1), [$p = 1.9 \times 10^{-6}$] and rs12511287 (4q27 (*IL-2/IL-21*), $p = 3.0 \times 10^{-4}$(Srivastava et al., 2012). Further metanalysis performed by the group showed significant associations of rs4147359 (10p15 (*IL2RA*), $p = 1.5 \times 10^{-8}$) and rs3197999 (3p21 (*MST1*), $p = 3.8 \times 10^{-12}$). Another multicentre study included PSC patients (N= 392), UC (987) and HC (N=2977) reported a novel loci associated with PSC and UC at 2q37 [rs3749171 at G-protein-coupled receptor 35 (GPR35), p= 3.0 X 10-^{29} and PSC alone at 18q21 [rs1452787 at transcription factor 4 (TCF4), p=2.61 X 10^{-28} (Ellinghaus et al., 2013).GPR35, a G-protein coupled receptor is known to modulate biliary and intestinal inflammation via the kynurenine pathway. TCF4 is involved in cell differentiation and growth and deficiency of TCF4 encoded transcription factor can cause partial blockage of T and B cell development.

Some studies have indicated a disrupted gut barrier and dysbiosis of the intestinal gut microbiota in PSC (Kummen et al., 2021, Pereira et al., 2017, Lapidot et al., 2021, Sabino et al., 2016). For example, using 16S rRNA gene sequencing in 543stool samples from PSC (N=85), UC (N=36),

and HC (N=263), distinguishing global microbial composition was seen in PSC compared to both UC ($p<0.01$) and HC ($p<0.001$)(Kummen et al., 2017). PSC was associated with a marked reduction in bacterial diversity. Veillonella genus, however, was markedly increased in PSC compared to UC ($p<0.02$) and HC ($p<0.001$). There was no difference in the microbiota of the patients with or without IBD. Another study of PSC with IBD (N=32), PSC without IBD (N=11), UC (N=32) and HC (N=31), reported low bacterial diversity in PSC patients. Veillonella, Enterococcus, Rothia, and Streptococcus were markedly increased in PSC and were independent of the presence or absence of concomitant IBD(Bajer et al., 2017). Adlercreutzia equolifaciens was significantly reduced in PSC without IBD ($p < 0.001$). A French group [PSC-IBD (N=27), PSC (N=22), IBD alone (N=33) and HC (N=30)], reported fungal dysbiosis in PSC patients, independent of IBD status(Lemoinne et al., 2020). A decreased proportion of Saccharomyces cerevisiae and an increased proportion of Exophiala were observed in PSC.

Lipopolysaccharides (LPS) are glycolipids derived from the outer membrane of gram-negative bacteria. They serve as markers of bacterial translocation. LPS-binding protein facilitates the association of LPS to soluble CD14 (sCD14). sCD14 signals the presence of gram-negative bacteria and activates monocytes. sCD14 concentration is used as a measure of host response to translocation. Norwegian group in PSC (n=166) compared to HC (N=100) measured sCD14, LPS, and LPS binding protein(Dhillon et al., 2019). They found raised levels of sCD14 (ng/mL) and LPS binding protein (ng/mL) in PSC (median 13662 vs 12339, $p = 0.01$ and 1657 vs 1196, $p < 0.001$, respectively). Higher levels of LPS binding protein (median 14030 vs 13097, $p=0.04$) and sCD14 (2045 vs 1490, $p<0.1$) were seen in those who had liver-related events (OLT or death) compared to transplant-free survivors. Findings may support the interruption of gut barrier and bacterial translocation in the pathogenesis of PSC. An enterocyte cytoplasmic protein intestinal fatty acid-binding protein (I-FABP) has been reported as a marker of enterocyte damage. Anti-gliadin IgA antibodies (AGA-IgA) and anti-F-actin IgA antibodies (AAA-IgA) serve as markers of mucosal damage and increased intestinal permeability(Piton and Capellier, 2016). Increased levels are seen in coeliac disease and cirrhosis-related portal hypertension (Reiberger et al., 2013). Hungarian Autoimmune Liver Disease Study Group in PSC (N=67), UC (N=172) and HC (N=53), evaluated F-actin (AAA IgA/IgG), gliadin (AGA IgA/IgG)], intestinal fatty acid-binding protein (I-FABP), LPS binding protein (LBP)], anti-OMP plus IgA and endotoxin core IgA antibody (EndoCAb). AAA IgA ($p<0.001$), AAA IgG ($p< 0.001$), and AGA IgG ($p = 0.01$) were significantly higher compared to UC and HC. AAA IgA positivity was associated with a higher frequency of anti-microbial antibodies anti-OMP plus IgA ($p=0.01$), EndoCAb IgA, $p < 0.001$), and I-FABP ($p= 0.01$). Poor disease outcome

was independently related to AAA IgA-positivity adjusted for cirrhosis [HR = 5.2 (95%CI 1.27-20.9), p = 0.022] and Mayo risk score (HR = 4.2 (95%CI 0.99-18.2), p= 0.05]. Findings further highlight the role of gut-barrier disruption in PSC.

Translocation of microbial metabolites and abnormal trafficking of gut lymphocytes have been hypothesized to trigger the activation of peribiliary inflammation. Mayo group studied hepatic macrophages (number and location), fibrosis, and serum biochemistry mice models of Acute Sclerosing cholangitis (induced by intrabiliary injection of the inhibitor of apoptosis antagonist BV6) and Chronic sclerosing cholangitis (*Mdr2*$^{-/-}$ knockout)(Guicciardi et al., 2018). Human PSC liver cells were examined using immunofluorescence. Increased macrophages in per-portal and intra-fibrotic tissue were observed in PSC compared to PBC and HCV. A significant increase in the CD68^{+}/CCR2^{+} cells, pro-inflammatory (iNOS^{+}) (FC=6), and anti-inflammatory (CD206^{+}) macrophages (FC=3.4) was observed in the peribiliary area of PSC livers compared to normal livers. Findings were replicated in the mice model. Peribiliary macrophage recruitment paralleling the liver injury was observed. This was reversed on resolution in the acute sclerosing cholangitis mice model, with CCR2 inhibition using Cenicivroc.

The study from Germany included PSC (N=135), PBC (N=99), AIH (29), NAFLD (8), IBD (8), ARLD (8), and HC (N=97). They analysed monocytes and T Cells from serum and liver(Kunzmann et al., 2020). PSC compared to PBC and HC, showed a significant increase in IL17-A-producing CD4+ T cells, upon ex vivo stimulation with phorbol myristate acetate/ ionomycin. This was independent of the presence or absence of concomitant IBD. PSC patient monocytes compared to HC upon microbe (Candida Albicans) stimulation produced more cytokines involved in Th17 cell differentiation: IL-6 (18900 vs 14700 pg/mL, p= 0.04) and IL-1β (46600 vs 36000 pg/mL, p = 0.037). Findings add further evidence that gut barrier disruption and microbial translocation-inducing macrophage response may be implicated in PSC pathogenesis.

Interleukin (IL)-17 production by T cells has been linked to inflammation and development of cholestatic fibrosis in PSC (Nakamoto et al., 2019, Borchers et al., 2009, Tedesco et al., 2018). A study from a German center culture bile obtained during ERCP in PSC patients (N=51)(Katt et al., 2013). Serum samples for pathogen peripheral blood mononuclear cell (PBMCs) stimulation were obtained from 46 patients. From the bile cultures staphylococcus, streptococcus, and candida albicans were the main isolates. Pathogenic bacterial stimulation of PBMCs induced significantly more IL17A-producing CD4+ cells in PSC patients compared to HC (2.2% vs 0.77%, p< 0.001). The change was not observed in PBC compared to HC and

there was no difference in PSC with or without IBD. This may indicate that IL17A-related T Cell activation is an independent phenomenon in PSC. No difference between PSC and HC was observed after stimulation with non-pathogenic (heat-killed E. Coli). Higher levels of IL17A-producing CD4+ cells after stimulation with Candida albicans (7.0% PSC vs 1.5% HC, $p<0.01$).

1.2.3 Symptoms and Diagnosis:

PSC is a complex condition with a variable progression that can lead to cirrhosis and its associated complications, as well as cholangiocarcinoma (CCA) and colorectal cancer (CRC). Most patients experience progressive fibrosis, which results in biliary strictures, recurrent bacterial cholangitis, cirrhosis, and ultimately, end-stage liver disease. PSC is increasingly being diagnosed at earlier stages, likely due to greater awareness of the disease, the use of non-invasive radiological tests, and routine screenings of liver function tests, especially in the general population and among patients with inflammatory bowel disease (IBD) (Takakura et al., 2017, Kuo et al., 2019). Nearly half of adult PSC patients present with persistent or intermittent symptoms at diagnosis. About one-fifth acquire symptoms within 5 years of diagnosis (Bambha et al., 2003). Common symptoms include right upper quadrant abdominal pain, fever, jaundice (often linked to acute cholangitis, which can recur), fatigue, and itching. In the later stages of the disease, patients may present with severe complications such as hematemesis and melena (indicating variceal upper gastrointestinal bleeding), confusion (hepatic encephalopathy), and abdominal swelling (ascites), all of which are related to complications arising from cirrhotic portal hypertension (Broome et al., 1996). Many patients also experience anxiety and depression, which can stem from the limited treatment options available and the heightened risk of cancer associated with the condition (Cheung et al., 2016, Ranieri et al., 2020, Isa et al., 2018).

Biochemical indicators are sensitive for diagnosing PSC but lack specificity. Approximately 75% of patients have elevated ALP and GGT. Increased Alanine transaminase (ALT) and Aspartate transaminase (AST) are also common and do not always indicate overlapping AIH (Boberg et al., 2011, Dyson et al., 2018). In individuals with PSC, the detection of serum autoantibodies such as ANA, anti-smooth muscle antibody (SMA), and perinuclear anti-neutrophil antibodies is very variable and has low diagnostic relevance for PSC (Sebode et al., 2018, Zeman and Hirschfield, 2010, Stinton et al., 2014, Lo et al., 1994, Lo et al., 1992) . Up to 15% of PSC patients may have elevated serum IgG4 levels, although very high levels of IgG4 are uncommon. In such cases, one should consider the possibility of IgG4-related sclerosing cholangitis, which is a distinct clinical entity with different outcomes (Lian et al., 2017, Boonstra et al., 2014a, Fischer et al., 2014).

PSC should be evaluated in all individuals with cholestasis, particularly those with IBD. MR-Cholangiography-based strictures are used to establish the diagnosis. In the presence of a normal cholangiogram, the diagnosis of small-duct PSC should be evaluated using histology from liver biopsy. Additionally, liver biopsy is indicated when an overlap syndrome is suspected. In the absence of IBD, careful exclusion of secondary sclerosing cholangitis is essential (Isayama et al., 2018, Liver, 2009, Chapman et al., 2019, Lindor et al., 2015).

MRI cholangiopancreatography is the initial diagnostic non-invasive modality for suspected PSC (Zenouzi et al., 2019, Ringe et al., 2019, Schramm et al., 2017). ERCP should only be used for therapeutic intervention including tissue sampling. Multiple studies demonstrate that the diagnostic accuracy of MRCP and ERCP are equivalent (Moff et al., 2006, Aabakken et al., 2017). Classical imaging features of PSC are multifocal stricturing with dilation, referred to as "beading". Limitations of MRCP include poor definition of peripheral ducts and missing early PSC (Venkatesh et al., 2021, Dave et al., 2010). A standard Ultrasound (US) or Computerised tomography (CT) is insufficient to exclude PSC (Swensson et al., 2019, Ruiz et al., 2014).

The need for a liver biopsy to diagnose PSC has decreased due to advances in modern imaging techniques. A liver biopsy should be considered if small-duct PSC (where MR-cholangiogram is normal) or overlap with AIH is suspected (Ludwig, 1991, Burak et al., 2003).

1.2.4 Prognosis and Cancer Risk in PSC:

Younger age at diagnosis, female gender, patients with PSC-AIH overlap, small-duct PSC, and chronically low-normal levels of ALP are associated with an improved prognosis(Dyson et al., 2018, Chazouilleres et al., 2022). Patients diagnosed before the age of 20 years, when compared to those diagnosed after the age of 60 years have a median transplant-free survival 2.5 times longer and 17 times lower CCA incidence(Deneau et al., 2017). Small-duct PSC also has a relatively later onset of cirrhosis and a reduced incidence of hepatobiliary cancer. (Weismüller et al., 2017, Björnsson et al., 2008, Al Mamari et al., 2013, de Vries et al., 2016). The poor prognostic factors include symptoms at presentation, jaundice, poor liver synthetic function, portal hypertension, and extensive biliary (intra/extrahepatic) involvement (Dyson et al., 2018). A multicenter study from Europe, North America, and Australia over 30 years in PSC patients (N=7121), reported HPB malignancy (N=721) in 75, 11%, 16%, and 22% at 5, 10, 15, and 20 years respectively. The majority of the malignancies were cholangiocarcinoma (N=594), other included HCC (N=59), gall bladder cancer (N=58), and pancreatic cancer (N=10). The incidence of **CCA** increases with advancing age. The incidence rate (per 100 patient-years) was 1.2 for < 20 years old, 6.0 for 21–30 years old, 9.0 for 31–40 years old, 14.0 for 41–50 years old, 15 for 51–60 years old, and 21 for those > 60 years. Risk of malignancy was lower in PSC-

AIH overlap vs classical PSC (HR=0.31, 95%CI 0.17-0.55, p<0.01), small duct PSC vs classic PSC (HR=0.19, 95%CI 0.07-0.5, p=0.001), Female vs males (HR=0.7, 95%CI 0.6-0.8, p=0.01), Crohn's ds vs UC (HR=0.7, 95%CI 0.5-0.9, p=0.01), No-IBD vs UC (HR=0.7, 95%CI 0.6-0.8, p<0.001). The risk of LTD compared with classic PSC was lower in small duct PSC (HR=0.3, p<0.01). Female sex was also associated with a lower risk of LTD (HR=0.7, p< .001). Another prospective study (N=215) compared outcomes in early (< 50 years) and late (> 50 years) age at diagnosis of PSC(Rupp et al., 2018). 14.9% of patients were in the late onset group. The proportion of females was lower in the early-onset group (28% vs 48%, p=0.02). Transplant-free survival at 24 years follow-up was 20.8 years in earlier onset vs 10.5 years in the late-onset group ($p < 0.0001$). The late-onset group had a higher rate dominant structure (84% vs 53%, p=0.01) and (48% vs 21%, P=0.03) respectively. A retrospective Japanese study of PSC patients (N-144) divided into two groups based on age at onset <44 years (N=91) and > 44 years (N=530, median follow-up of 6.7 years did not find any difference in the two groups in terms of biliary tract related cancers (HR= 0.89, 95%CI 0.17-4.5, p=0.8)(Watanabe et al., 2021). They reported non-significantly higher all-cause mortality in the> 44-year age group (HR=1.6, 95%CI 0.9-2.85, p=0.12). A multicenter study in a paediatric-onset PSC population (N=781) with, a median age of 12 years reported long-term outcomes(Deneau et al., 2017). On multivariate analysis, they reported better prognostic factors being PSC-IBD (HR=0.6, 95%CI 0.5-0.7, p=0.004) and small duct PSC (HR=0.7, 95%CI 0.5-0.9, p=0.03). Gender (p=0.4), Age (<12 years vs >12 years, p=0.7), and PSC-AIH overlap (p=0.4) did not have any impact on long-term outcomes. A study from Oxford United Kingdom (N=139) PSC patients compared patients who achieved sustained reduction in serum ALP (SAP) (N=55) to < 1.5 ULN to those who did not (N=84)(Al Mamari et al., 2013). SAP group at diagnosis had lower levels of ALP (p=0.01), AST (P<0.0001), bilirubin (p<0.0001) and higher levels of albumin (p=0.004). 38% in no-SAP compared to 6% in the SAP group reached endpoint (variceal bleed, OLT, CCA, or death) (OR=10.5, 95%CI 3.0-37, p=0.002). Similar trends were reported in a study from Mayo Clinic (N=87), where 35 patients achieved complete normalisation of ALP and 52 did not. 33% of patients with persistent raised ALP compared to 14% with normalised ALP reached the endpoint (CCA, OLT, or death), p=0.02(de Vries et al., 2016).

Bacterial cholangitis in patients with PSC occurs in around 5% at the time of diagnosis and roughly one-third develop this complication along the course of the disease. Candida in bile is an indicator of a poor prognosis (Goldberg et al., 2013, Rudolph et al., 2009). In comparison, bacterial cholangitis was not associated with poor prognosis. A German group reported a study in PSC patients (N=171) followed up over 20 years. 97 (56%) developed dominant bile duct stricture (DS) and a cumulative of 500 endoscopic dilatations were performed(Rudolph et al.,

2009). At 18-year transplant-free survival in the DS group was 25% compared to 75% in the no-stricture group (p=0.01). Transplant-free survival was shorter in those who had DS at the point of entry to the study compared to those who developed DS in the follow-up period (p=0.1). Transplant-free survival was significantly shorter in patients with Candida in bile compared to sterile bile (p = 0.02) and enterobacteria in bile (p = 0.03). There was no difference between the candida group and the staphylococci group (p = 0.22).In a retrospective study in a cohort of 171 PSC patients on the transplant waiting list, 39% experienced cholangitis before listing and 28% whilst on the waiting list(Goldberg et al., 2013). On multivariate competing-risk models, a history of bacterial cholangitis was not associated with an increased risk of waitlist removal or death (HR=0.67, 95%CI 0.65–0.70, p < 0.001).

PSC patients at the time of diagnosis may have dominant strictures which can be asymptomatic. Another 40% will develop dominant strictures during the course of the disease(Bowlus et al., 2023). In a German prospective study (N=106), median follow-up of 5 years, 10 patients (11%) had dominant biliary stricture at entry to the study(Stiehl et al., 2002). 42 (38%) developed dominant strictures over the follow-up period. Kaplan–Meier analysis for estimate of stenosis formation, showed significantly lower rates in stage 1 compared to stage 2-4 disease (p<0.01). High-grade strictures with pre-stenotic dilatation on imaging, dominant stricture on ERCP and rapid advancement of a stricture (raising suspicion of CCA) are related to poorer outcomes and lowered survival (Dyson et al., 2018, Björnsson et al., 2004, Hilscher et al., 2018, Stiehl et al., 2002). According to the new definition of dominant stricture (DS), DS is defined as narrowing of any length in the extrahepatic or first-order ducts with worsening cholestatic symptoms and biochemistry, difficulty in passing standard 5F catheter during ERCP or improvement of symptoms and/or biochemistry (>20% ALP drop) after 2-4 weeks of ERCP intervention. A Finnish prospective study evaluated 228 PSC patients who underwent ERCP. Dominant stricture was seen in 37% of patients – 17% (NDS) and 25% traditional definition structure (TDS). NDS was more advanced stricture with higher cholestatic enzymes and associated symptoms (p<0.01).

Model name (N)	Variables included	Measured outcome	Reference
PREsTo (n=509) Lower risk: < 20%, Higher risk: ≥ 20%	Age, duration of disease, Bilirubin, Albumin, AST, ALP, Haemoglobin, platelets, and sodium	Hepatic decompensation measured by ascites, variceal hemorrhage, or encephalopathy	(Eaton et al., 2020) rtools.mayo.edu/PRESTO_calculator/
UK-PSC (n=1001) Lower risk: < 1.46 Higher risk: ≥ 1.46	Age, bilirubin, albumin, ALP, haemoglobin, variceal bleed, Cholangiogram	Death or LT	(Goode et al., 2019) http://www.uk-psc.com/resources/the-uk-psc-risk-scores/
Amsterdam-oxford (n=692) Lower risk: < 1.58 Higher risk: ≥ 1.58	Age, bilirubin, albumin, AST, ALP, platelets, Cholangiogram (small vs large duct involvement)	PSC-related death / LT	(de Vries et al., 2018) https://sorted.co/psc-calculator/
Tischendorf (n=273)	Age, bilirubin, albumin, splenomegaly/ hepatomegaly, cholangiogram findings	Death or LT	(Tischendorf et al., 2007)
Ponsioen (n=174)	Age at diagnosis, cholangiogram findings	Liver-related death/ LT	(Ponsioen et al., 2002)
Boberg (n=330)	Age at diagnosis, bilirubin, albumin	PSC-related death / LT	(Boberg et al., 2002)
Revised Mayo Score (n=405)	Age, bilirubin, albumin, AST, variceal bleed	Death	(Kim et al., 2000c)
Other earlier scores included Broome (n=305)(Broome et al., 1996), Farrant (n=126)(Farrant et al., 1991) and Wiesner (n=174)(Wiesner et al., 1989).			

Table 2: PSC prognostic models

Prognostic models in PSC, variables included compared to measured outcomes. (ALP: Alkaline Phosphatase, AST: Aspartate aminotransferase, LT: Liver transplant)

Risk of malignancy in PSC:

Patients with PSC are at increased risk of **CCA, Colorectal, and gall bladder cancer**. PSC cirrhosis increases the likelihood of developing HCC, but this is comparable to other causes of cirrhosis (Boonstra et al., 2013, Aune et al., 2021, Bergquist et al., 2002). The **risk of CCA** in PSC is 100-400 times higher than that of the general population. The highest incidence is in the first year after a PSC diagnosis (2.5%). Thereafter risk is 1%–1.5% per year. Advancing age, rising bilirubin levels, male sex, dominant stricture, and concomitant IBD are associated with increased risk of CCA (Rudolph et al., 2010, Chalasani et al., 2000, Gulamhusein et al., 2016a, Tischendorf et al., 2007). A Dutch study (N=590), with a median follow-up of 92 months, reported CCA in 7% of patients (Boonstra et al., 2013). Median (range) age at diagnosis of CCA was (47, 21-87 years), and time from PSC to CCA diagnosis was (6, 0-36 years). Compared to the general population risk of developing CCA in PSC was 400-fold (SIR=398, 95% CI 246-608). 10, 20, and 30-years cumulative risk of CCA after was 6%, 14%, and 20% respectively. Risk factors for CCA were older age at PSC diagnosis (HR=1.02, p=0.04) and occurrence of CRC in a time-dependent manner (HR=4.57, p=0.04). In a study from London (N=128), with a mean follow-up of 10 years, 62% of patients had a dominant biliary stricture. The mean survival was much lower in those with dominant strictures compared to those without (13.7 years vs 23 years). This difference was related to a 26% risk of CCA which developed in dominant structure patients only(Chapman and Williamson, 2017). Similar results were reported in a multicentre international study (N=2616) of PSC patients of which 721 developed HPB malignancy (Weismüller et al., 2017). 23% (N=594) were cholangiocarcinoma, 2.25% (N-59) HCC and 2.25% (N=58) were gallbladder cancer. The risk was lower in small duct PSC compared to classic PSC (HR=0.2, p=0.001), PSC-AIH overlap compared to PSC alone (HR=0.31, p<0.01), female vs male (HR=0.68, p=0.01), Crohn's disease vs UC (HR=0.7, p=0.01).

The incidence of **gallbladder cancer** in PSC is around 1.1 per 1000 person-years. Up -to 15% of people with PSC have gallbladder polyps, which may indicate a premalignant stage (Said et al., 2008, Bowlus et al., 2019, van Erp et al., 2020). Polyps greater than 1 cm in size have a higher risk of dysplasia (Eaton et al., 2012, Torabi Sagvand et al., 2018). A Swedish study evaluated gall bladder abnormalities in PSC patients (N=285)(Said et al., 2008). Gallstones were found in 25% and 25% had cholecystitis. The prevalence of gallstones was higher among patients with extrahepatic PSC compared to intrahepatic PSC (28% vs 17%, p= 0.05). Similarly, the frequency of cholecystitis extrahepatic PSC was higher than intrahepatic PSC (30% vs 9%, p < 0.0001). Mass lesion (mean size 2cm) in the gall bladder was found in 6% of patients. 56%

of these lesions were gall bladder cancer (overall= 3.5%). Another study of PSC patients (N=236) based on imaging found gall bladder polyps in 11%, size (mean, range= 8, 2-27mm) with the time of PSC diagnosis to the finding of GB polyp (mean=10 years) (Sagvand et al., 2018). 2.5% (N=6) were found to have gallbladder cancer.

The **risk of CRC** in patients with PSC is 10 times that of the general population and five times that of individuals with IBD without PSC. In a meta-analysis of 1022 patients (16 studies) by Zheng et al the risk of CRC or dysplasia was assessed to be three times higher in people with PSC with IBD than in those with IBD alone (OR = 3.24 + 95% CI = 2.14–4.90) (Zheng and Jiang, 2016). Similar findings were reported by Soetniko et al in a 564-patient metanalysis from 11 studies (Soetikno et al., 2002). Patients with PSC-UC had a higher risk of CRC compared to UC alone (OR=4.8, 95%CI3.6-6.4]. A Swedish large cohort study (N=604) of PSC patients with a median time of follow-up was 5.7 years, reported a 7.4% cumulative risk of CRC(Bergquist et al., 2002). In their study, CRC was observed only in patients with concomitant IBD. In a study from Rotterdam in PSC (N=211), 10 and 20-year risk of CRC was reported to be 9% and 22% respectively(Claessen et al., 2009). The median time from diagnosis of PSC to diagnosis of CRC was 8.3 years (range 0–18.2). 10 and 20-year CRC risk in PSC-IBD was much higher compared to PSC alone (14% vs 2% and 31% vs 2% respectively, p=0.008). 10 and 20-year dysplasia risk in PSC-IBD was much higher compared to PSC alone (15% vs 2.4% and 30% vs 21% respectively, p=0.02). 63% of these CRCs were in the right colon. Interestingly, patients with IBD who develop PSC during the course of the disease pose a higher risk of CRC compared to IBD alone. A population-based study from England explored the risk of cancer and death in IBD patients with onset of PSC (N=2558) compared to those with IBD only (N=284560) over 10 years (Trivedi et al., 2020). Development of PSC was associated with increased risk of CRC (HR=2.43, p<0.001), lower median age at CRC diagnosis (59 vs 69 years, p<0.001) of cholangiocarcinoma (HR=8.4, p< .001), HCC (HR=21.00, p<.001), pancreatic cancer (HR=5.3, p< .001), and gallbladder cancer (HR=9.1, p< .001) and increased risk of death (HR=3.2, p<0.001).

1.2.5 PSC and IBD:

Inflammatory bowel disease (IBD) is associated with PSC in up to 80% of patients. PSC patients with IBD tend to be younger at diagnosis. A UK study (N=53 PSC) over 20 years reported IBD in 62% of patients (90% UC and 10% CD)(Kingham et al., 2004).The UK Clinical Practice Research Datalink 15-year study (N=250), reported 54 patients had IBD compared to 2% in matched controls. Patients with IBD were younger than those without IBD (mean age 50 vs 57 respectively, p=0.002)(Liang et al., 2017b). An epidemiolocal study from the United

States (22 cases) over 25 years, reported IBD in 75% of patients (75% UC and 20% CD)(Bambha et al., 2003). A 5-year epidemiological study from the United States (N=169), reported IBD in 65% of patients (88% UC and 12% CD). PSC patients with IBD were younger (mean 41 years vs 49-year non-IBD patients, p=0.005) and male (73% vs 51% females, p=0.005)(Toy et al., 2011).

PSC-related colitis (UC) tends to be predominantly right-sided. A Dutch study of 579 PSC patients reported IBD in 66% of patients (75% UC and 25% CD). Pancolitis was present in 95% of the UC patients compared to 62% in controls (p<0.001). Left-side colitis and rectal sparing were found to be less frequent in PSC-related UC compared to matched controls (p<0.001)(Boonstra et al., 2012b). PSC with IBD clinically tends to do better compared to PSC without IBD. Data from Cleveland clinic PSC patients (N=222), 75% with UC and 25% without UC, reported that on sex, year of PSC diagnosis, and Mayo-PSC risk score, proportional hazards analysis UC was not found to be associated with liver-related adverse outcomes (OLT or death), (HR=0.9, 95%CI 0.6-1.34, p=0.6)(Navaneethan et al., 2012). PBC patients with UC were younger compared to those without IBD (mean 38 vs 47 years respectively, p<0.001). PBC without IBD had the more frequent requirement of OLT (Kaplan–Meier curve for proportion: Log-rank $P = 0.004$) and lesser transplant-free survival (Log Rank $P < 0.001$). UK Liver Transplant Centre reported 96 PSC patients with IBD with a median follow-up of 144 months(Marelli et al., 2011). 47% of these required liver transplants. They reported in the OLT group, UC was more quiescent (p=0.002) and required fewer steroid courses (p=0.02) with fewer flare-ups (p=0.04). Histological inflammation on colonic biopsies was more in the non-transplant group (p=0.006) with more frequent high-grade dysplasia and colorectal cancer (p=0.01).

1.2.6 PSC overlap:

5-10% of adult patients can have PSC overlapping with AIH (Mago and Wu, 2020, Czaja and Carpenter, 2017). Rochester group in PSC (N=211), using the International Autoimmune Hepatitis Group numerical scoring system for definitive and probable diagnosis reported definitive and probable AIH in 1.4% and 6% of patients respectively(Kaya et al., 2000). A retrospective Dutch study of 113 PSC patients over 22 years reported that 8% (N=9) of patients had PSC-AIH overlap. In 5/9 patients PSC-AIH overlap was diagnosed after a mean interval of 3.3 years from the initial diagnosis of PSC(van Buuren et al., 2000).PSC-AIH overlap patients tend to be younger. Italian group in a prospective 2-year follow-up of 41 PBC patients reported PSC-AIH overlap in 7 patients(Kaya et al., 2000). The mean age in PSC-AIH group was 21 years compared to 32 years in classic PSC, p<0.01. ALT, AST and IGG were higher in overlap

group [(357.0 ± 26.5 vs 83.7 ± 60.7 U/L, p < 0.005), (191.0 ± 14.8 vs 48.9 ± 34.5 U/L, p < 0.005) and (25.6 ± 4.7 vs 12.9 ± 6.0 mg/dl, p < 0.0001) respectively]. A UK major liver transplant centre in their study reported outcome data on 238 patients with AIH, 16 PSC-AIH overlap, and 10 PBC-AIH overlap(Al-Chalabi et al., 2008). PSC-AIH patients were younger (mean age 27 years) compared to AIH (46 years) and PBC-AIH (55 years), p=0.0007. Significantly lower 10–20-year survival was seen in the PSC-AIH overlap group compared to AIH alone (log-rank p=0.04, HR=2.08). However, when compared to classic PSC, PSC-AIH overlap tends to have better transplant-free survival. A large international collaborative retrospective study of 37 centres from Europe, North America, and Australia, reported outcomes in 7121 patients (90% classic PSC,3.5% small-duct disease PSC and 6.5% PSC-AIH overlap)(Weismüller et al., 2017). Overall median transplant-free survival was 14.5 years (95%CI 13.6-15.2 years). PSC-AIH overlap had a lesser risk of liver-related events (OLT or death) in male patients, (aHR=0.73, 95%CI 0.56-9.14, p=0.01). There was no difference among the female patients (aHR=1.19, 95%CI 0.91-1.54, p=0.2).

1.2.7 Management of PSC:

Currently, there is no approved drug therapy for PSC. According to recent EASL guidelines, there is a weak recommendation for the use of UDCA (at a dose of 15-20 mg/kg/d) to improve liver chemistry, as a surrogate marker for prognosis (Liver, 2022). Therefore, management focuses on identifying and treating complications. Patients with refractory cholangitis and ESLD due to decompensated cirrhosis are recommended for LT.

Medical management

- **Ursodeoxycholic acid (UDCA)**

UDCA is the drug most studied in PSC (Poupon, 2012). Low-dose UDCA (13–15 mg/kg/day) has demonstrated a 12-month improvement in ALP. However, liver histology or transplant-free survival did not show any significant improvements (Lindor, 1997). With intermediate-dose UDCA (17–23 mg/kg/day) no significant reduction was observed in need for LT, risk of CCA, and overall mortality (Silveira and Lindor, 2008b, Olsson et al., 2005). Higher doses (28–30 mg/kg/day) of UDCA are not regarded as safe (Imam et al., 2011). Olsson etal, randomized placebo-controlled of 17-23mg/kg UDCA (N=110) and placebo (N=109), over 5 years did not find any beneficial effects of UDCA. Trends were seen in the non-significant improvement of ALP in the UDCA group. Combined end points of LT and death were no different in UDCA compared to the placebo group (7.2% vs 10.9%, p=0.37) (Olsson et al., 2005). High-dose

UDCA was linked to an elevated risk of colorectal neoplasia in patients with PSC and ulcerative colitis (Eaton et al., 2011). In their study, they reported a higher risk of CRC in the UDCA group compared to placebo (HR=4.4, 95%CI 1.3-20, p=0.02). One metanalysis which included 8 RCT (N=465), reported trends towards improvement in liver histology with UDCA in PSC [OR=9.1, 95%CI 0.98-86, p=0.05]. They did not find any significant effect on symptoms (pruritus and fatigue), need for LT, and death(Shi et al., 2009). Another meta-analysis of 8 RCTs (N=567) found no beneficial effect of UDCA at low or high doses. 5 trials used standard (10-15 mg/ kg dose) and 3 trials used higher doses (17-30 mg/kg). No difference was found in histological progression (OR=0.9, 95%CI, 0.35–2.5), pruritus (OR= 1.5, 95% CI, 0.3–7.0), risk of developing CCA (OR=1.7, 95% CI, 0.6–5.1) and mortality (OR=0.6, 95% CI, 0.4–1.4)(Triantos et al., 2011). BSG PSC guidelines do not recommend UDCA for routine treatment of newly diagnosed PSC (strength of recommendation: STRONG; quality of evidence: GOOD). UDCA is also not recommended for prevention of CRC(Chapman et al., 2019).

Other immunosuppressive agents like prednisolone (Boberg et al., 2003, Schramm et al., 1999), Azathioprine(Schramm et al., 1999), Mycophenolate (Sterling et al., 2004), Methotrexate (Knox and Kaplan, 1994) and tacrolimus (Talwalkar et al., 2007); and antibiotics like metronidazole (Färkkilä et al., 2004), penicillamine (LaRusso et al., 1988) have been trialled, but have not shown to be effective.

- **Bacterial cholangitis**

Bacterial cholangitis is common in PSC and is an important complication of the disease. This usually occurs in patients with high-grade biliary strictures (Karlsen et al., 2017, Bangarulingam et al., 2009, Navaneethan et al., 2015). Bacterial cholangitis can be the first presentation in 6% of patients(Kaplan et al., 2007). The first episode of bacterial cholangitis in PSC should be investigated with MR-cholangiography. ERCP +/- stenting in PSC is a major risk factor for cholangitis. High-grade strictures with severe acute cholangitis carry high mortality. Urgent biliary decompression is required. Therefore, antibiotics should be routinely administered in PSC patients undergoing ERCP (Aabakken et al., 2017, Björnsson et al., 2000). Occasionally patients with complex cholangiopathy and recurrent episodes of cholangitis, require rotating long-term antibiotics. Recurrent cholangitis which does not respond adequately to antibiotic therapy can be an indication for LT. In some countries, patients are awarded extra Model of End-stage Liver disease (MELD) points for this indication whilst on the waiting list (Burra et al., 2016, Andersen et al., 2015b, Goldberg et al., 2013). However, in the U.K. this is not an accepted practice at present (Liver, 2022).

1.2.8 Liver transplant in PSC:

Around 5-15% of the LTs across North America and Europe are done for PSC (Martin and Levy, 2017, Berenguer et al., 2021, Adam et al., 2018). Indications include Complications of ESLF related to advanced cirrhosis, recurrent bacterial cholangitis, Intractable pruritus, and selected cases of early CCA (in trials) (Klose et al., 2014, Boyd et al., 2017, Goldberg et al., 2013, Sapisochin et al., 2020). The rate of recurrent PSC (r-PSC) has been reported between 10-35% at 6 months to 5 years and around 12% will require re-LT at 10 years. Male sex, primary immunosuppression with tacrolimus, allograft rejection, steroid-free induction protocols, extended criterion grafts, and poorly controlled IBD are the factors that increase the risk of r-PSC (Vera et al., 2002, Steenstraten et al., 2019, Lindström et al., 2018). Data from the European Liver Transplant Registry (between 1980 and 2017), 6443 transplants were done for PSC in 159 centers, making 4% of transplants done for all indications(Berenguer et al., 2021). 1, 5, 10-, 15-, 20-, and 30-year graft survival rates were 84%, 71%, 58%, 50%, 31%, and 12% respectively. On multivariate analysis for patient survival, factors associated with higher mortality loss were recipient age (HR=1.025, $p < 0.001$), male sex recipient (HR=1.142, $p = 0.03$), donor age (HR=1.008, $p<0.001$), non-DBD graft (HR=1.47, $p= 0.003$), presence of CCA (HR=4.1, $p < 0.001$), and reduced size graft (HR=1.26, $p = 0.031$). Between 1980-2015, recurrence was diagnosed in 259/ 1549 (16.7%) on a median follow-up of 5 years (Visseren et al., 2021). 1-, 5-, 10-, and 20-years patient survival after the first PSC-OLT was 89%, 80%, 73%, and 57%, respectively. rPSC had a significant negative impact on patient survival (HR=6.7,95% CI 4.9–9.1). 1, 5, 10, and 20-years graft survival for the first PSC-OLT was 80%, 70%, 60%, and 41%, respectively. rPSC had a significant negative impact on graft survival (HR=2.3, 95%CI 1.5–3.3). rPSC patients underwent significantly more re-transplants (OR=3.6, 95%CI 2.7–4.8). The Nordic Liver Transplant Registry between 1982 and 2013 registered 15% of the OLTs were done for PSC (Fosby et al., 2015). 1,5,10 and 20-year graft survival were 87%, 75%, 64% and 44% respectively. Japanese registry reported outcomes in LDLT. 114 PSC LDLT were performed over 12 years(Egawa et al., 2011). The 5 and 10-year graft survival was 70% and 34% respectively. rPSC was diagnosed in 27% (N=26) at 8-79 months. 5 and 10-year recurrence rates were 32% and 52% respectively. The rate of graft loss was higher in rPSC (69%) compared to those without (23%). Multivariate analysis showed that high MELD scores [>24] (HR=3.7, 95%CI 1.6-8.4, p=0.002), first-degree-relative donors (HR=3.1, 95%CI 1.14-8.3, p=0.02), postoperative CMV infection (HR=3.3, 95%CI 1.4-7.6, p=0.005), and early (<12 months) biliary anastomotic complications (HR=4.2, 95%CI 1.64-10, p=0.003) were significant risk factors for rPSC. A meta-analysis of 14 eligible cohort studies (N=2159) of patients

transplanted for PSC reported 17.7% rPSC (Steenstraten et al., 2019). Factors associated with significant risk of rPSC were presence of IBD (HR=1.73 (95%CI 1.17-2.54), advancing donor age (HR=1.24, 95%CI 1.07-1.45 per 10 advancing years), acute rejection (HR= 1.94, 95%CI 1.32-2.83), higher MELD score (HR=1.05, 95%CI 1.02-1.08) and CCA (HR=2.42, 95% CI: 1.20-4.86). Colectomy before OLT was found to be protective (HR=0.65, 95% CI 0.42-0.99). Another retrospective study of 69 patients transplanted for PSC over 14 years reported an association of rPSC with, acute cellular rejection (OR=8.7, 95% CI, 1.5-49, p=0.01), and steroid-resistant rejection (p= 0.01) (Alexander et al., 2008).

In 2006, MELD exception point (+2) was agreed for those with recurrent cholangitis defined as ≥2 non-iatrogenic episodes of culture-proven bacteraemia or septic complications over consecutive 6 months without a correctable structural lesion by Organ Procurement and Transplantation Network (OPTN) consensus (Gores et al., 2006). There was a significant increase in the number of acceptances of exception applications post consensus (80% vs 66%, p<0.001), and those meeting the criterion were more likely to be transplanted (78% vs 63%, p0.04)(Goldberg et al., 2012). In Norway, 17% (24/138) of the PSC-related OLT were done for recurrent bacterial cholangitis as presented in a study over 5 years(Andersen et al., 2015a). However, in U.K. less than 5% of PSC transplants are done for this indication(Karlsen et al., 2017).

In UK Cholangiocarcinoma is not an accepted indication for OLT ((http://odt.nhs.uk/pdf/liver_selection_ policy.pdf). A Canadian study (N=10, 8 PSC) of incidental CCA in explant reported 30% post-OLT 3-year survival (Ghali et al., 2005). Similar results were reported from the U.K. (N=9, 7 PSC) with incidental CCA in explant. 56% developed recurrent CCA in a median interval of 2 years, and there was 66% 3-year survival (Ali et al., 2011b). German study (N=47) patients transplanted for Hilar Cholangiocarcinoma (hCCA) reported a median survival of 3 years, with 3 and 5-year survival rates of 31% and 22% respectively. hCCA recurred in 34% of cases (Kaiser et al., 2008). A meta-analysis of 20 studies (cohort studies) (N=428) reported slightly better outcomes in OLT for CCA with prior chemo-radiotherapy (Cambridge et al., 2021). Pooled 1,3 and 5-year survival was 71%, 48%, and 32% respectively for OLT without neoadjuvant therapy compared to 83%, 66%, and 65% respectively in the neoadjuvant therapy group. Pooled 3-year recurrence was 52% without and 24% with neoadjuvant therapy.

1.3 AMA positive with normal LFTs (AMANL) patient:

One of the most important diagnostic characteristics of primary biliary cholangitis is the presence of anti-mitochondrial antibodies (AMAs). Up to 95% of patients with PBC test positive for AMAs, making AMA positivity a key diagnostic criterion in all international guidelines (Hirschfield et al., 2018, Younossi et al., 2019, Lindor et al., 2019, Liver, 2017, You et al., 2022). AMA are antibodies directed against 2-oxo-acid-dehydrogenase complexes involved in mitochondrial energy metabolism. The major antigens include the E2 subunit of the pyruvate dehydrogenase complex (PDC-E2), and the branched-chain 2-oxo acid dehydrogenase complex (OGDC-E2)(Vergani et al., 2004, Gershwin et al., 1988, Moteki et al., 1996, Leung et al., 1995).Utilizing the most up-to-date diagnostic methods, approximately 5% of well-documented PBC patients do not react to any of the mitochondrial antigens (Hirschfield and Heathcote, 2008, Vergani and Bogdanos, 2003). Among the general population prevalence of AMA antibodies is reported to vary from 0.07% to 9.9%(Chen et al., 2016, Turchany et al., 1997).

A subset of patients encountered in clinical practice are those in whom AMA antibody is positive, but liver blood biochemistry (ALP) is normal. According to the findings of several studies some patients who tested positive for AMA but had normal serum ALP levels did not exhibit any clinical signs of having PBC (Metcalf et al., 1996, Sun et al., 2019).

Mitchison et al. (1986) first reported 29 patients (28F and 1M) with AMAs detected at a titer of >/= 1:40 when screening for other autoimmune disease. None of the patients had any symptoms of liver disease. Liver biochemistry was normal (normal bilirubin and ALP [ULN 110 IU/litre]) in all of these patients. GGT (ULN <40 IU/litre for women and <70 IU/litre for men) was raised in 14 patients. However, on liver biopsy, 24 of the patients had features that were either suggestive (N=12) or conclusive (N=12) for the diagnosis of PBC. The mean follow-up was 8.7 years from the time of detection of AMA to recruitment in the study. 12/16 females followed for >4 years developed abnormal liver chemistry (11 raised ALP and 5 raised AST of which 4 have associated raised ALP). Five patients developed symptoms of pruritus (n=4) and fatigue (n=1). These patients were followed up for 10 years. Five patients died during this period of the causes unrelated to liver disease (median time to death 11.7 years). Among the 24 survivors, the median follow-up was 17.8 years. Of these 3/4 developed symptoms of PBC and 4/5 developed persistent cholestatic liver chemistry. 16 patients had raised ALP, 7 ALT, and 1 raised bilirubin (ULN 34 µmol/l). The median time to first raise ALP was 5.6 years. None of

the patients developed portal hypertension or liver cirrhosis, indicating slow progression of disease in this subset(Mitchison et al., 1986, Metcalf et al., 1996).

A study from China, by Shunyan et al., reported 80% of patients with AMA positivity and normal ALP had histological evidence of PBC on liver biopsy. The study recruited 169 patients, (87.6% females, mean age 46 years, median AMA 1:320 and elevated IgM in 53%). The normal range for liver biochemistry in this study was ALP 40–150 U/I, ALT & AST 10–40 U/I, and GGT 10–50 U/I. 67 had a liver biopsy of which 55 (82%) showed histological evidence of PBC(Sun et al., 2019). Within the normal ALP range levels were higher for those with histological features of PBC (0.61 × ULN) compared to those with normal histology (0.41 × ULN), p=0.001. Multivariate regression analysis, showed higher (within normal) levels of ALP (>0.475 × ULN) were associated with histological changes of PBC (OR =21, CI 2.4–182, p = 0.006).

Similar findings were reported in a Swiss study of 30 patients with PBC-positive serology and normal ALP (90% females and median age 53 years). GGT in 16, ALT in 11, and AST in 1 patient were raised above the ULN. In their study, 80% (20/30) of the patients had histology suggestive or conclusive of PBC. Of these 2 were typical, 16 were consistent and 6 suggestive of histological features of PBC. 22/24 patients with positive liver histology were on UDCA therapy (with normal ALP before the start of UDCA). Currently, GGT is not included in the diagnostic criterion of PBC. They observed significant improvement [median (range) pre-treatment vs post-treatment 1.46 X ULN (0.14-6.8) vs 0.43 (0.14-3.4), (p=0.0018)] in GGT levels of these patients with UDCA therapy. Study limitations included small sample size, short follow up and retrospective design. (Beretta-Piccoli et al., 2021).

Another prospective French study of 720 AMA-positive patients divided into three groups of established PBC diagnoses (G1, N=216), new PBC diagnosis (G2, N=275), and AMA positive with normal liver chemistry (G3, N=229), followed up for 7 years, reported prevalence of 16.1/100,000 in G3. 3/4 of the patients were females. Their median age and AMA titer were 58 years and 1:160 respectively. 46% had evidence of autoimmune non-liver-related disease and 6% had cirrhosis. Follow-up data was available in 41% of the G3 patients. Patients in G3 compared to G2 were relatively younger with lower AMA positivity titre. 10% (9/92) followed-up patients developed PBC, with cumulative incidence rates of 2% (95% CI= 0-7), 7% (95% CI= 2-15), and 16% (95% CI= 6-29), at 1, 2 and 5 years respectively. Though the deaths were unrelated to PBC, only 75% compared to 90% in healthy controls 5-year survival was observed in this group (Dahlqvist et al., 2017).

In a study by Gulamhusein et al., first-degree relatives (FDRs) of patients who tested positive for AMA and had normal liver function tests, were found to have a relatively lower risk of developing primary biliary cholangitis (PBC). Overall, only 3% of the FDRs of diagnosed PBC patients had received a PBC diagnosis themselves. However, during a 5-year follow-up, 4% of the FDRs of patients with confirmed PBC developed the disease. In comparison none of the FDRs who were AMA-positive with normal liver chemistry developed PBC (Gulamhusein et al., 2016b).

APASAL recommends regular follow-up and early initiation of UDCA in patients who are AMA-positive with normal liver chemistry and histological diagnosis of PBC(You et al., 2022).

1.4 Bile acid metabolism:

The description of bile acids dates far back in history. “Temperament theory” was described by Hippocrates in the third century BC. According to this theory human body is composed of four “humours” (fluids), which influence health and disease. Two of these humours were biles- “Yellow bile and black bile”, in addition to phlegm and blood (Goodacre and Naylor, 2020).

Bile Acid synthesis and regulation:

Cholesterol metabolism results in the formation of BAs as the end product. BAs are formed from cholesterol by the oxidative hydroxylation process (Russell, 2003a, Hofmann, 1999). 200-600 mg/ day of bile acids are produced in human liver. A nearly equal amount of bile acids is excreted in the faeces. In the human body, 3 grams of bile acids are recirculated 4-12 times/ day. Cholic acid (CA) and Chenodeoxycholic acid (CDCA), and their Glycine [Glycocholic acid (GCA), Glycochenodeoxycholic acid (GCDCA)] and Taurine conjugates [Taurocholic acid (TCA) and Taurochenodeoxycholic acid (TCDCA)] are the primary bile acids. CA and CDCA are amphipathic molecules that have strong detergent properties. Glycine and Taurine conjugate increases their solubility at physiological pH and aids the formation of sodium salts called “Bile salts”. Most bile acids in humans are conjugates of glycine or taurine and exist in an approximated (Glycine: Taurine-conjugate) ratio of 3:1 (Bortolini et al., 1997).

Two distinct biosynthetic pathways and 17 enzymes are involved in the synthesis of bile acids from cholesterol (Chiang, 1998). These enzymes are located in mitochondria, peroxisomes, endoplasmic reticulum, and cytosol. All bile acids are C-24 Cholanic acids. The enzymes catalyse oxidative cleavage of carbon and modification of the steroid ring of cholesterol to produce C-24 bile acids (Russell, 2003a, Bortolini et al., 1997). The two pathways are: classic and alternative (also known as acidic pathway). In the human liver, cholesterol 7-hydroxylase (CYP7A1) [a rate-limiting enzyme] initiates the classic pathway, which synthesises two primary bile acids, CA and CDCA. CA synthesis requires microsomal sterol 12-hydroxylase (CYP8B1). In the absence of 12-hydroxylase, CDCA is produced. The acidic pathway (accounting for 9-10% of human bile acid production) is initiated by the mitochondrial cytochrome P450 enzyme sterol 27-hydroxylase (CYP27A1)(Duane and Javitt, 1999). This enzyme is found in macrophages and most tissues (Russell, 2003a, Chiang, 1998).

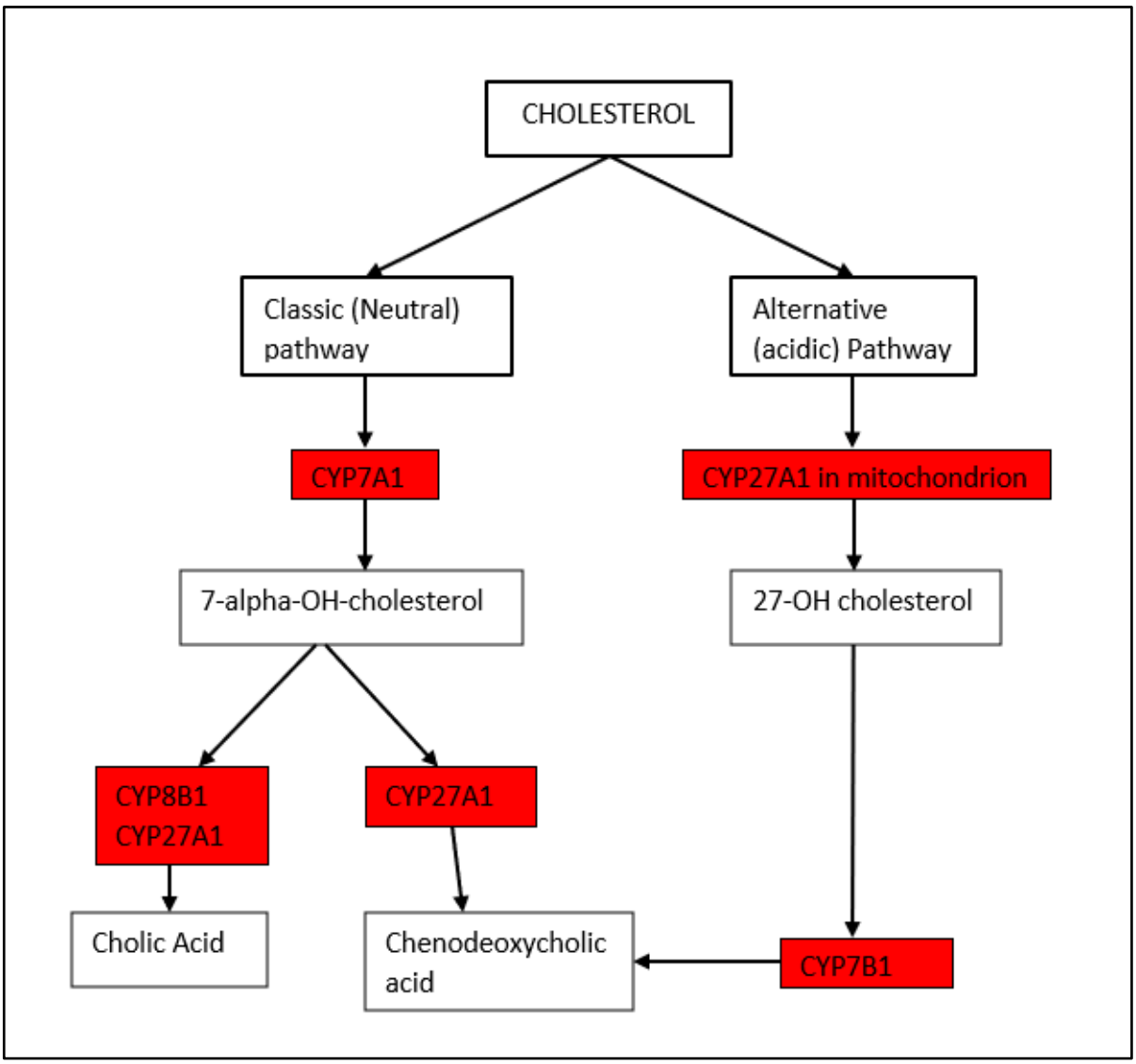


Figure 2: Bile acid synthesis (Classic and acidic pathways)

Bile acids are produced from cholesterol in the liver through two main pathways. The classical pathway begins with the rate-limiting enzyme CYP7A1, which synthesizes the two primary bile acids in humans: cholic acid (CA) and chenodeoxycholic acid (CDCA). The enzyme CYP8B1 is essential for the synthesis of CA, alongside mitochondrial CYP27A1, which facilitates the oxidation of the steroid side chain. In contrast, the alternative pathway starts with CYP27A1, followed by the action of CYP7B1.

Bile contains BAs, cholesterol, phospholipids, drugs, and metabolites. Bile is stored in the gall bladder and upon stimulus by cholecystokinin (causing gall bladder contraction), after taking a meal is released into the small intestine where bile salts (mainly sodium salts), aid digestion and absorption of nutrients. Most of the bile acids are recirculated to the liver from the intestine via portal blood, through a very strong enterohepatic circulation cascade operating in the human body (Hofmann, 2009). 95% of the BAs are reabsorbed at the terminal ileum brush border epithelium. DCA absorption, however, occurs in the colon.

In the distal intestine, primary BA conjugates of CA and CDCA are first deconjugated and then converted respectively to secondary bile acids Deoxycholic acid (DCA) and lithocholic acid (LCA), by the action of bacterial 7-dehydroxylase (Ridlon et al., 2006). The majority of LCA is eliminated in the faeces, while small amounts are circulated back to the liver. Sulfation is the primary pathway for hydrophobic bile acid detoxification in humans (Kakiyama et al., 2014). Recirculated LCA undergoes sulphate conjugation before being released back into the bile(Hofmann, 2004). C6-hydroxylated BAs (Muricholic acid) are only found in trace amounts in adult urine and faeces (Goto et al., 1992, Dawson and Karpen, 2015).

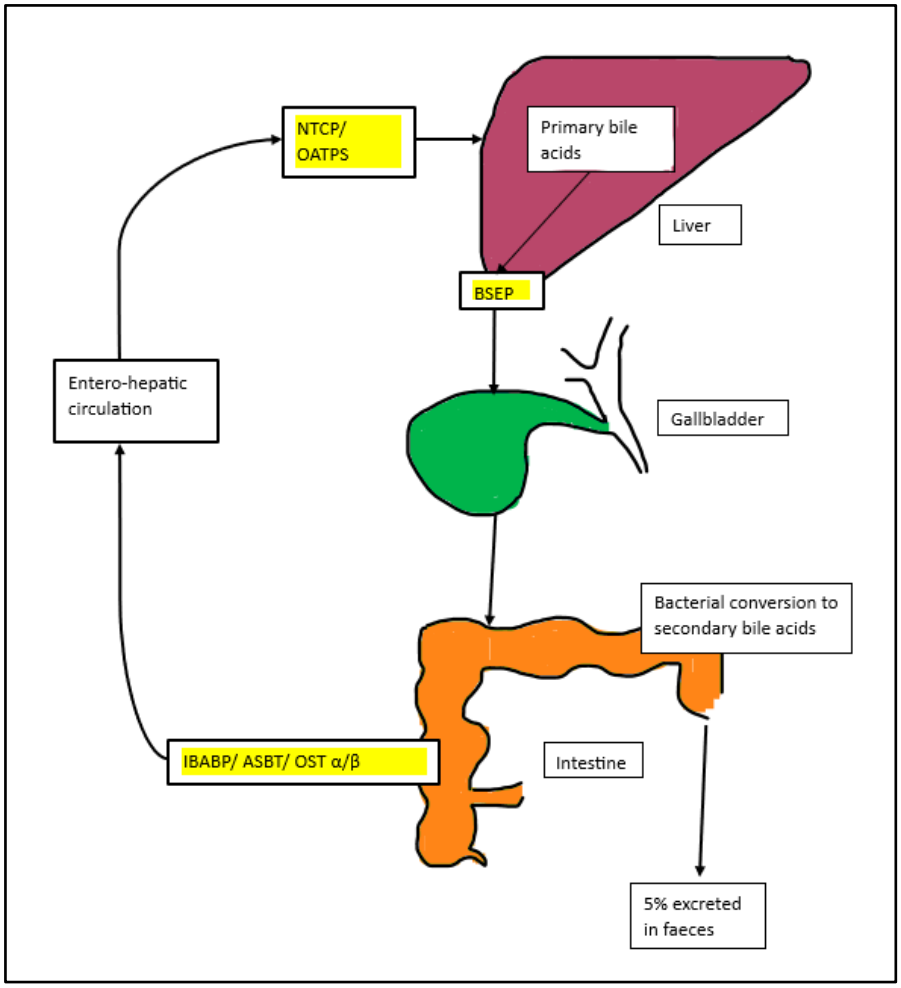


Figure 3: Enterohepatic circulation of bile acids

After the liver synthesizes bile acids (BAs) and secretes them into the bile, these acids undergo a process known as enterohepatic circulation. The main canalicular transporter responsible for bile acid transport is BSEP (ABCB11). Once the bile acids are reabsorbed in the ileum by ASBT, they exit enterocytes through the organic solute transporters OSTα/β and are then transported back to the liver via the portal blood. The reuptake of conjugated bile acids from the portal blood into hepatocytes through NTCP and OATPs completes the enterohepatic circulation.

Various mechanisms contribute to the regulation of bile acid synthesis. Bile acids are recirculated to the liver through enterohepatic circulation, where they inhibit the CYP7A1 enzyme, exerting a regulatory negative feedback mechanism. Studies have reported instabilities in CYP7A1 mRNA transcription (3'-untranslated regions) and apoB editing complex (Apobec-1) (Myant and Mitropoulos, 1977, Li et al., 1990, Xie et al., 2009). One theory suggests that the activity of CYP7A1 is regulated by the availability of cholesterol, by steroid response element binding protein-2 (SREBP-2) in response to intracellular oxysterol levels. High levels of oxysterol inhibit, and low levels stimulate cholesterol synthesis (Yabe et al., 2002, Engelking et al., 2004, Edwards et al., 2000). CYP7A1 expression has also been shown to be depressed by glucagon and induced by insulin and glucose (Song and Chiang, 2006, Lundåsen et al., 2003, Li et al., 2010, Li et al., 2006, Li et al., 2012).

Nuclear receptors and bile acids regulation:

The human genome contains 48 nuclear receptor genes. There are three types of NRs- Type 1 (endocrine), Type 2 (adopted) and Type 3 (Orphan- no known ligand). BAs act mainly on 3 NRs. These are PXR (pregnane X receptor), FXR (Farsenoid X receptor) and VDR (Vitamin D Receptor) (Makishima et al., 1999, Thomas et al., 2008, Makishima et al., 2002). Free and conjugated hydrophobic BAs (CDCA, LCA, DCA and CA) activate FXR. FXR is not activated by UDCA and MCA (hydrophilic BAs). CDCA is the most efficacious FXR ligand. Whereas LCA and 3-Keto LCA are the most effective PXR and VDR ligands (Wang et al., 1999, Francis et al., 2003).

Effective concentration 50 (EC50), is also known as half maximal effective concentration. This is the concentration of a substance that induces a response halfway between baseline and maximum after a defined exposure time under a specified set of conditions(Holford, 2017). EC50 is used to define the agonistic effect. Similarly, IC50 Inhibitory concentration 50 is used to define antagonistic effect. The measure is used to define the potency of a compound to produce an effect on NR and its co-regulator. The higher the potency, the lower the concentration of the compound will be required to achieve effect. For example, GW4064, was identified as the first high-affinity nonsteroidal FXR agonist using human FXR expression vectors and CV-1 cells transfected with mice showed EC50-values of 90 and 80 nM respectively (Maloney et al., 2000).

1. Farsenoid X receptor (FXR):

FXR in humans inhibits CYP7A1 gene transcription. This is done via two different pathways: one in hepatocytes and one in enterocytes. FXR in the gene promotor region, binds to IR1 (inverted repeat with one-base spacing) to activate transcription of target genes. An in vitro study using HepG2 cells demonstrated that FXR/RXR-α heterodimer specifically binds to the Inverted repeat (IR-1) element in the BSEP promoter. The heterodimer is essential for bile acids to fully transactivate the BSEP promoter. Mutational analysis showed this heterodimer-mediated transactivation happens with specific binding to the IR-1 site. A W469A point mutation and AF-2 deletion (TXR transactivation deficient mutations) failed to transactivate the BSEP promoter (Ananthanarayanan et al., 2001). In hepatocytes, SHP (small heterodimer partner) is induced by FXR. Primary bile acids CA and CDCA bind to FXR. Isoxazole GW4064 has shown to be a selective and 1000-times more potent activator of FXR using CV-1 cells in vitro studies. The respective EC50 values of GW4064-activated human and mouse FXR are 90 and 80 nM. An in vitro study using rat liver cells demonstrated that GW4064 treatment induced a 6-fold increased expression of SHP-1 compared to vehicle treatment. There was a 4-fold decrease in the expression of CYP7A1 mRNA in GW4064 treated livers. Results were replicated in human hepatocytes when treated with GW4064 and CDCA (a natural FXR ligand) (Goodwin et al., 2000). SHP-1 leads to inhibition of hepatocyte nuclear factor (HNF4) and/ or liver-related homologue-1 (LRH-1), which are NRs (Lee et al., 2000). These NRs bind to the promoter region of CYP7A1, and their inhibition leads to inhibition of CYP7A1.

FXR causes bile acid efflux into bile by stimulating BSEP (bile salt export pump), phosphatidylcholine by MDR 2-3 (multidrug resistance protein), Cholesterol via ABCG5 / G8 (ATP binding cassette) and glucuronidase- and sulphated-bile acids by MRP-2 (MDR related protein)(Plass et al., 2002). BSEP utilising ATP hydrolysis, is the principal BA efflux pump and is located in the canalicular membrane of hepatocytes. The post-prandial efflux of BAs stimulates the secretion of FGF-19 into the blood from the intestine, to inhibit BA synthesis in the liver. This is supported by the observation that in human serum a diurnal peak in FGF-19 is seen in the afternoon (15:00 hours) and at night (21:00 hours). In cholestasis, studies have reported FGF-19 expression in serum and hepatocytes. Higher levels of serum FGF-19 and FGF-19 mRNA in the liver, with lower CYP7A1 mRNA were seen in cholestasis and reversal of levels was seen in patients who received a biliary stent (Zollner and Trauner, 2009, Tu et al., 2000, Lundasen, 2006). FGF-4 (fibroblast growth factor) in hepatocytes via activation of ERK1-2 pathway inhibits CYP7A1 (Shin et al., 2003, Lefebvre et al., 2009, Zhang and Edwards, 2008). Schaap etal, reported the effect of extrahepatic cholestasis on FGF19. 22

patients with suspected pancreatic or periampullary malignancy with obstructive jaundice underwent peri-operative liver biopsy. Ten patients had pre-operative biliary stenting and ten patients underwent surgery within one week (without stenting). Control liver biopsies were obtained from non-jaundiced patients with pancreatic malignancy (N=10). Plasma FGF19 was found to be higher in the non-drained cholestatic group in comparison to those who were drained (FC=6, p=0.004) and the control group (FC=8, p=0.04). Similarly, FGF19 mRNA levels from liver cells were higher in the cholestatic (non-drained group) compared to those who received stent (FC=31, p>0.001) and control group (FC=374, p>0.001). The mRNA levels of CYP7A1 were lower in cholestatic group compared to stent group (FC= -7.0, p=0.005) and control group (FC= -24, p <0.001)(Schaap et al., 2009).

Intestinal FGF-19 induced by FXR activates FGFR-4. In the mouse genome, there is no FGF-19 gene. The closest gene sharing 50% amino acid identity is FGF-15. In an animal study mice were treated with GW4604, CA and vehicle (Inagaki et al., 2005). RNA was extracted from the liver, small intestine and colon. In GW4604 treated mice no FGF-15 mRNA was seen in the liver cells; however, increased levels were seen in the ileum. Further, increased expression of SHP mRNA was seen in both liver and intestinal cells. BDL mice showed an increase in CYP7A1 mRNA in the liver (FC=3) and reduced FGF15 expression in the ileum. When BDL mice were treated with GW4604, FGF15 mRNA in the ileum increased (FC=50), SHP mRNA in the liver increased (FC=40) and CYP7A1 mRNA decreased in the liver (FC= -10). BAs in the ileum are transported across the basolateral membrane into portal circulation by FXR-mediated stimulation of IBABP (ileum bile acid binding protein), and OSTα/β (organic solute transporter). NTCP (Sodium-dependent taurocholate cotransport peptide), stimulates bile acid reabsorption into hepatocytes from portal blood. In a negative feedback loop, FXR inhibits NTCP. As an adaptive response to cholestasis, FXR induces BA reflux via MRP 3 on hepatocyte and enterocyte sinusoidal membrane(Frankenberg et al., 2006, Denson et al., 2001, Jansen and Sturm, 2003)

2. Pregnane X receptor (PXR):

The Pregnane X receptor (PXR) is a NR. Structurally PXR has the following parts - NH2 terminal ligand independent activation function domain (AF-1), DNA binding domain (DBD), hinge domain, C-terminal ligand binding domain (LBD) and activation function 2 domain (AF-2) (Moore et al., 2003). PXR activation after binding to its ligand results in it translocating to the nucleus from the cytoplasm of the cell. In vitro studies in mouse liver showed that wild-type PXR was mainly expressed in cytoplasm. Treatment with 5-pregnen-3β-ol-20-one-16α-

carbonitrile (PCN), a PXR-specific antibody, resulted in the translocation of PXR to the nucleus and increased CYP3A1 expression (Squires et al., 2004). PXR then forms a heterodimer with RXR and this binds to DNA response elements. PXR plays a key role in phase 1 and 2-xenobiotic metabolism. PXR activation leads to transcription of CYP genes, and upregulation of CYP450 family enzymes which promotes phase 1 detoxification (JM, 1998). PXR modulates phase 2 metabolism by regulation of key enzymes UDP-glucuronosyl transferase (UGT), glutathione S-transferase (GST) and sulfotransferase (SULT) (Sonoda et al., 2002, Bock, 2010, Alnouti and Klaassen, 2008, Knight et al., 2008). Concerning PBC, PXR has its role in solubilisation, detoxification and execration of bile acids. Using mice model Xie etal, demonstrated that PXR mediates the CYP3A gene. LCA is a metabolic substrate for CPY3A hydroxylation and PXR confers resistance to LCA toxicity by inducing CYP3A. PXR null mice demonstrated loss of resistance to LCA and xenobiotic-induced toxicity after PCN administration (P=0.004) (Xie et al., 2001). PXR-induced CYP3A expression is essential for bile acid hydroxylation and excretion. PXR also modulates the effect of multidrug resistance-associated protein 2 (MRP2) and organic anion transport protein 2 (OATP2) which mediate the efflux of conjugated BAs into biliary canaliculi across the apical membrane of hepatocytes (Kast et al., 2002). In vitro study demonstrated that PXR activates transcription of UGT1A1, which facilitates glucuronidation-mediated clearance of bilirubin. VP-hPXR transgenic mice compared to WT mice showed enhanced clearance of conjugated bilirubin after experimental administration of bilirubin (p=0.001) (Xie et al., 2003).

A study assessed the changes in PXR in early (2 weeks) and late-stage (4 weeks) cholestasis in BDL mice. The CYP3A1 and CYP3A2 mRNA were significantly increased in mild cholestasis compared to controls ($p<0.01$). In severe cholestasis, there was a significant drop in levels of CYP3A1-2 compared to controls ($p<0.05$) and mild cholestasis ($p<0.01$). This was parallel with changes in the expression of CYP3A protein levels increasing in early cholestasis ($p<0.01$) and decreasing in late cholestasis ($p<0.05$). Similarly significant increase in PXR mRNA and protein expression was seen in mild cholestasis ($p< 0.001$) which reduced significantly in severe cholestasis compared to controls ($p<0.05$) and mild cholestasis ($p<0.01$) (Gabbia et al., 2017). A study from Zollner etal reported a study in late-stage PBC (N=11, 6 stage III and 5 stage IV) in comparison to no liver disease controls (N=7). In the liver cells CYP7A1 mRNA levels were significantly reduced in late-stage PBC (10%) compared to controls (18%), $p<0.001$). Though statistical significance was not reached, a moderate reduction in mRNA levels of PXR (40–60%) in late-stage PBC was observed (Zollner et al., 2007).

3. Takeda G protein-coupled receptor 5 (TGR5):

Takeda G protein-coupled receptor 5 (TGFR5) is a G-protein coupled receptor (GPCR). TRG5 has been demonstrated to contribute to the pathogenesis of various liver diseases. The secondary BAs: LCA, TLCA, CA, DCA and CDCA activate GPCR, which in turn via adenyl cyclase induces cAMP signalling (Maruyama et al., 2006, Watanabe et al., 2006). The highest levels of TGFR5 are found in the colon. Other than this TGFR5 is found in Hepatic Kupffer cells, brown adipocytes, liver sinusoid epithelium, gall bladder epithelium, kidney, spleen, pancreas, and macrophages (Keitel et al., 2007, Keitel et al., 2009, Keitel et al., 2008).

TGR5 activation leads to an increase in intracellular cAMP which triggers downstream signaling. A study of H69 cells (simian virus 40 transformed human liver cholangiocytes) showed the strongest expression of TGR5 in ciliary membranes (Masyuk et al., 2013). They used TGR5 agonists (TLCA and LCA), selective agonists, (INT-777 and oleanolic acid and UDCA as non-ligand negative control to study the cholangiocyte functional response to ciliary TGR5 signalling. TGR5 agonist-induced opposite effects in ciliated and non-ciliated cells. In ciliated cells coupling of TGR5 to Gαi protein cAMP levels and EKR signally was increased resulting in inhibition of proliferation. In non-ciliated cells coupling of TGR5 to Gαs protein cAMP levels and EKR signally was reduced resulting in activation of proliferation of cholangiocytes.

TGR5 protects cholangiocytes from BA-induced toxicity, via its anti-inflammatory effects. TGR5 stimulation by BAs leads to ligand binding via TGR5 ligand binding to coupling to a Gα(s)(Keitel and Häussinger, 2013). This results in increased intracellular cyclic AMP levels via stimulation of adenylate cyclase. cAMP in turn triggers chloride secretion via the cystic fibrosis transmembrane conductance regulator (CFTR, ABCC7) chloride channel. The anion exchanger 2 (AE2) mediates chloride/bicarbonate exchange across the apical membrane of cholangiocytes(Hohenester et al., 2012). This leads to the formation of a protective bicarbonate film called a bicarbonate umbrella.

TGR5 knockout mice have a smaller and altered BA pool and develop more severe liver injury after Cholic acid (BA) feeding or common bile duct ligation(Reich et al., 2016). LCA diet also resulted in a more severe liver injury in TGR5 knockout mice. TLCA and TGR5 agonists induced cholangiocyte proliferation via reactive oxygen species-mediated epidermal growth factor receptor transactivation and subsequent Erk1/2 phosphorylation in wild type. Therefore, low TGR5 expression by increased susceptibility to BA toxicity led to cholestatic liver injury. TGR5 promotes the integrity of the tight junction and protects from liver injury due to hydrophobic bile acids after partial hepatectomy. In TRG5 knockout mice, higher levels of

hydrophobic BAs and hepatocyte necrosis were seen after partial hepatectomy(Péan et al., 2013, Merlen et al., 2020).

In human CCA cells, TGR5 was overexpressed and associated with activation of TGR5-dependent proliferation via EGFR and ERK1/2(Reich et al., 2016). Apoptosis was inhibited by TGR5-dependent CD95 receptor serine phosphorylation. TGR5 upregulates the expression of the mortalin gene, resulting in downward inhibition of tumour suppressor p53(Li et al., 2020). Therefore, TGR5 activation may promote the proliferation of malignant transformed cholangiocytes.

Hepatic fibrosis (leading to cirrhosis) is a significant stage in the natural history of different liver conditions. Hepatic sinusoidal endothelial cells (HSCs) are activated during liver injury and promote hepatic repair and proliferation of hepatocytes(Greuter and Shah, 2016). Continuous activation of HSCs during various chronic liver conditions leads to fibrosis. Platelet-derived growth factor B (PDGF-B) is a strong activator of HSCs and levels of which are shown to be raised in early fibrosis. PBGF-B binds to its receptor (PDGFR-β) which plays a key role in hepatic fibrosis by HSCs activation(Yoshida et al., 2014, Wang et al., 2016). A study of TGR5-mediated portal pressure regulation showed that PDGFR-β levels were higher in LCA-treated TGR5 knock-out mice(Klindt et al., 2020). It is plausible that TGR5 loss casing BA imbalance may lead to oxidative stress and liver fibrosis. Reduced permeability of limbal epithelial stem cells (LESC) and reduced nitric oxide (NO) production contribute to portal hypertension. TGR5 activates endothelial nitric oxide synthase (eNOS) and induces NO production(Keitel et al., 2007). This results in reduced secretion of ET-1 in LSEC which is a strong vasoconstrictor.

Nuclear factor kappa light-chain enhancer of activated B cells (NF-*k*B) activation has been shown to play a role in the pathogenesis of inflammatory and autoimmune conditions. NF-*k*B in response to LPS and TNK, translocated to the nucleus and activated target genes. TGR5 is a negative regulator of NF-*k*B. TGR5-/- mice showed more severe liver inflammation and necrosis in response to LPS-induced inflammation. The levels of inflammatory serum markers (ALT, AST, IL-1*B*, IL-2, IL-6, IL-10) were significantly higher in TGR5-/- mice compared to WT (Wang et al., 2011).

4. Sphingosin-1-phospahte receptor (S1P):

S1P is a membrane-derived lysophospholipid. S1P acts as extracellular signalling molecules, signalling of which is induced by S1P receptors (1-5). Sphingosine-1-phosphate receptor (S1P)

is a Gαi protein-coupled receptor. S1P2 is expressed in hepatocytes and is stimulated by tauro-conjugated (TDCA, TAC and TUDCA) and glycol-conjugated (GCA and GDCA) bile acids. These conjugated BAs activate ERK1&2 and AKT pathways in a GPCR-dependent manner (Dent et al., 2005). In Vitro, a study using human embryonic kidney cells (HEK293) demonstrated that TCA significantly activated S1P2 but not S1P1,3-5. In rat hepatocytes, TCA induced ERK1/2 and AKT signalling, which was reversed by an S1P2 antagonist (JTE-013). In S1P2-/- knock-out mice significant reduction in activation of ERK1/2 (30%) and AKT pathway (44%) compared to WT mice was observed ($p<0.01$)(Studer et al., 2012). Bile acid activation of S1P2 via ERK1/2 phosphorylation stabilises SHP and leads to inhibition of CYP7A1, a key enzyme in primary bile acid synthesis (Song et al., 2009). S1P/ S1P3 signalling has also been suggested to play a role in cholestasis-induced liver fibrosis. In vitro study in BDL mice model of cholestasis-induced fibrosis showed a marked increase in S1P levels ($FC=2$, $p<0.05$) and S1P3 receptor levels compared to sham-mice. Further, they demonstrated that suramin (a selective $S1P_3$ receptor antagonist) administration markedly reduced BDL-induced liver fibrosis (as measured by hepatic collagen and hydroxyproline content) (Li et al., 2009).

SIP2 signalling stimulates glycogenesis, and inhibits gluconeogenesis and lipolysis, resulting in reduced serum TGs and glucose. Through activation of epidermal growth factor receptors, SIP2 activates insulin receptors and activates IRS-1 (insulin receptor substrate). The end-of-chain result is the activation of a key glycogenesis enzyme, glycogen synthase kinase 3β. By phosphorylation process, AKT also inhibits the transcription factor FOX01. This inhibits 2 of the gluconeogenesis enzymes, glucose-6-phosphatase, and phosphoenolpyruvate carboxykinase. SIP2 also stimulates FXR. This causes inhibition of fatty acid synthesis mediated by steroid response element binding protein 1c and fatty acid oxidation mediated by peroxisome proliferator-activated receptor α/γ (Song et al., 2009, Miao et al., 2009, Puigserver et al., 2003).

1.5 Metabolomics:

1.5.1 Introduction:

Over the last few decades, Metabolomics has become an important tool in the understanding of the nature and development of several diseases in biology(Nicholson et al., 1999, Emwas et al., 2013). The end products of any cellular regulatory process are called metabolites, the levels of which are regulated by environmental and genetic stimuli. This set of metabolites synthesized by a biological system defines its Metabolome (Holmes et al., 2008, Nicholson et al., 1999). By measuring changes in metabolite concentrations in the biofluids the range of biochemical effects of a disease can be determined (Veselkov et al., 2011).

Another term that is used interchangeably with Metabolomics is Metabonomics. Metabonomics is defined as the comprehensive and simultaneous profiling of metabolites and their effective changes resulting from different conditions such as diet, lifestyle, genetic or environmental factors (Nicholson et al., 1999, Fiehn, 2002). Metabonomics is thus the quantitative measurement of the dynamic multipara metric metabolic response of living systems to pathophysiological stimulation or a genetic modification.

These high-profile technologies have improved our understanding of the biopathology and natural history of various disease processes and have the potential to identify therapeutic points of intervention to improve clinical outcomes and patients' quality of life.

Symptoms in PBC as we know can be quite debilitating; metabolomic studies can help to better understand the pathogenesis of disease development and progression; which in turn can inform drug discovery and therapy of this rare disease and related symptoms.

1.5.2 Understanding the basic concept of Metabolomic analysis:

Analytic approaches in Metabolomics:

The two main approaches are targeted and untargeted Metabolomics (Sarmad et al., 2023, Roberts et al., 2012, Schrimpe-Rutledge et al., 2016, Wawrzyniak et al., 2023).

Targeted Metabolomics is the measurement of a specified group of chemically annotated and characterized group of metabolites in a given sample.

Targeted Metabolomic	
Advantages	**Limitations**
1. Detection and precise quantification of known compounds 2. Specific hypothesis with metabolites related to pre-defined one or more pathways.	1. Requires compounds to be known and available in purified form for calibration and analysis, and their limited availability makes this approach currently less viable.

Table 3: Targeted Metabolomics

Untargeted Metabolomics is the measurement of all the measurable analytes (known and unknown) in a given sample.

Untargeted Metabolomics	
Advantages	**Limitations**
1. No fixed hypothesis 2. Possible to measure an unrestricted number of metabolites and compare them between the samples. 3. Metabolites are not precisely quantified. They are measured in relative quantity change.	1. Possible bias toward detection of most abundant metabolite. 2. Instrument and technique-dependent variability

Table 4: Untargeted Metabolomics

1.5.3 Pathway of Metabolomic Analysis:

Metabolomic study analysis consists of the following steps(Smith et al., 2006, Dunn et al., 2011, Veselkov et al., 2011, Emwas et al., 2015, Emwas et al., 2013):

1. **Sample preparation:** Samples are homogenized and pulverized to fine particles to increase the exposed surface area to extract chemical buffer.
2. **Data Acquisition:** Combines different compound separation and detection techniques to generate a data set.
    - *Compound separation techniques*: Gas chromatography, high-performance liquid chromatography, ultra-high-performance liquid chromatography and capillary electrophoresis

- *Compound detection techniques*: Mass spectrometry or nuclear magnetic resonance (NMR)

3. **Data Processing:**
    - Data acquired as raw signals- chromatogram, spectra, or NMR data.
    - Pre-processing the raw data involves noise reduction, retention time correction, peak detection, and chromatogram alignment.
    - Metabolites in Untargeted studies are identified using databases such as the Human Metabolome Database or the Metabolite and Tandem MS Database (METLIN)
    - Data integrity check performed.
    - Data matrices preparation
    - Data normalization
4. **Statistical analysis** is performed in two stages:
    - A combination of various uni- and multi-variate analysis techniques and mining techniques are used to identify metabolites with significant changes and to separate those which are functionally related.
    - Significantly expressed metabolites are ranked using p-values. Cut-off thresholds (arbitrary) are applied to separate top-K ones from the list.
5. **Data Interpretation and Integration:**
    - Enrichment analysis investigates the over / under-expression of defined metabolite groups to define significant and coordinated changes in them to suggest a biological pathway.
    - Pathway analysis involves visualization and description of interactions among genes, proteins, and metabolites.

1.5.4 Nuclear magnetic resonance (NMR) and Mass Spectrometry:

The two most widely used platforms for metabolomic profiling are Nuclear magnetic resonance and Mass spectrometry. NMR spectroscopy uses the spin active nuclei to absorb or re-emit radiation of a certain frequency when placed in a magnetic field which helps study the molecular structure, molecular motion, and chemical environment. MS, on the other hand, is used in conjunction with Liquid Chromatography (LC) or Gas Chromatography (GC) where chromatographic columns are packed with small particles and ultra-high pressure is used for chromatographic separation.

Nuclear Magnetic Resonance

NMR is a highly versatile spectroscopic technique. Alongside its potential to provide structural information, it also presents a considerable potential for quantitative analysis. Hence, its applications in a wide range of disciplines, ranging from chemistry laboratory to human biology and medicine (Hollis, 1963, Lindon et al., 2000, Zhang et al., 2008). 1H-NMR generates untargeted metabolic profiles for the discovery of biomarkers associated with disease risk from study samples.

Understanding the concept of NMR:

Spectroscopy is the study of the interaction of radiation (electromagnetic) with matter. An electromagnetic wave has a magnetic field perpendicular to that electric field defined by wavelength and frequency (Hz) (no of waves per second). The energy of EMV is defined as hμ. When a matter is exposed to EMV matter can respond by scattering the energy, absorbing the energy, or simply letting it through unchanged. The matter does not have continuous energy levels, instead has scattered energy levels called quantized energy. The matter will respond to the EMV if there is a difference in the energy levels inside the molecule for molecules to get shifted from ground to excited state. The various properties of the molecule like structure, rotation, vibration, and electronic state can be studied. Depending upon the wavelength EM spectrum consists of Radio waves (103), Microwaves (10-2), Infrared waves (10-5), Visible spectrum (0.5 X 10-6), Ultraviolet rays (10-8), X-rays (10-10) and Gamma rays (10-12). A specific wavelength of the radiation is chosen to interact with matter based on the property being studied. Any matter is made up of atoms. An atom consists of a central nucleus surrounded by electrons, the nucleus of an atom consists of protons and neutrons collectively called nucleons. Protons are positively charged, and neutrons have no charge. Both protons and neutrons have a spin that is equal and represented as 1/2h. Overall nucleus has a positive charge and has spin associated with it. Protons and neutrons are paired which cancels the spin. Thus, the nucleus after the pairing of all protons and neutrons will have a spin which is called the net spin. The NMR technique relies on the atom having a net spin value that is not zero. When a sample is exposed to a magnetic field the nuclei in the atom align in a ground or excited state. When energy is applied to this sample (usually radio waves), the nuclei move from the ground to an excited state (called as resonance). ***Hence the terminology Nuclear Magnetic Resonance (NMR).*** The frequency in NMR is measured in MHz which is derived from the strength of the magnetic field measured in Tesla (T).

In summary: *a source of radiation is shown on the matter → Matter absorbs some radiations and transmits some → Transmitted radiations are picked up by the detector → This is then analysed and recorded as a spectrum which is a plot of frequencies of radiation which have been absorbed by the matter.*

NMR Spectroscopy
Advantage: • Minimal sample processing required. • Low per-sample cost • Non-targeted analysis • Non-destructive, No chemical extraction or derivatization • High reproducibility, quantitative information, and in-vivo metabolism can be monitored. • ~50 metabolites identified/quantified (μM sensitivity) • Abundant metabolite databases
Limitations: • Low sensitivity • Relative Low throughput (10-15 mins/ sample) • Relative high cost of equipment

Table 5: Advantages and limitations of NMR

Mass Spectrometry

Mass spectrometry methods- with liquid (L-MS) or gas (G-MS) phases are used for targeted detection and quantification of specific biomarkers. Human biological fluids can be studied using both NMR and MS. Metabonomics has been successfully used to identify the biomarkers in a variety of liver diseases, including non-alcoholic fatty liver disease (NAFLD), alcohol-related liver disease, viral hepatitis, cirrhosis, Autoimmune hepatitis, cholangiocarcinoma and hepatocellular carcinoma (HCC) (Beyoğlu and Idle, 2013, Wang et al., 2014a).

The mass spectrometer had four basic components- An inlet system, an ion source, a mass analyser, and a detector. Samples are placed in the inlet source as solids or as a mixture of gas or liquid. Sample molecules are subjected to ionizing radiation. Ions are formed from the molecules without decomposition or fragmentation in the source. These ions acquire kinetic energy and move out of the source. The mass-to-charge ratio (m/z) of these passing ions is measured by a calibrated analyser and recorded as a spectrum. Separation is based on lipid's physicochemical properties. In the positive ion mode protonated and/or alkali adduct analyte molecules are generally observed in the mass spectra. In the negative ion mode operation peaks corresponding to deprotonated analyte molecules are observed.

Liquid chromatography/ MS (LC/MS)-

- Ion suppression can be eliminated as metabolites can elute at different retention time
- Chromatographic peaks provide a more accurate tool for metabolite quantification compared to MS alone.
- Sample derivation is not required
- Metabolites with diverse chemical structures can be measure
- Metabolites with increasing size can be measured. (Metz et al., 2007, Dunn and Ellis, 2005, Buchholz et al., 2001)

Gas chromatography MS (GC/MS)-

- Most suitable for volatile metabolites
- Needs chemical derivation to improve volatility of molecules
- Limited application in terms of size and polarity of the molecules

Mass Spectrometry
Advantage: • High sensitivity • High throughput (1-20 mins/sample) • Targeted analysis • Several hundred metabolites can be measured in a single sample. • Coupled with GC/LC allow a wide number of platforms to enable comprehensive metabolite analysis. • GC-MS: ~70 metabolites identified/quantified (<μM sensitivity) • LC-MS: ~300 metabolites identified/quantified (nM sensitivity)
Limitations: • Substantial sample processing required. • Need for chemical extraction or derivatization. • Incomplete metabolite databases

Table 6: Advantages and limitations of MS

1.6 Metabolomics in PBC- what do we know?

1.6.1 Literature review:

Key points of metabolomic studies in PBC are summarised in Table 7

Author	Study Type	Study details	Assay Type	Analysis Type (software package)	Main Findings
Yang Etal, 2018	Murine Study	30 male mice induced cholestasis by administering alphanaphthyl isothiocyanate (ANIT), 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC), and lithocholic acid (LCA).	1. Ultra-performance liquid chromatography-linked electrospray ionization quadrupole time-of-flight mass spectrometry for plasma samples 2. Haematoxylin and Eosin staining of liver tissue	Unsupervised principal components analysis (PCA) and supervised orthogonal partial least squares discriminant analysis (OPLS-DA) by SIMCA-P (13.0)	• Serum bile acids were increased (TCDCA, TH/UDCA, T-α/β-MCA)- all three models • Serum arginine levels were decreased- all three models • Serum Lysophosphatidylcholine (LPC) and carnitine levels increased- ANIT and DDC models • Liver Protoporphyrin IX increased- DDC model
Wang etal, 2014	Human study	Serum and urinary metabolic profile in PBC (N=41) compared to AIH (N=39), AIH/PBC overlap (N=18), DILI (N=14), and HC (N=23)	1-H NMR spectroscopy	Multivariate Analysis using SIMCA-P (11.0) (PLS-DA)	9 markers were integrated to produce a model with high specificity to discriminate AIH from PBC -citrate, glutamine, acetone, pyruvate, β-hydroxyisobutyrate, acetoacetate, histidine, dimethylamine, and creatine.
Bell etal, 2015	Human study	Serum metabolome of PBC (N=18) compared to PSC (N=21) and HC (N=10)	Mass spectrometry • UHPLC-MS • GC-MS	Statistical analysis of log-transformed data using R-package	1. Raised BA levels in PBC and PSC 2. FFAs (linoleate, linolenate, palmitoleate, and oleate increased in PBC and PSC (PBC > PSC) 3. Ketone bodies acetoacetate, 3-hydroxybutyrate, and acylcarnitines increased in PBC and PSC 4. Amino acids (glutamate, serine, phenylalanine, aspartate, and ornithine) and 9 dipeptides - lower in PSC 5. Three fibrinogen cleavage peptides- higher in PSC
Vignoli etal, 2019	Human study	Serum and urine metabolomes of PBC (N=23), compared to Coeliac disease (N=21) and HC (N=17)	1-H NMR spectroscopy	R- package OPLS-DA	1. Serum pyruvate, citrate, glutamate, glutamine, serine, tyrosine, phenylalanine, and lactate- higher in PBC vs HC 2. Urine hippuric acid and trigonelline- lower in PBC vs HC 3. Serum pyruvate, citrate, phenylalanine, and formate - higher in PBC vs CD 4. Urine pyruvate and p-cresol sulfate- lower in PBC vs CD
Tang etal, 2015	Human study	Serum and urine metabolomes of PBC (N=32), compared to HC (N=32)	Mass spectrometry UPLC/Q-TOF MS	PCA and PLS-DA using SIMCA-P software (Umetrics AB)	1. PBC urine -of the 18 potential biomarkers, 11 increased and 7 decreased 2. PBC serum -of the 20 potential biomarkers, 9 increased and 11 decreased 3. Serum BAs and prostaglandins raised in PBC 4. Serum carnitines decreased in PBC 5. Urine deoxyguanosine increased in PBC

Lian etal, 2015	Human study	Serum and urine metabolomes of PBC (N=20), compared to AIH (N=19) and HC (N=25)	Ultraperformance liquid chromatography-mass spectrometry (UPLC-MS)	Multivariate statistical analysis using SIMCA-P (12.0) PLS-DA	1. GCDCA increased in PBD 2. FFA, LPC, PC, and SM decreased in PBC
Hao etal, 2017	Human study	Serum metabolomes of PBC (N=29), compared to HBV cirrhosis (N=30) HC (N=41)	1H-NMR	Simca-P (11.5) (Umetrics AB) and MetaboAnalyst 3.0	Four metabolites [4-hydroxyproline, 3-hydroxyisovalerate, citraconate, and pyruvate] diagnostic model-AUROC 0.937 (95% CI: 0.868–0.976)
Kim etal, 2019	Human study	Serum and urine metabolomes of HV (N=24) received UDCA 400mg, 800mg or 1200mg for 2 weeks Comparison pre and post UDCA	Mass spectrometry LC/QTOFMS GC/TOFMS	Unsupervised PCA analysis Multivariate and pathway analysis- using MetaboAnalyst 4.0.	Post UDCA therapy (1200mg): 1. Flavonoid compounds (fertaric acid, ferulic acid-sulfate and vanillin)- increased in urine 2. L-threonine, alanine, fumaric acid, lysine and histidine-reduced in urine 3. Glutamyl-phenylalanine-decreased in serum 4. p-hydroxy phenethyl, trans-ferulate, cholesteryl ferulate and cinnamyl alcohol- increased in serum 5. FA (Lyso-PC and PE)- reduced in serum 6. Linoleic acid- increased in serum

Table 7: Summary of PBC Metabolomic studies

Detailed review of the studies:

A murine study (Yang et al., 2018b) investigated the mechanisms and the metabolic changes in cholestatic liver damage. Liver damage was induced in 30 male mice by administering alphanaphthyl isothiocyanate (ANIT), 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC), and lithocholic acid (LCA), 3 classic mouse models for PBC and PSC. The animals were subsequently culled, and serum and liver tissue samples were collected. Liver tissue was stained using Haematoxylin and Eosin (H&E) stain. Plasma metabolites were analysed using ultra-performance liquid chromatography-linked electrospray ionization quadrupole time-of-flight mass spectrometry (UPLC-ESI-QTOFMS). Histologically, H&E staining showed obvious biliary degeneration and necrosis in mice treated with ANIT, Porphyrin plugs blocked bile ducts, ductular proliferation and cholangitis with periductal fibrosis was seen in mice treated with DDC, and necrosis and neutrophilic-granulocytic infiltrate was seen in mice treated with LCA. Several bile acids were increased (TCDCA, TH/UDCA, T-α/β-MCA), and arginine levels were decreased in the plasma of all three mouse cholestasis models compared to controls. The increased bile acid levels are commonly known indicators of cholestatic liver injury. Lipid

metabolism was disrupted in both ANIT and DDC models which caused an increase of Lysophosphatidylcholine (LPC) and carnitine levels, whereas these were depleted in LCA. LCA is an endogenous secondary bile acid that shows significant toxicity in the body and is thought to convert LPC to PC through oxidative stress, depleting LPC levels. Protoporphyrin IX was significantly increased in the liver of the DDC model, which is consistent with the histopathological findings of the DDC model. Increased PPIX has been found to cause liver damage by disturbing endogenous metabolites, which leads to ROS formation and bile duct blockage. The metabolic changes in these three distinct models that cause cholestatic damage via different mechanisms may help to understand the pathophysiology of different types of cholestatic liver damage in PBC and PSC in humans.

Several metabolomic studies compared the serum and urinary metabolic profile in PBC patients compared with other diseases, as well as healthy controls. These studies have identified distinct metabolomic signatures for PBC and showed that these can be used to aid its diagnosis and to differentiate between different causes of liver disease. A study by Wang etal, compared the plasma metabolic profile of 39 patients with AIH, 41 patients with PBC, 18 patients with PBC/AIH overlap syndrome (OS), 14 patients with DILI, and 23 healthy controls (Wang et al., 2014b). They used proton nuclear magnetic resonance spectroscopy coupled with a partial least-squares discriminant analysis to identify significant differences in metabolites between the groups. This study found nine metabolomic biomarkers that were integrated to produce a model with high specificity to discriminate AIH from other diseases including PBC and OS. These metabolites included citrate, glutamine, acetone, pyruvate, β-hydroxyisobutyrate, acetoacetate, histidine, dimethylamine, and creatine. High levels of plasma glucose, pyruvate, lactate, acetate, and acetoacetate in AIH patients can be explained by increased metabolic activity causing an increase in non-aerobic metabolic pathways, a finding that has also been observed in other studies of metabolomics of PBC patients. The higher citrate content in AIH, but not in PBC or healthy controls, points to an involvement of the TCA cycle, which citrate is the starting molecule for. This may point to an increase in aerobic energy metabolism in AIH which does not occur in PBC. Interestingly, this study did not observe changes in bile acid levels in AIH patients that were observed in PBC patients, which fits with our knowledge about the pathophysiology of the latter and confirms that bile acids may be useful biomarkers in differentiating the two conditions. High levels of creatine in AIH compared to PBC also reflect an energy metabolism disorder in the former condition, as creatine would normally be taken up by muscles and phosphorylated to phosphocreatine. Overall, this study comparing the metabolomic profiles in AIH, PBC, and healthy controls shows that metabolomics can be a

useful tool in understanding the similarities and differences in the pathophysiology of these conditions and can be used to differentiate between them.

In another metabolomic study by Bell, the metabolomic signatures of 18 PBC patients, 21 PSC patients, and 10 healthy controls were compared (Bell et al., 2015b). They used mass spectrometry and an untargeted metabolomics approach and identified 420 metabolites of interest. The number of metabolites that differed significantly between PBC and control; PSC and control; and PBC and PSC groups were 101, 115 and 56 respectively ($p \leq 0.05$). Principal component analysis (PCA) demonstrated significant differentiation between the cholestatic diseases and controls but significant overlap between PSC and PBC. Random forest plot classification analysis, with an accuracy of 95%, was able to distinguish between PBC and PSC. Metabolites of lipid metabolism, lipid peroxidation and inflammation; and bile acid metabolism, were found to significantly discriminate between the two disease groups and controls. Based on biochemical changes identified in PBC and PSC patients' serum compared to healthy controls, metabolic pathways that can be linked to the pathophysiology of these cholestatic diseases were explored. Levels of bile acids were elevated in PBC or PSC, including glycocholate, taurocholate, glycochenodeoxycholate, and taurochenodeoxycholate. There was also a greater than 23-fold elevation in glycoursodeoxycholate and tauroursodeoxycholate in the PBC and PSC groups, which was more pronounced in patients on UDCA therapy due to its content of exogenous bile acids. Increased levels of bile acids are characteristic of cholestatic diseases, due to biliary injury resulting in hepatocyte accumulation of bile acids. Elevated cholesterol levels were also found in PBC and PSC, which can be related to reduced excretion of cholesterol, which is mainly done via bile acids. Alterations in lipid metabolism were also observed in PSC or PBC compared to controls, including elevations in free fatty acids linoleate, linolenate, palmitoleate and oleate. The degree of elevation was more pronounced in PBC patients compared to PSC patients. Elevations in ketone bodies [acetoacetate and 3-hydroxybutyrate (BHBA)], and several acylcarnitines were observed. This points towards reduced fatty acid oxidation and ketogenesis, the result of which is an increase in circulating free fatty acids and reduced utilization of ketones. Several metabolic markers of oxidative stress and inflammation were increased in PBC and PSC when compared to controls. These included kynurenines and 12-hydroxyeicosatetraenoic acid (HETE), 13-hydroxyoctadecadienoic acid (HODE) + 9-HODE, free radical scavenger biliverdin, and 7β-hydroxycholesterol. This is in line with the inflammatory pathophysiology underlying PBC and PSC, although differences in the metabolites point to differences in pathways underlying this change. Finally, metabolites related to protein metabolism were altered in PBC and PSC compared to controls. Compared

to PBC levels of several free amino acids (glutamate, serine, phenylalanine, aspartate, and ornithine) and 9 dipeptides were found to be significantly lower in PSC. In PSC compared to PBC there were significant elevations in the levels of three fibrinogen cleavage peptides. PBC observed distinguishingly raised serum levels of 7-α-hydrocholesterol, 7-β-hydroxycholesterol and cortisol was elevated, which may reflect differences in lipid peroxidation and pituitary-adrenal axis in PBC compared to PSC. These represent potential biomarkers for the differentiation of PBC and PSC.

Vignoli et al, conducted a metabolomics study of the serum and urine of 23 PBC patients compared to 17 healthy controls (HC) (Vignoli et al., 2019). They also compared the PBC metabolic profile with 21 coeliac disease patients, an immune-related disorder frequently associated with PBC. The group used H NMR (proton nuclear magnetic resonance), and metabolite NMR signals were profile matched using AMIX 3.8.4 and BBIOREFCODE, freely available databases and published literature when available. Discrimination accuracy between PBC and HC using NMR was 78.9-84.6% (84.6% with CPMG, 84.5% with NOESY and 78.9% with diffusion spectra) and 76.9% for urine. Furthermore, the NMR metabolomic profiling was able to cluster PBC compared to CD patients. The classification accuracies in the discriminations between the two groups were 91.7% for serum and 77.7% for urine.

In the comparison between serum CPMG spectra of PBC and HC, PBC demonstrated altered levels of pyruvate, citrate, glutamate, glutamine, serine, tyrosine, phenylalanine, and lactate compared to HC sera. PBC patient urine showed lower levels of trigonelline and hippuric acid for HC. This shows that PBC patients have a distinct metabolic profile compared to HCs. Tyrosine and phenylalanine levels were increased in PBC compared to HCs. This is thought to be related to the liver's role in amino-acid catabolism, which is disrupted. Tyrosine is a key precursor for the synthesis of certain neurotransmitters (including dopa, dopamine, adrenalin, and noradrenaline), its high serum levels may reflect decreased synthesis of neurotransmitters, which may explain fatigue symptoms in PBC. Increased lactate and pyruvate levels are related to disruption in the mitochondrial respiratory chain, due to decreased aerobic respiration due to AMA autoantibody causing increased anaerobic muscle metabolism. The resultant increased serum lactate may be a contributor to symptoms of peripheral fatigue in PBC. PBC patients had reduced urinary p-cresol sulphate, hippuric acid, and trigonelline. These metabolites are direct by-products of intestinal microbiota. This may suggest alterations in the microbiome of PBC patients, which may play a role in the pathogenesis of PBC, for example via immune implications. PBC patients did not show any significant alterations in lipoprotein lipids and

subclasses, compared to HC. When compared with CD patients, PBC patients demonstrated a distinct metabolic fingerprint as well.

Tang et al., in their study, performed a serum and urine metabolomic study of 32 PBC patients and 32 HCs using a liquid-chromatography mass spectrometry-based technique, followed by PCA to display the metabolomic profile of PBC patients, as well as find models to distinguish PBC from HCs (Tang et al., 2015b). This study identified 20 serum biomarkers, and 18 urine biomarkers, that were predictably different in PBC patients versus HCs, and therefore can be used in models for the diagnosis of PBC. Levels of serum bile acids were increased in PBC patients versus controls, including chenodeoxycholic acid, deoxycholic acid, glycocholic acid, Taurocholic acid, and Tauroursodeoxycholic acid. The levels of these bile acids correlated with the progression of PBC. Levels of carnitines [propionyl and butyryl carnitine] were decreased in PBC patients compared to HCs, and the level of decrease correlated with the progression of PBC. Bile acids are a product of cholesterol metabolism and are excreted by the liver. With biliary hepatocyte injury in PBC, this is reduced, leading to abnormally increased bile acid levels in the blood. Carnitines in turn are known to be involved in long-chain fatty acid metabolism, but their involvement in the pathophysiology of PBC is not understood. Theories proposed include decreased hepatic fatty acid metabolism, as carnitines are involved in their oxidation, which may have pathological consequences on the liver. This study also found increased levels of serum prostaglandin in PBC patients, a well-recognised inflammatory mediator. This is in line with other studies which found increased levels of metabolites involved in prostaglandin production in PBC patients (Hayashi et al., 2001, Chiricolo et al., 1989). Prostaglandin is involved in mediating chronic inflammation, via cytokine signalling, amplifying chemokines and recruiting pro-inflammatory cells. PBC patients also had increased levels of urine deoxyguanosine, oxidatively damaged nucleobase of DNA, compared to HCs. Oxidative stress has been shown to cause cellular senescence, one of the mechanisms underlying the pathogenesis of PBC (Bell et al., 2015b). This study revealed the urine and serum metabolomic profile of a cohort of PBC patients, mainly involving bile acids and carnitines, biomarkers that can be used to formulate a diagnostic model for PBC.

A study by Lian et al. performed a serum metabolomic study using ultraperformance liquid chromatography-mass spectrometry, on 20 patients with PBC, 19 patients with AIH, and 25 HCs to identify the metabolomic profile of each group and develop a diagnostic model for the diagnosis of AIH and PBC (Lian et al., 2015b). Differentiating between AIH and PBC is sometimes clinically challenging due to variable clinical profiles with a degree of overlap, but important as the management of these conditions is very different. Results from the

metabolomic study were analysed using PCA, PLS-DA, and OPLS-DA. The blood metabolic phenotypes generated by AIH and PBC patients had very different profiles. This study identified metabolites of 5 main groups, which, like in other metabolomic studies in PBC, pertain to changes in bile acids and lipid metabolism. In particular, glycochenodeoxycholic acid was significantly increased in the PBC group compared with that in the AIH and the HC groups. With regards to lipid metabolism, this study found decreased serum levels of free fatty acid, lysolecithin, phosphatidylcholine; and sphingomyelin in PBC patients compared to AIH and HCs. The authors generated a PLS-DA model, where the R^2Y and Q^2 values were 0.991 and 0.943, respectively. The area under the ROC curve was 1 with a sensitivity of 100% and a specificity of 100%. This model was externally validated on 12 independent samples, including 4 PBC patients, 4 AIH patients, and 4 AIH-PBC patients. The model accurately diagnosed all PBC and AIH cases and found that the 4 AIH-PBC metabolic profiles were different to the PBC and AIH cluster, indicating that these patients have different metabolic profiles. A simplified model using OPLS-DA also had 100% sensitivity and specificity for diagnosing PBC and AIH.

Hao et al., performed a serum metabolomic study of 29 PBC patients and 41 HC (Hao et al., 2017). They also analysed serum metabolites of 30 patients with Hepatitis B virus-induced cirrhosis. They found and validated a diagnostic model using 4 metabolites that were sensitive and specific in diagnosing PBC and differentiating it from AIH and HBC. The altered metabolites were associated with glucose, amino acid, and fatty acid metabolism. Serum was analysed using high-resolution NMR. Pattern recognition models were used for pairwise comparison of datasets. A pairwise comparison between the PBC, and HBC patients found that 25 metabolites can accurately differentiate between the two groups. A pairwise comparison between the PBC and healthy controls found 33 metabolites that also distinguish these two groups. The principal component analysis (PCA) score plot showed visual discrimination between the HBC patients and healthy controls. The orthogonal partial least squares discriminant analysis (OPLS-DA) model was used to recognise the metabolites. Pathway analysis showed fifteen distinguishing metabolic pathways. These included pathways of taurine, glycine, phenylalanine, pentose phosphate, pyrimidine; serine and threonine; arginine and proline, pyruvate, and citrate; glycerolipid, butanoate, glycolysis or gluconeogenesis, lysine biosynthesis, valine, leucine, and isoleucine biosynthesis; cysteine and methionine, and primary bile acid biosynthesis to be influenced by PBC. A four-metabolite [4-hydroxyproline, 3-hydroxyisovalerate, citraconate, and pyruvate] diagnostic model was developed using Logistic regression and ROC analysis. 4-hydroxyproline is an amino acid involved in stabilising collagen, with its content being correlated with the degree of fibrosis present and increased

serum and urine levels having been associated with fatigue and depression. 3-hydroxyisovalerate is a by-product of ketogenesis, and its increased levels may reflect an increase in non-aerobic metabolic energy pathways in PBC. Citraconate is a compound that is derived from citrate and is a competitive inhibitor of fumarate reduction. Finally, pyruvate is a metabolite that is part of the metabolism of carbohydrates, proteins, and fats. Pyruvate dehydrogenase, an enzyme that catalyses steps in these metabolic pathways where pyruvate is involved, is targeted by AMA. This may contribute to the pathogenesis of PBC. The area under the curve (AUC) of the optimised model involving these 4 metabolites was 0.937 (95% confidence interval (CI): 0.868–0.976). The corresponding sensitivity and specificity were 69.23% and 92.69% respectively. This model was validated using samples collected from different patients (21 PBC, 7 AIH, 9 HBC), and in the validation set, AUC was 0.890 (95% CI:0.743-0.969), with respective sensitivity and specificity of 95.24 and 75.00%. This study revealed metabolites, and corresponding pathways, that may be of significance in the pathogenesis of PBC, and it constructed a model that may have good diagnostic performance for PBC.

Metabolomics can also be a powerful tool in their risk stratification and prognostication abilities. Mindikoglu group studied 34 metabolites that had previously been validated in their prognostic abilities in other liver diseases, in 13 patients with PBC-induced cirrhosis and 26 patients with PSC-induced cirrhosis (Mindikoglu et al., 2019). Plasma metabolites were detected using ultrahigh-performance liquid chromatography/tandem mass spectrometry. Plasma levels of 25 of 33 previously identified metabolites were significantly increased in patients with high compared with low liver and kidney disease severity. The top five plasma metabolites had greater predictive accuracy than the MELD-Na score, myo-inositol, N-acetylputrescine, trans-aconitate, erythronate, and N6-carbamoylthreonyladenosine. Furthermore, several bivariate combinations of the MELD-Na score and plasma metabolites had a greater accuracy in predicting 1-year mortality than the MELD-Na score alone. Combined with the results of their previous study in Hepatorenal dysfunction in 134 patients with cirrhosis of varying aetiology, this study confirmed that there is a degree of metabolic overlap in cirrhosis caused by cholestatic and non-cholestatic liver diseases, with specific differences and similarities needing further mapping and subsequent relating to common and distinct aspects in liver diseases' pathophysiology (Mindikoglu et al., 2018). Importantly, this study shows the future application of metabolomics in increasing the prognostic abilities of scoring systems such as the MELD-Na, which may have valuable clinical applications including the equitable allocation of donor livers.

Metabolomics is also a promising tool in pharmacology, as it aids in studying the mechanisms by which drugs mediate their therapeutic effects. Kim et al. performed a metabolomics study of the serum and urine of 24 healthy males. Participants received UDCA at a dosage of 400 mg, 800 mg, or 1200 mg daily for 2 weeks to investigate the dose-dependent effect of UDCA(Kim et al., 2019). Urinary and plasma global metabolomics analyses were conducted using a liquid chromatography system coupled with quadrupole-time-of-flight mass spectrometry (LC/QTOFMS) and gas chromatography-TOFMS (GC/TOFMS). Unsupervised multivariate analysis (PCA-X) was performed to identify distinguishing markers before and after UDCA treatment. PCA was performed to examine differentiation in overall metabolite profiles between groups. 40 differential metabolites were identified in plasma and urine samples. The relative intensity values of metabolites identified in the urine and serum indicated an alteration in amino acid and fatty acid levels with an increase in phenol-containing products in post-UDCA administered samples. Similar patterns of metabolite alterations were identified in both plasma and urine samples involving four major pathways, amino acid, fatty acid, flavonoid metabolism; and TCA cycle. This study found that several metabolites that may be classified as amino acids were decreased with UDCA therapy. Amino acids are metabolised in the liver, and increased levels reflect decreased metabolic activity in the liver, but also has been suggested to be hepatotoxic. Plasma phospholipid levels, including phosphatidylcholine (PC), lyso-PC, and phosphoethanolamine levels were reduced following UDCA treatment. Their increased levels reflect reduced canalicular flux, which is improved following UDCA therapy. Understanding the metabolites and metabolic pathways associated with hepatoprotection by UDCA may have clinical applications in predicting those PBC patients who may or may not respond to UDCA, and therefore establish non-UDCA responders on alternative therapies early and potentially preventing a degree of PBC progression and liver damage.

1.6.2 Conclusion:

Several metabolomic studies have been conducted on PBC patients, to try and bring light to its pathophysiology, improve the diagnostic accuracy of non-invasive tests, understand the mechanisms of action of mainstream therapies, and improve models of disease risk stratification and prognostication. These individual small studies shed some light on these areas and offer a glimpse of the potential that metabolomics has in furthering our understanding of PBC and improving clinical tools. However, this field of work is still in the early stages and will require considerable further studies to enable significant progress to be made in PBC.

1.7 Study outline:

1.7.1 Study Synopsis:

Study design: Nested Cohort study of PBC patients. Further case-control study, with PBC patients as cases and PSC, AMANL and HV as controls.

Study aim: To better understand the factors underpinning the development of PBC in 'at risk' groups with a view to the future development of preventative and risk stratification strategies; and mechanisms and therapeutic targets.

Study objectives:

1) To collect blood, urine, and stool samples from healthy volunteers, PBC patients, patients with the anti-mitochondrial antibody (specific for PBC) with normal liver blood tests ("pre-PBC") and cholestatic disease control patients with Primary Sclerosing Cholangitis (PSC).

2) To study the samples using metabolic profiling (Metabolomics) to identify novel molecular biomarkers and metabolic pathways underlying disease risk

3) To provide a framework for identifying appropriate preventative, interventional or management programmes at the individual or population level.

Study configuration: Appropriate patients will be identified by the clinical teams in the recruiting centre-Newcastle.

Outcome measures: As this is a non-interventional study, there are no outcome measures.

Sample size estimate: This pilot proof of concept study will recruit up to 170 adult participants.

1.7.2 Inclusion and Exclusion Criterion:

No pre-registration investigations were necessary. If the patient met the diagnostic entry criteria (determined by results of routine clinical diagnostics) recruitment was carried out without any study-specific pre-registration evaluations. ***Healthy controls were not required to provide any 'baseline' blood samples*** to confirm they do not have PBC.

Inclusion criteria for each study group

For healthy volunteers:

1. Age ≥18 years of age

2. Able to give informed consent

3. No known diagnosis of PBC, PSC or other liver disease.

4. No other known medical problems

For PBC patients:

1. Age ≥18 years of age

2. Able to give informed consent

3. Established diagnosis of PBC

Diagnosis of PBC as demonstrated by at least two of the following criteria:
i. presence of anti-mitochondrial antibodies (AMA) or PBC-specific anti-nuclear antibodies at a titre of >1:40.
ii. History of elevated serum alkaline phosphatase (ALP) levels
iii. Liver biopsy consistent with PBC if a biopsy has been performed.

4. No concomitant liver disease

For PSC patients:

1. Age ≥18 years of age

2. Able to give informed consent

3. Established radiological or histological diagnosis of PSC with abnormal liver biochemistry (raised ALP)

4. No concomitant liver disease

For patients with AMA +ve but normal liver function tests:

1. Age ≥18 years of age

2. Able to give informed consent

3. Confirmed positive AMA and normal ALP, in the absence of UDCA therapy.

Exclusion criteria (applicable to all study groups)

1. Age <18 years of age
2. Unable or unwilling to give informed consent.

Withdrawal criteria

Patients recruited will be withdrawn if:

• The patient requests withdrawal from the study

• The patient's diagnosis is changed, placing them outside the inclusion criteria

If a participant wishes to be withdrawn from the study, he/she will contact the investigator at the centre where he/she was recruited. If the participant requests that his/her donated biomaterials and data be removed from the study, the investigator will identify the relevant data and biomaterials and dispose of them appropriately. Identifiable data will not be considered in the analyses. Study documentation, including consent forms and the CRF, will be retained securely.

Consent:

Staff should be GCP trained and be on the delegation log.

Consent procedure:

- Screen the outpatient clinic for all eligible patients. Send out the patient information. This allows the participant more time to read the information.
- Patients can also be recruited on the day in the clinic:
  - Give the participant sufficient time to read over the PIS.
  - Answer any questions or queries about the study.
  - Consent the participant.
- Participants can also be recruited on the wards if they are an inpatient.

1.7.3 Recruitment Flow Chart:

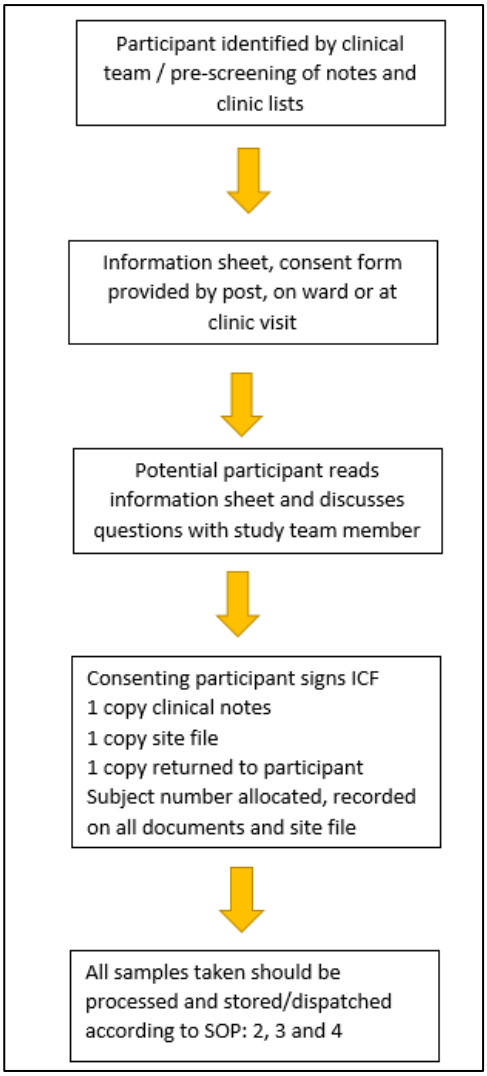


Figure 4: Recruitment flow chart

1.8 Methodology:

1.8.1 Data collection:

Healthy volunteers were asked to:

- Provide demographic details (age, sex, ethnicity, address)
- Provide information on over-the-counter medications, food, and alcohol intake in the three days before sample collection.

  (See Appendix 1)

Patient participants were asked to:

- Provide demographic details (age, sex, ethnicity, address)
- Clinical details of their liver disease or when found to have positive AMA
- Treatment history
- Past medical history
- Provide information on over-the-counter medications, food, and alcohol intake in the three days before sample collection.

  (See Appendix 2)

This data collection was completed in paper form (Case Report Form, CRF). All participant data was collected in coded form to preserve participant confidentiality. The forms were formatted using the FORMIC system so they could be scanned electronically into a spreadsheet.

1.8.2 Collection, storage, and transport of samples:

Sample Collection

This research study involves the collection of blood (serum), urine and stool samples from the participants. During the three days before the sample collection, participants were asked to:

- To maintain a food diary and limit their alcohol intake to a minimum (ideally less than 6 Units/day)
- Avoid paracetamol (if possible), cherries, grapefruit, berries, walnuts, liquorice, and vanilla (as these substances are known to cause interference with metabonomic analyses).

Participants collected a stool sample during the three days before attending the research study visit and brought the sample to the study centre. Blood and urine samples will be collected at the study centre.

The following samples were collected from each study participant:

Blood: A total volume of 12ml of blood was taken. The time of blood collection was noted on the CRF. (See Appendix 3)

Urine: 15-20 ml of urine in a plain additive-free universal container with a screw cap for metabonomic analysis. The urine sample should ideally be the second void of the day. Female participants should not provide a urine sample during the menstrual period. The collection time should be recorded on the CRF. On the day of the research study visit, he or she should pass urine as normal on waking and then, if possible, avoid passing urine again until he/she has arrived at the study centre. (See Appendix 4)

Stool: Single stool sample in a plain additive-free container for metabonomic analysis. The patients were provided with a stool collection kit so that he/she could bring a stool sample to the research study visit. The patient collected a stool sample during the three days leading up to the scheduled research study visit. The stool sample was stored in the patient's freezer and brought frozen to the research study visit using the cooler bag provided. The collection time was recorded on the CRF. (See Appendix 5)

1.8.3 Storage and Transport of Samples:

All samples were stored at -80 degrees Celsius. Samples collected at the Newcastle study centre will be stored at the Newcastle Biomedical Biobank (NBB) which holds a Research Licence (number 12534) and stores collections of human tissues in compliance with the ethical and legal framework of the Human Tissue Act of 2004 (HTA).

All samples were stored in linked anonymised form (linked to stored tissue but donor not identifiable to researchers). Samples were labelled with coded data which may be transferred between collaborating centres for analysis. Personal information (name, date of birth, contact details, NHS, and local identifier numbers) was held separately in secure electronic storage at the recruiting sites (NHS sites). The biobank(s) and the research laboratory at Imperial College London only had the coded samples and no personal information about the donor. Samples were transported on dry ice to Imperial College London for metabonomic and microbiomic analyses which were performed under the supervision of academic consultants. Samples remaining after analysis will be stored in the Imperial College London biobank for use in ethically approved studies in future.

(See Appendix 6)

Processing of samples:

Samples and CRF forms were linked in an anonymised form. All samples were given a unique reference number, recorded in the study registry documents.

Study number allocation:

Allocate patient study numbers using the following three components:

Study initials: MB - followed by a 5-digit code.

The first two digits identify the site at which recruitment took place:

- 01 - Newcastle

The final 3 digits are allocated to participants sequentially within the site.

For example: MB-01-034 will be the 34th patient recruited at Newcastle.

1.8.4 Sample processing methodology:

Using techniques of ^{1}H NMR spectroscopy and ultra-high performance liquid chromatography-mass spectrometry (UHPLC-MS), a total of 107 serum, 107 faecal samples and 105 urine samples from 107 study participants were analysed. Sample preparation, quality control and analysis were done according to the described protocols (Lewis et al., 2016, Sarafian et al., 2015, Dona et al., 2014). In brief, both the MS and NMR assays were used to maximise the capture of a wide range of metabolites including micro- and macromolecular analytes; hydrophilic and lipophilic metabolites; and bile acid species.

- **Mass Spectrometry experiments:**

UHPLC-MS assays for serum and faecal extract analysis were adapted specifically for the separation of lipophilic analytes or the separation of bile acids (including non-conjugated, taurine and glycine conjugated and sulphated compounds) by two different optimised reversed-phase chromatography (RPC) assays. UHPLC-MS assays for urine analysis were adapted for broad small molecule coverage using another RPC assay. These data sets were produced: serum lipid positive (S LPOS), lipid negative (S LNEG), bile acid negative (S BANEG); faecal extract lipid positive and negative, and bile acid negative (FE LPOS, FE LNEG and FE BANEG respectively); urine small molecule positive (U RPOS), and negative (U RNEG) by coupling the MS- assays to positive- and/or negative-mode ionisation.

- **Nuclear Magnetic Resonance experiments:**

With NMR, a standard 1-dimensional (1D) ^{1}H NMR profile analysis was performed using the 1D-nuclear magnetic resonance nuclear Overhauser effect spectroscopy presat pulse sequence with water presaturation for both serum and urine samples. An additional spin-echo analysis was done for serum samples using the 1D Carr-Purcell-Meiboom-Gill (CPMG) pre-sat pulse sequence to better visualise the small metabolite signals.

Data acquisition:

Data was acquired from each assay to produce global profiling (untargeted) and targeted data sets. The two approaches work in a complementary manner. An untargeted data set provides a comprehensive analysis of all measurable metabolites in a given sample and generates multiple variables per analyte. The identities of many of these analytes can be unknown. The targeted extraction, however, works on a predefined set of metabolites. The targeted preannotated data sets are easily interpretable. The method has its limitations in that the metabolites are limited to the predefined set only. Targeted extraction for UHPLC-MS was performed using PeakPantheR (Wolfer et al., 2021) and for NMR was performed using the in vitro diagnostics platform (IVDr) from Bruker Biospin (www.bruker.com).

Details of sample processing methodologies:

1) Mass Spectrometry

A) Serum sample:

Sample sorting:

1. Samples were sorted according to the established analysis order
2. During this period samples remained frozen at -80°C

Sample formatting:

1. Sorted samples were thawed overnight at 4°C
2. 80 sample sets were formatted in 96 (2mL, Eppendorf) deep-well polypropylene plates
3. Columns 11 and 12 were left for the addition of pooled QC samples
4. Silicone cap mats were applied to seal the plates

5. Sealed plates were centrifuged at 4°C for 10mins at 3486×g using Eppendorf 5810R centrifuge; rotor A-2-DWP-AT.

Sample Aliquoting:

1. Centrifuged samples were aliquoted
2. If solid fat particles were observed they were removed using the tip of clean pipette
3. An eight-channel 15-1200µL pipette (Eppendorf Xplorer Plus) was used to aspirate and dispense supernatant to 96-well polypropylene plates
4. Silicone caps were used to seal the plated
5. Sealed plates were stored at -80°C. (Izzi-Engbeaya et al., 2018)

- ***Serum MS Lipid RPC profiling:***

1. Samples were thawed at 4°C for 2h
2. Thawed samples were diluted with H2O (1:1 v/v)
3. For protein precipitation one part of the diluted sample was mixed with four parts of isopropanol containing a mixture of reference standards
4. Plates were heat sealed and mixed at 1400rpm at 4°C for 2h
5. Homogenous supernatant was separated by centrifuging at 3486×g and 4°C for 10mins
6. 125µL of supernatant was aspirated and dispensed into 96-well polypropylene plates
7. Plates were heat sealed, and centrifuged for 5mins at 3486×g at 4°C
8. 2µL of prepared sample was injected in the chromatographic system using full loop mode
9. Lipidomic profiling was conducted using a 2.1 × 100mm BEH C8 column, thermostatted at 55°C
10. Solvent A - 50:25:25 mixture of H2O: ACN: IPA (5mm ammonium acetate, 0.05% acetic acid, and 20µM phosphoric acid)
11. Solvent B - 50:50 ACN: IPA (5mm ammonium acetate and 0.05% acetic acid)
12. A: B (99:1) flow rate set at 0.6mL/min
13. MS parameters for lipid analysis were set as - sample cone voltage 20/25V, capillary voltage 1.5/2kV, source temperature 120°C, desolvation temperature 600°C, desolvation gas flow 1000L/h, and cone gas flow 150L/h.
14. Data was collected in centroid mode with a scan range of 50-2000m/z and a scan time of 0.07-0.15s
15. LockSpray mass correction was performed using a 200pg.µL-1 leucine enkephalin solution (m.z-1 556.2771) at a flow rate of 10µL/min

16. Lockmass scans were collected every 60 and averaged over 3 scans.

- ***MS serum BA (Negative ion mode) profiling:***

1. Pooled study reference samples were prepared using equal parts of study samples and used as quality control
2. A series of study reference sample dilutions were created to the concentrations of 100%, 80%, 60%, 40%, 20%, 10% and 1% by diluting with LC-MS grade water / 2-propanol (1:4 v/v) and used for assessment of linearity of analyte response at the beginning and end of each analysis set.
3. For UHPLC–MS profiling samples were prepared by aliquoting 50 µl of serum onto 96-well plates. 60 µl of LC-MS grade water and 10 µl of internal standard solution, followed by 360 µl of ice-cold LC-MS grade methanol was added to each well.
4. After mixing for 2 mins on a plate mixer at 1400 rpm at 2–8°C), samples were incubated at −20°C for 4 h
5. Samples were then centrifuged at 3486 X g at 4°C for 10 min
6. 125 µl of clear supernatants from each sample were transferred to an analytical 96-well plate
7. The plate was heat sealed and placed in an autosampler at 4°C for the analysis
8. BA profiling was performed using ACQUITY UHPLC–MS (Waters Ltd.) coupled to a Xevo G2-S Q-ToF mass spectrometer with an electrospray ionization source operating in negative ion mode (ESI–; Waters)
9. Mass spectrometry parameters were capillary voltage (1.5 kV), cone voltage (60 V), source temperature (150°C), desolvation temperature (600°C), desolvation gas flow (1000 L/h), and cone gas flow (150 L/h).
10. Data acquisition was done using Masslynx software (Waters). (Lewis et al., 2016, Sarafian et al., 2015)

B) Urine sample:

Sample sorting:

1. Samples were sorted according to the established analysis order
2. The order was preloaded into an in-house laboratory information management system (LIMS)
3. Samples were then sorted into sets of 80 using aluminum racks (9x9 position format)
4. During this period samples remained frozen at -80°C

Sample formatting:

1. Sorted samples were thawed overnight at 4°C
2. 80 sample sets were formatted in 96 (2mL, Eppendorf) deep-well polypropylene plates
3. The sample was checked against a LIMS-generated map of sample identifiers and positions in the 96-well plate
4. Columns 11 and 12 were left for the addition of pooled QC samples
5. Silicone cap mats were applied to seal the plates
6. Sealed plates were centrifuged at 4°C for 10mins at 3486×g using Eppendorf 5810R centrifuge; rotor A-2-DWP-AT for the removal of suspended particulate matter.

Sample Aliquoting:

1. Centrifuged samples were aliquoted
2. An eight-channel 15-1200µL pipette (Eppendorf Xplorer Plus) was used to aspirate and dispense supernatant to 96-well polypropylene plates
3. Silicone caps were used to seal the plated
4. Sealed plates were stored at -80°C.(Lewis et al., 2016)

- ***Urine sample MS profiling:***

1. Samples were thawed at 4°C
2. Samples were diluted with 150 µL of ultrapure water
3. 75 µL of IS solution was added to all samples
4. Plates were heat sealed and mixed at 850 rpm at 4°C for one minute using a MixMate (Eppendorf) and centrifuged for 10 minutes at 3486 × g.
5. The supernatant was split between two analytical microwell plates, one for positive and one for negative ion mode
6. Plates were loaded into a 2777C sample manager (Waters Corp., Milford, MA, USA) and held at 4°C under a constant flow of nitrogen until analysis
7. MS instrument ion source and ion guide settings were- capillary = 1.5/1.0kV (+/-), cone = 20V, source offset = 80V, StepWave 2 offset = 10V, and cone gas flow = 150 L/hr.

2) NMR Spectroscopy:

Sample preparation for NMR spectroscopy of serum and urine. (Dona et al., 2014)

Reagents used:

1. Potassium dihydrogen phosphate [KH_2PO_4], 99.99% ACS, anhydrous (Product code: Sigma-Aldrich 229806)
2. Potassium hydroxide [KOH], ≥85% KOH (Product code: Sigma-Aldrich P1767)
3. Disodium hydrogen phosphate [Na_2HPO_4], 99% anhydrous, (Product code: Sigma-Aldrich W239901)
4. Deuterium oxide [D_2O], 99 atoms % D (Product code: Sigma-Aldrich 435767)
5. Sodium azide [NaN_3], 99.5% (Product code: Sigma-Aldrich S2002)
6. 3-trimethyl-silyl- [2,2,3,3- 2H4] propionic acid [TSP], sodium salt 98 atom % D (Product code: Sigma-Aldrich 269913)

Reagent preparation:

Important consideration- Reagents used are of analytic grade. The volume of the reagents used can vary, however relative ratios of the reagents used must be maintained.

Buffer for urine samples:

1. Prepare 100 mL of 1.5 M KH_2PO_4 buffer by dissolving 20.4 g of the reagent in 80 mL of D_2O.
2. Dissolve 100 mg of TSP and 13 mg of NaN_3 in 6 to 10 mL of D2O.
3. Mix both solutions using sonication.
4. Adjust the pH to 7.4 by adding KOH pellets.
5. Transfer the solution to a 100 mL volumetric flask and adjust the volume with D2O.

Buffer for serum samples:

1. Prepare 500 mL of 0.075 M NaH_2PO_4 buffer by dissolving 5.32 g of NaH_2PO_4 in 380 mL of water.
2. Add 0.4 g of TSP and shake until the powder is dissolved.
3. Add 5 mL of 4% NaN_3 aqueous solution and shake.
4. Add 100 mL of D_2O.
5. Adjust the pH to 7.4 by adding 1M HCl/NaOH solutions.
6. Transfer to a 500 mL volumetric flask and fill up to 500 mL with water.

Gilson robot preparative protocols for calibration of urine and serum samples:

1. Wash the needle by purging the push buffer (pure water).
2. Aspirate gas gap.
3. Aspirate buffer (60uL – urine, 300uL – serum).
4. Dispense buffer in NMR tube.
5. Aspirate gas gap.
6. Aspirate sample (540 uL – urine, 300uL –serum).
7. Dispense the sample in an NMR tube.
8. Following preparation of an entire rack the tubes are sealed and manually mixed.
9. Samples are then set up for analysis.

NMR experimental set-up:

Temperature calibration:

1. A 99.8% deuterated methanol (MeOD) standard sample in a sealed 5 mm NMR tube is used to calibrate the temperature before each run of samples.
2. The temperature calibration is to ensure that urine samples are run at exactly 300 K and serum samples at exactly 310 K.
3. The MeOD NMR tube is inserted into the magnet manually and allows time (~ 5 min) to equilibrate.
4. Following this, the probe is automatically tuned matched and locked to deuterated methanol and automatically shimmed.
5. A standard 90° proton parameter set is used to run an experiment with 2 scans using a pulse length of 1 μs.
6. If required the target temperature of the probe is adjusted and the procedure is repeated until the actual calculated temperature of the sample is 300 ± 0.05 K.
7. The temperature is recorded and later stored in the relevant parameter set for running under automation.
8. The temperature for a urine sample set should be adjusted to 300 K (corresponding to a chemical shift difference of 1.526 ppm)
9. The temperature for a serum sample set should be adjusted for a real temperature of 310 K (corresponding to a shift difference of 1.428 ppm).

Water suppression:

1. A standard 2 mM sucrose sample (containing 0.5 mM TSP, 2 mM NaN_3 in 90% H_2O:10% D_2O) is loaded into the magnet and is used to check the performance of the water suppression functionality.
2. Firstly, the centre frequency (O1) is optimized by using a 1D NMR experiment with presaturation, a long relaxation delay and 1 scan.
3. Then water suppression performance is evaluated by acquiring a full cycle (8 scans) experiment and relatively long delays (~ 10 s).

The signal-to-noise value must be higher than 300 (as measured on the anomeric proton of sucrose), the resolution better than 15 % (a measure of the height of the minimum of the anomeric proton as a percentage of the entire peaks signal) and the water hump not bigger than 40/80 Hz (as measured at 100 % and 50 % of the TSP signal intensity respectively).

Quality Assurance:

Any spectrum run under automation that does not fit the criteria described below should be either rerun immediately or alternately removed from a dataset before biological biomarker discovery can proceed.

1. **Line width:** The homogeneity of the magnetic field during urine spectrum acquisition should be assessed using the TSP resonance. The resulting signal should be symmetrical and at half height, the line width should be < 1 Hz after a line broadening of 0.3 Hz has been applied to the data.
2. **Baseline:** The spectral noise (from a line through zero relative intensity) should have no residual trend outside the -0.5 – 10 ppm range for urine and outside the -5 – 15 ppm range for serum.
3. **Water Peak Saturation:** The residual water resonances should have an area consistent with a concentration of less than 10 mmol (pure water is 110M in hydrogen). A urine NMR spectrum should not be affected by the residual water resonance outside of the range of 4.7 – 4.9 ppm. As the serum is run at 310 K the residual water resonance shifts to a higher ppm value but this should not affect the resulting spectrum outside of 4.6 – 4.8 ppm.
4. **Phase Error:** Checking the zero-order phase manually (after automatic processing) around the TSP signal, there should be less than a 0.2° distortion in the value

A) <u>Serum NMR Profiling:</u>

1. Serum samples were centrifuged at 12000g at 4°C for 5 mins
2. 350 µl of sample was added to 96-well plates
3. 300 µl of plasma was loaded into 4'' 5 mm NMR tubes and mixed with 300 µl of H2O: D2O buffer
4. Sample preparation was done using 215 Gilson liquid handling robot
5. Samples were loaded onto a refrigerated Sample-Jet robot (Bruker Corporation, Germany) and kept at 6 C
6. 1D NMR profiling was acquired using the 1D-NOESY presat pulse sequence, a spin-echo experiment using the 1D-CPMG presat pulse sequence and J-res 2D experiments were run in automation at 310 K in a Bruker Avance III HD 600 spectrometer working at 14.1 T equipped with a BBI probe.
7. Processing of the spectra was done in automation using Top-Spin 3.6 (Bruker Corporation, Germany).
8. For targeted NMR analyses, quantification of 41 metabolites and 112 lipoprotein parameters was performed by an in vitro diagnostics platform (IVDr) from Bruker Biospin (www.bruker.com).(Jiménez et al., 2018)

B) <u>Urine NMR profiling:</u>

1. Urine samples were centrifuged at 12000g at 4°C for 5 mins
2. 600 µl of sample was added to 96-well plates
3. 540 µl of plasma was loaded into 4'' 5 mm NMR tubes and mixed with 60 µl of D2O buffer
4. Sample preparation was done using 215 Gilson liquid handling robot
5. Samples were loaded onto a refrigerated Sample-Jet robot (Bruker Corporation, Germany) and kept at 6 C
6. 1D NMR profiling was acquired using the 1D-NOESY presat pulse sequence and J-res 2D experiments were run in automation at 300 K in a Bruker Avance III HD 600 spectrometer working at 14.1 T equipped with a BBI probe.
7. Processing of the spectra was done in automation using Top-Spin 3.6 (Bruker Corporation, Germany).
8. For targeted NMR analyses, quantification of 50 metabolites was performed by in vitro diagnostics platform (IVDr) from Bruker Biospin (www.bruker.com).(Jiménez et al., 2018)

3) Faecal Bile Acid analysis:

Faecal extraction:

1. Faecal samples were lyophilized for 24 hours using a VirTis Benchtop BTP 8ZL freeze dryer (BPS, UK).
2. Skirted 2mL microtubes suitable for bead beating were filled with 0.5mm glass or Zirconia beads (0.5/0.7/1/1.4 mm in diameter
3. Weight of each bead-filled tube as recorded
4. Bead-containing tubes were filled with 5-15mg of freeze-dried sample material and weighed again
5. Bile acids were extracted using a 2:1:1 (vol) mixture of water, acetonitrile and 2-propanol (10 mg/1 mL dried sample: solvent)
6. 1ml of the extraction solution was added to per 10mg of freeze-dried stool sample and tubes placed in biospec bead beater
7. The bead beating cycle was 3x30 sec with 15 sec pauses in between each
8. Microtubes were centrifuged for 10 minutes at 22,000 x g
9. 500µl of the supernatant was placed in a micro-centrifuge filter (0.22um PTFE, Costar) and centrifuged for 10 minutes at 22,000 x g
10. Filtrated was stored at -80ºC for analysis. (Mullish et al., 2018)

Faecal Bile acid profiling:

1. Pooled study reference samples were prepared using equal parts of faecal filtrates and used as quality control
2. A series of study reference sample dilutions were created to the concentrations of 100%, 80%, 60%, 40%, 20%, 10% and 1% by diluting with LC-MS grade water / 2-propanol (1:4 v/v) and used for assessment of linearity of analyte response at the beginning and end of each analysis set
3. For UHPLC–MS profiling samples were prepared by aliquoting 75 µl of filtered faecal extracts onto 96-well plates. 75 µl of LC-MS grade water and 75 µl of internal standard solution, followed by 75 µl of ice-cold LC-MS grade methanol was added to each well.
4. After mixing for 2 mins on a plate mixer at 1400 rpm at 2–8°C), samples were incubated at −20°C for 4 h

5. Samples were then centrifuged at 3486 X g at 4°C for 10 min
6. 125 µl of clear supernatants from each sample were transferred to an analytical 96-well plate
7. The plate was heat sealed and placed in an autosampler at 4°C for the analysis
8. BA profiling was performed using ACQUITY UHPLC–MS (Waters Ltd.) coupled to a Xevo G2-S Q-ToF mass spectrometer with an electrospray ionization source operating in negative ion mode (ESI–; Waters)
9. Mass spectrometry parameters were capillary voltage (1.5 kV), cone voltage (60 V), source temperature (150°C), desolvation temperature (600°C), desolvation gas flow (1000 L/h), and cone gas flow (150 L/h).
10. Data acquisition was done using Masslynx software (Waters).
11. Targeted extraction of metabolites was performed using the PeakPantheR package. (Lewis et al., 2016, Sarafian et al., 2015)(Wolfer et al., 2021)

LIPIDOMICS:

Lipidomics is the study of lipids present in biological samples. Given the close association of lipids with diseases related to metabolic syndrome, it plays a key role in mechanistic studies, risk prediction, and therapeutic monitoring. Lipidomics can identify lipid markers of disease and help understand the biochemical mechanisms of lipids. Additionally, it aids in diagnosing disease phenotypes by providing insights into the lipid composition of cells, tissues, and biofluids. Abnormal lipid metabolism is frequently observed in patients with primary biliary cholangitis (PBC). Limited studies have revealed lipid profiles in the plasma of patients with PBC through untargeted lipidomics. Glycomics, on the other hand, is the study of glycans. Glycomics can have several disadvantages. Glycomics analysis often loses information about the protein carriers of glycans. Glycomics analysis techniques are generally low throughput. Glycomics analysis is only applicable to small sample amounts.

1.8.5 Analysis:

Using multivariate data analysis software, SIMCA-16 (Sartorius), **Principal Component Analysis (PCA)** was performed for NMR and MS data to visualise inherent clustering and outliers. **Orthogonal projection to latent structure (OPLS-DA)** was performed to maximise class differentiation and minimise class-unrelated variability. R^2 gives the goodness of fit of the model. A cross-validated Q^2 gives a quantitative measure of the predictability of the model. Q^2

>0.5 is generally accepted as a good model. The cross-validated analysis of variance (CV-ANOVA) corresponds to a null hypothesis of equal predictive residuals between comparative models. A P-value of <0.05 was considered significant, suggesting the model is superior compared to those chosen at random. Pareto-scaling was applied to metabolic variables before analysis. S-plot loadings were used to determine metabolites contributing to class separation. Variable importance in projection (VIP) score estimates the influence of individual variables on the OPLS model. A higher the value of VIP score indicates a greater contribution to group separation. A VIP score of >/= 1.0 is considered significant and was included in the final analysis. For each PCA component (with VIP >1) one-way ANOVA was performed on scores comparing the two study groups using graph pad Prism software 9.0 (© 2023 GraphPad Software). Mann- Whitney test was used to compare the two groups and p-value <0.05 was taken to be significant.

1.8.6 Summary of raw data output:

Urine NRM profiling:

A total of 107 study samples (one excluded due to width-line failure) and 4 reference samples were analysed. Untargeted analysis obtained 18910 raw features. Targeted metabolomics analysis identified 49 qualifying features. All the samples were detected in one batch.

Serum MS Lipid Metabolic Profiling:

Data was acquired in two separate modes -positive ion (LPOS) and negative ion (LNEG).

- **LPOS mode:** A total of 105 study samples (one excluded due to width-line failure) and 10 reference samples were analysed. Untargeted analysis obtained 1800 raw features. Targeted metabolomics analysis identified 289 qualifying features. All the samples were detected in one batch.
- **LNEG mode:** A total of 105 study samples (one excluded due to width-line failure) and 11 reference samples were analysed. Untargeted analysis obtained 605 raw features. Targeted metabolomics analysis identified 52 qualifying features. All the samples were detected in one batch.

Serum MS Bile Acid Metabolic Profiling:

A total of 87 study samples and 11 reference samples were analysed. Untargeted analysis obtained 223 raw features. Targeted metabolomics analysis identified 39 qualifying features. All the samples were detected in one batch.

Contributions:

I did participant recruitment (screening and consent), bio-fluid sample collection, sample processing and cold storage, demographic and clinical data collection and recording, and analysis of raw data using SIMCA software.

Sample analysis and raw data generation were done in collaboration with Imperial College London (Dr Takis Panteleimon and Caroline Sands).

Chapter 2: Baseline Characteristics of the Study Population

2.1 Demographics of study population:

PBC (N=51)

51 patients with PBC with a median age of 64 years were included in the final analysis. 49 (96.1%) patients were on UDCA. 2 patients were not on UDCA therapy due to the issue of intolerance. The daily dose [median (range)] of UDCA was 1000 (500-1500) mg. Weight corrected (per kg) daily dose of UDCA [median (range)] was 13.8 (7-16) mg/kg/day. 41/49 (84%) were UDCA responders as per Toronto criteria (reduction in alkaline phosphatase to less than or equal to 1.67 times the upper normal limit), and 8/49 (16%) were non-responders. In terms of symptoms 16/51 (31.4%), 19/51 (37.3%) and 8/51 (15.7%) had pruritus, fatigue, and cognitive impairment respectively. 40 patients were AMA positive (Figure 5). 16 patients were ANA positive. Of these 8 were positive for both AMA and ANA.

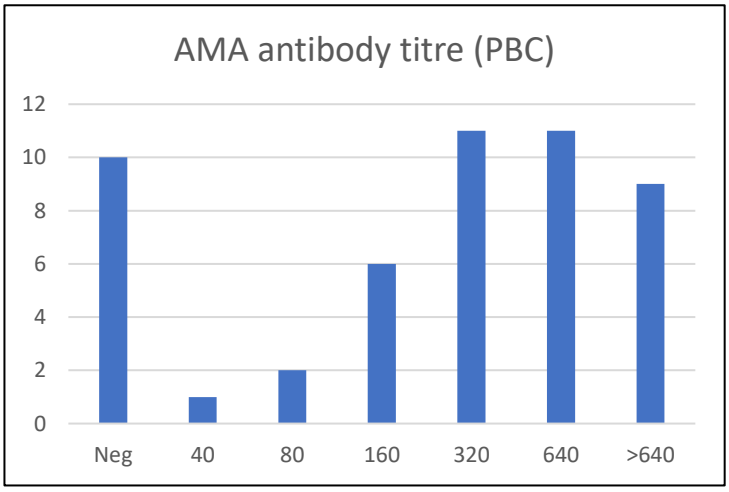


Figure 5: AMA titres in PBC patients

(N=51), X-axis: AMA titer and Y-axis: no of patients in each group.

- ***PBC with Pruritus:***

16 patients with PBC who had pruritus as one of their symptoms, with a median age of 59 years were included in the final analysis. Patient-reported pruritus as documented by the physician in medical notes, was included in this group. Pruritus was attributed to PBC after ruling out alternative medical conditions or medication used which could have contributed. No formal

tools were used to assess the severity. Based on history (occasional/ constant itch), and effect on the quality of life (e.g. interference with nighttime sleep and/or resulting in daytime somnolence), it was judged to be mild -severe by the physician. Most patients were on or had received bile acid sequestrants: Questran (Cholestyramine) and/ or Colesevalam for itch. In this group, seven patients were on rifampicin and 2 patients were on naltrexone for pruritus, reflecting a moderate to severe itch. One patient went on to have a liver transplant for intractable pruritus. 15 (93.8%) patients were on UDCA. The daily dose [median (range)] of UDCA was 1000 (500-1500) mg. Weight corrected (per kg) daily dose of UDCA [median (range)] was 14.4 (5-16) mg/kg/day. 11/15 (73%) were UDCA responders as per Toronto criterion, and 4/15 (27%) were non-responders. 10 (62.5%) and 2 (12.5%) respectively had fatigue and cognitive impairment as an additional symptom. 8 patients were AMA positive and 4 were ANA positive.

- ***PBC with Fatigue:***

19 patients with PBC had fatigue as one of their symptoms, with a median age of 64 years were included in the final analysis. Patient-reported pruritus as documented physician in medical notes, was included in this group. Fatigue was attributed to PBC after ruling out alternative medical conditions or medication used which could have contributed. Patients were assessed and corrected (where applicable) for treatable causes, namely Vitamin B12/ D deficiency and hypothyroidism. No formal tools were used to assess the severity. 18 (94.7%) patients were on UDCA. The daily dose [median (range)] of UDCA was 1000 (500-1500) mg. Weight corrected (per kg) daily dose of UDCA [median (range)] was 13.5 (5-16) mg/kg/day. 16/18 (89%) were UDCA responders as per Toronto criterion, and 2/18 (11%) were non-responders. 10 (52.6%) and 3 (15.8%) respectively had pruritus and cognitive impairment as an additional symptom. 15 patients were AMA positive and 17 were ANA positive. Three patients received modafinil for their fatigue. Three patients received experimental Rituximab (a monoclonal B-cell antibody) for treatment of their fatigue as part of a randomised placebo-controlled “RITPBC” trial, which was before recruitment in our study.

- ***PBC with Cognitive Impairment:***

8 patients with PBC had cognitive impairment as one of their symptoms, with a median age of 61 years were included in the final analysis. Patient-reported pruritus as documented physician in medical notes, was included in this group. Cognitive impairment was attributed to PBC after ruling out alternative medical conditions or medication use, hepatic encephalopathy and

structural brain lesion (negative CT/ MRI) which could have contributed. No formal tools were used to assess the severity. 8 (100%) patients were on UDCA. The daily dose [median (range)] of UDCA was 1000 (500-1500) mg. Weight corrected (per kg) daily dose of UDCA [median (range)] was 14 (12-15) mg/kg/day. 8/8 (100%) were UDCA responders as per the Toronto criterion. 6 patients were AMA positive and 7 were ANA positive.

PSC (N=21): 21 patients with PSC with a median age of 52 years were included in the final analysis. 20 (95.2%) patients were on UDCA. The daily dose [median (range)] of UDCA was 1000 (500-1500) mg. Weight corrected (per kg) daily dose of UDCA [median (range)] was 14 (12-16) mg/kg/day. 14/21 (66.7%) had IBD.

AMANL (N=15): 15 patients with positive AMA and normal LFTs with a median age of 57 years were included in the final analysis.

Age and liver chemistry is summarised in (Table 8). Lipid chemistry, INR and MELD is summarised in (Table 9) [all values are given in median (range)].

Group	Sex F/M	Age	Bilirubin	ALP	ALT	GGT	AST	Albumin
PBC (N=51)	48F/3M	64 (43-80)	7 (3-22) *n=50*	109 (36-602) *n=50*	26 (9-207) *n=50*	54 (14-557) *n=33*	29 (16-132) *n=21*	45 (35-50) *n=50*
PBC Pruritus (N=16)	15F/1M	59 (43-77)	7 (3-22) *n=15*	114 (36-602) *n=15*	26 (10-207) *n=15*	59 (22-557) *n=9*	36 (16-132) *n=8*	46 (36-50) *n=15*
PBC Fatigue (N=19)	6F/2M	64 (45-79)	7 (4-22) *n=19*	93 (36-408) *n=19*	19 (10-103) *n=19*	37 (22-557) *n=12*	29 (16-65) *n=9*	46 (41-49) *n=19*
PBC COG-I (N=8)	18F/1M	61 (51-72)	8 (6-9) *n=8*	100 (58-387) *n=8*	28 (11-81) *n=8*	34 (17-557) *n=6*	32 (22-54) *n=3*	45 (43-49) *n=8*
PSC (N=21)	7F/14M	52 (20-78)	12 (4-51) *n=21*	302 (61-707) *n=21*	48 (11-192) *n=21*	240 (10-676) *n=18*	31 (13-198) *n=21*	43 (28-50) *n=21*
AMANL (N=15)	14F/1M	57 (36-78)	7 (3-18) *n=15*	79 (31-123) *n=15*	14 (9-42) *n=15*		25 (12-30) *n=7*	44 (36-49) *n=15*
HV (N=20)	11F/9M	56 (27-79)						

Table 8: Summary of demographics and liver function tests in study groups

(Bilirubin, ALP-Alkaline phosphatase, ALT-Alanine aminotransferase, GGT-Gamma-glutamyl transferase, AST-Aspartate aminotransferase and albumin [median + range]) in PBC- Primary biliary cholangitis, PSC-Primary sclerosing cholangitis, AMANL-Antimitochondrial antibody positive with normal liver tests and HVs-Healthy volunteers.

Group	Sex F/M	Age	Chol	HDL	LDL	Non-HDL	INR	MELD
PBC (N=51)	48F/3M	64 (43-80)	5.3 (3.3-10.2) *n=32*	1.45 (0.7-3.4) *n=28*	1.75 (0.9-2.7) *n=28*	3.2 (1.7-8.2) *n=29*	1 (0.9-1.2) *n=50*	6 (5-13) *n=50*
PBC Pruritus (N=16)	15F/1M	59 (43-77)	5.5 (3.9-10.2) *n=7*	1.6 (1.0-2.4) *n=6*	2.0 (0.9-2.7) *n=15*	3.2 (2.8-8.2) *n=9*	1.0 (0.9-1.2) *n=15*	6.0 (5.0-8.0) *n=15*
PBC Fatigue (N=19)	6F/2M	64 (45-79)	5.6 (4.2-10.2) *n=12*	1.5 (0.8-3.4) *n=10*	1.85 (1.1-2.7) *n=10*	3.4 (2.4-8.2) *n=12*	1.0 (0.9-1.2) *n=19*	6.0 *(5-10)* *n=19*
PBC COG-I (N=8)	18F/1M	61 (51-72)	5.6 (4.2-10.2) *n=6*	*1.15* (1.0-2.0) *n=4*	2.3 (1.0-3.0) *n=4*	4.05 (3.0-8.0) *n=4*	0.9 *(0.9-1.2)* *n=8*	6.0 (6.0-8.0) *n=8*
PSC (N=21)	7F/14M	52 (20-78)	4.95 (3.4-7.1) *n=14*	1.0 (0.3-5.4) *n=13*	1.9 (0.9-3.2) *n=13*	3.1 (1.8-5.2) *n=18*	1..0 (0.9-1.2) *n=21*	6 (6-11) *n=21*
AMANL (N=15)	14F/1M	57 (36-78)	4.8 (2.8-6.7) *n=13*	1.25 (1-4) *n=12*	1.25 (1-2) *n=12*	3.4 (2-6) *n=12*	1.0 (0.9-1.1) *n=15*	6 (6-11) *n=15*
HV (N=20)	11F/9M	56 (27-79)						

Table 9: Summary of Lipid profile, INR and MELD score in study groups

(Cholesterol, HDL-High density lipoproteins, LDL-Low density Lipoproteins, and non-HDL), INR-International normalised ratio, and MELD-Model of end-stage liver disease score [median + range], in PBC- Primary biliary cholangitis, PSC-Primary sclerosing cholangitis, AMANL-Antimitochondrial antibody positive with normal liver tests and HVs-Healthy volunteers.

2.2 Comparison of Metabolomic Profile of Primary Biliary Cholangitis and Healthy Volunteers

2.2.1 Results of analysis:

The final analysis included a total of 51 patients with PBC and 20 HVs. There was a statistically significant difference in the median age (64 years for PBC and 56 years for HV) between the two groups (p=0.02). Patients with PBC were relatively older as compared to HVs. PCA comparison followed by OPLS-DA gave a robust differentiating model, which was subsequently cross-validated with multivariate CV-ANOVA analysis. Significant discrimination, as depicted by p-value <0.05, was seen in the two groups on serum BAs and lipids, and faecal BAs metabolites, an overview of which is presented in (Table 10). The resonances **increased in serum BAs of PBC patients** were metabolites of UDCA; and secondary bile acid LCA and Murocholic acid. The resonances **increased in serum lipids of PBC patients** included metabolites of Lysophospholipid, Phosphatidylcholine, Sphingomyelin, and fatty acids. The resonances **increased in faecal BAs of PBC patients** were UDCA, Lithocholic and DCA metabolites.

Overview of Analysis:

		A	R^2X	Q^2	p-value CV-ANOVA
Serum	MS Bile acid (NEG)	2	0.621	0.522	3.61032e-05
	MS Lipid (NEG)	4	0.735	0.565	0.003
	MS Lipid (POS)	6	0.673	0.499	0.001
	NMR BiLISA	3	0.758	0.679	0.01
Urine	NMR QUANT	2	0.719	0.254	0.54
	MS (NEG)	3	0.46	0.259	0.37
	MS (POS)	2	0.491	0.399	0.52
Faecal	MS BA (NEG)	5	0.712	0.495	8.01948e-06

Table 10: Summary of PCA analysis in PBC vs HV

PCA-X coordinates [A= no of principal components with R^2X and Q^2 values] and corresponding multivariate analysis CV-ANOVA p-values for serum, urine, and faecal assays of PBC patients compared with HVs. R^2 predicts goodness of fit and Q^2 is goodness of prediction of the model. Significant p-values (<0.05) are highlighted in green.

Serum Analysis comparing PBC patients with HVs

- **Serum Bile Acid (MS-Negative ion mode) PBC vs HV**

A total of 40 patients with PBC and 16 HVs were included in the final analysis. A 2-component PCA model (R^2X=0.621, Q^2=0.522, good model) attained visual discrimination between patients with PBC and HV in serum bile acids on mass spectrometry. This was confirmed subsequently on a 2-component OPLS DA model (Table11), with the following statistics: (1+1+0), R^2X=0.528, R^2Y=0.595, Q^2=0.40, CV- ANOVA p=3.61032e-05. Permutation testing demonstrated that the model was valid in that the cut-offs for R^2 and Q^2 were lower in the randomly permutated model (999 permutations) (Figure 6). Metabolites with VIP > 1 included Glycoursodeoxycholic Acid-3-Sulfate, Glycoursodeoxycholic Acid, Tauroursodeoxycholic Acid, Ursodeoxycholic Acid, Ursodeoxycholic Acid-3-Sulfate, Murocholic Acid, 3-alpha-Hydroxy-7 Ketolithocholic Acid and Glycodeoxycholic Acid (Table 12). Resonance **increased in PBC patients** included metabolites of UDCA (Glycoursodeoxycholic Acid-3-Sulfate, Glycoursodeoxycholic Acid, Tauroursodeoxycholic Acid, Ursodeoxycholic Acid, Ursodeoxycholic Acid-3-Sulfate) and secondary bile acid- Muricholic acid (Table 13).

				CV-ANOVA p-value
PCA- X Coordinates	A	R^2X	Q^2	
	2	0.621	0.522	3.61032e-05

OPLS-DA	A	R^2X	R^2Y	Q^2
Coordinates	1+1+0	0.528	0.595	0.40

Table 11: PCA and OPLS-DA coordinates of PBC (n=40) vs HV (n=16) (Serum bile acids, MS-negative ion mode).

A= no of principal components, R^2 predicts goodness of fit and Q^2 is goodness of prediction of the model. Significant p-values (<0.05)]

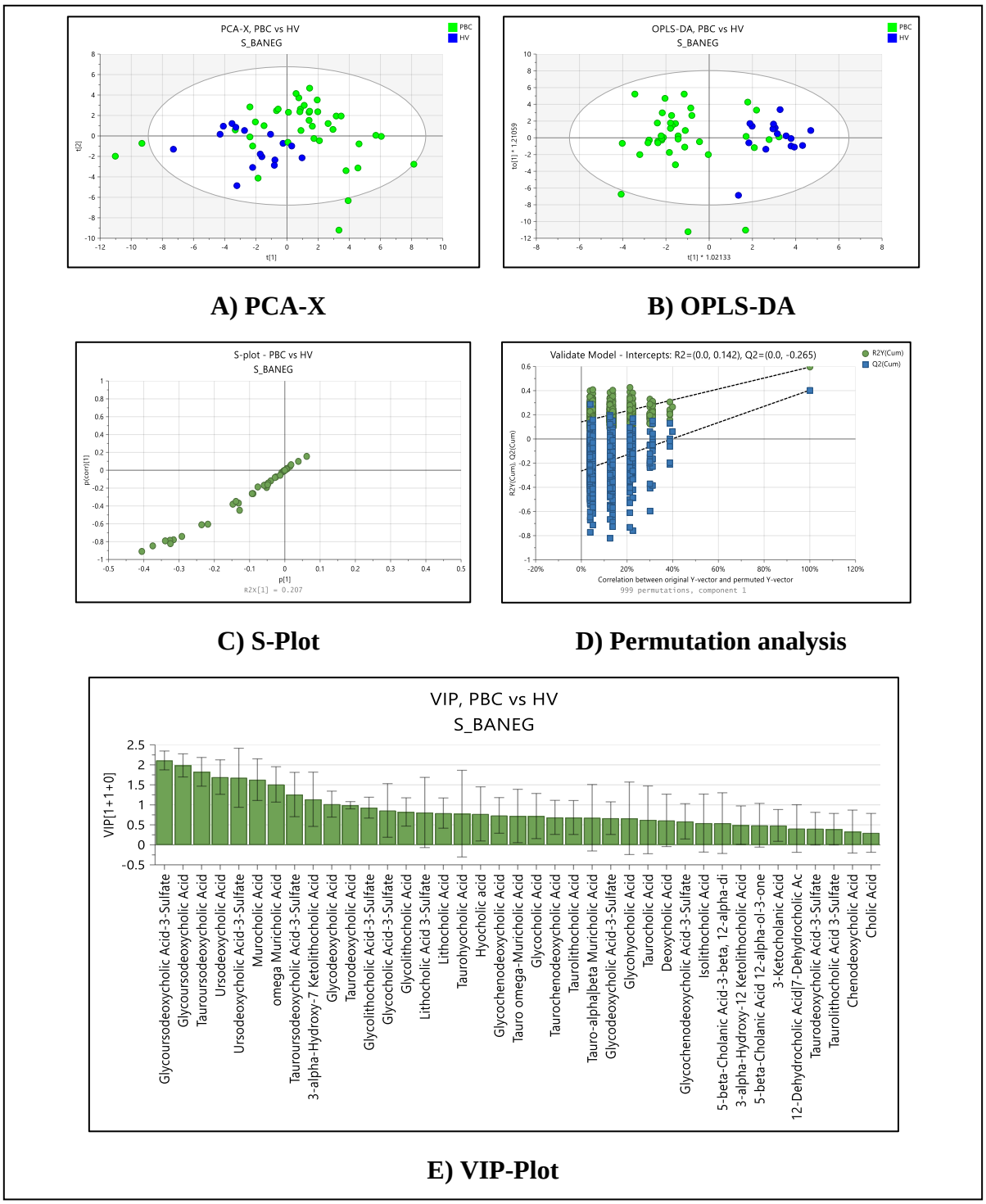


Figure 6: Serum Bile acid profile model PBC (n=40) vs HV (n=16)

These multivariate models demonstrate discrimination of patients with PBC and HV. (A) Principal components analysis (PCA) scores plot [2-component model $R^2X=0.621$, $Q^2=0.522$]. (B) Orthogonal projection least squares discriminant analysis (OPLS-DA) scores plot [(1+1+0), $R^2X=0.528$, $R^2Y=0.595$, $Q^2=0.40$]. (C) S-line loading plot (D) Permutation analysis (999 permutations) (E) VIP-scores plot

Metabolites with a VIP score of ≥ 1 were used to predict their influence on class separation between the two study groups (Table 12):

Glycoursodeoxycholic Acid-3-Sulfate	2.11
Glycoursodeoxycholic Acid	1.98
Tauroursodeoxycholic Acid	1.82
Ursodeoxycholic Acid	1.69
Ursodeoxycholic Acid-3-Sulfate	1.67
Murocholic Acid	1.62
3-alpha-Hydroxy-7 Ketolithocholic Acid	1.13
Glycodeoxycholic Acid	1.01

Table 12: Serum Bile Acid metabolites with VIP >1 PBC (n=40) vs HV (n=16).

	PBC (median + IQR)	HV (median + IQR)	p-value Mann-Whitney
Glycoursodeoxycholic Acid-3-Sulfate	178530 (30082 – 332558)	1674 (1055 – 2649)	<0.0001
Glycoursodeoxycholic Acid	475315 (139011 – 1166084)	6692 (1353 – 16304)	<0.0001
Tauroursodeoxycholic Acid	10517 (3640 – 45737)	177.4 (0.000 - 829.8)	<0.0001
Ursodeoxycholic Acid	118301 (17172 – 427461)	3191 (1666 – 6021)	<0.0001
Ursodeoxycholic Acid-3-Sulfate	17308 (5114 – 44957)	747.4 (288.6 – 2360)	<0.0001
Murocholic Acid	62290 (16513 – 179113)	2203 (1084 – 7924)	<0.0001
3-alpha-Hydroxy-7 Ketolithocholic Acid	879.1 (412.3 – 2152)	409.1 (0.000 - 962.9)	0.10
Glycodeoxycholic Acid	33812 (10261 – 85730)	50120 (21963 – 72530)	0.64

Table 13: Mass Spectrometry- serum bile acids in PBC and Healthy Volunteers

Mass spectrometry observed serum bile acid metabolites with intensity differences (Median +/- IQR) associated with patients with PBC [n=40] and HV [n=16]. Statistical significance was determined by a non-parametric Mann-Whitney test, comparing ranks. Significant p-values (<0.05) are highlighted in green. Resonances increased in patients with PBC were collection of metabolites of UDCA and secondary BA- Murocholic acid.

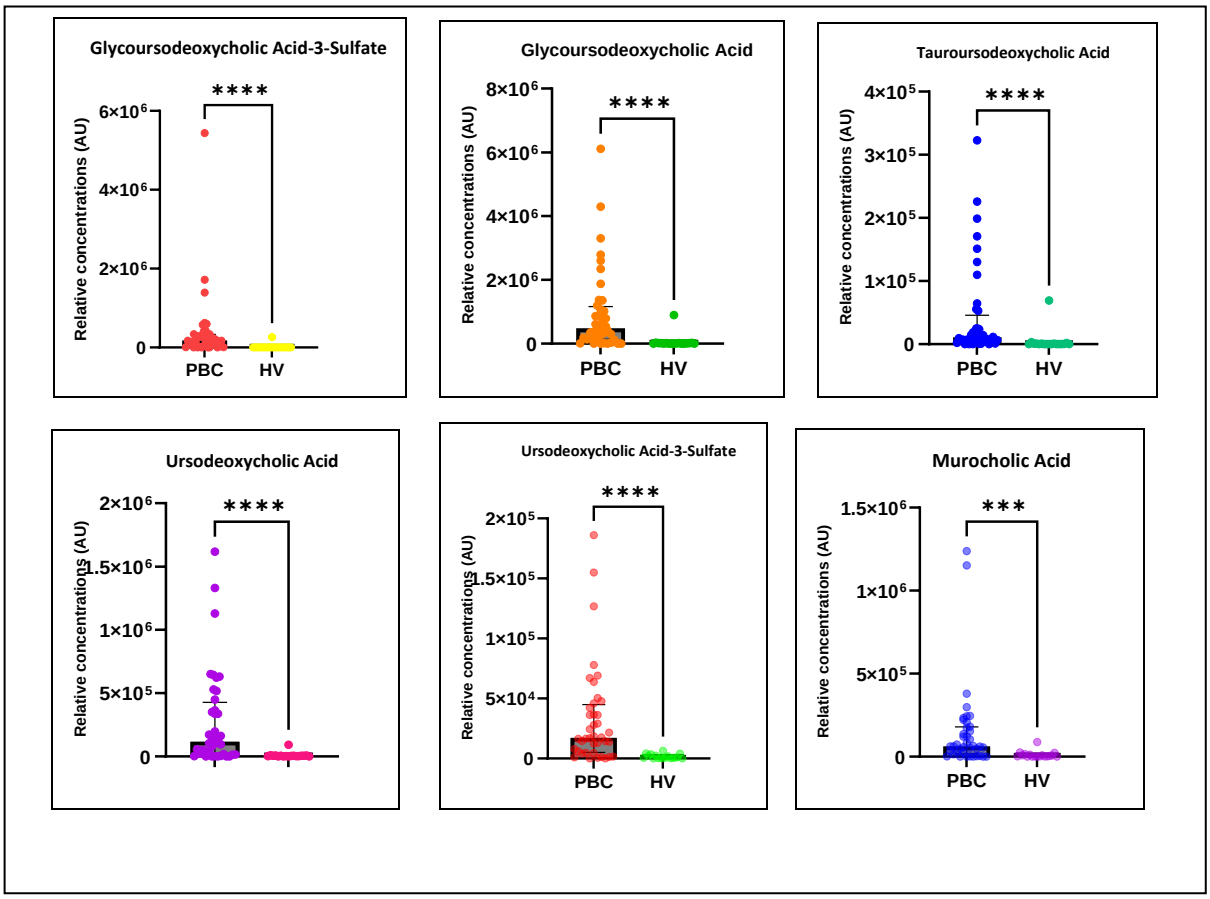


Figure 7: Targeted Metabolomics: Panel to compare individual bile acid metabolites measured in serum in patients with PBC [n=40] and HV [n=16].

The X-axis shows the relative serum concentrations expressed in Arbitrary Units (AU). p-value: ns= no significant, <0.05*, <0.01**, <0.001***, <0.0001****.

- **Serum MS Lipids (Negative ion mode) PBC vs HV**

A total of 50 patients with PBC and 18 HVs were included in the final analysis. A 2-component PCA model (R^2X=0.735, Q^2=0.565, good model) attained visual discrimination between patients with PBC and HV, in serum lipids on mass spectrometry in negative ion mode. This was confirmed subsequently on a 2-component OPLS DA model (Table 14), with the following statistics: (1+1+0), R^2X=0.547, R^2Y=0.414, Q^2=0.211, CV-ANOVA p=0.003. Permutation testing demonstrated that the model was valid in that the cut-offs for R^2 and Q^2 were lower in the randomly permutated model (999 permutations) (Figure 8). Metabolites with a VIP score of ≥ 1 were used to predict their influence on class separation between the two study groups (Table 15). Resonance **increased in PBC** patients included collection of metabolites of Lysophosphatidylcholine and Fatty acids (Table 16).

				CV-ANOVA p-value
PCA- X Coordinates	A	R^2X	Q^2	
	4	0.735	0.565	0.003

OPLS-DA	A	R^2X	R^2Y	Q^2
Coordinates	1+1+0	0.547	0.414	0.211

Table 14: PCA and OPLS-DA coordinates of PBC (n=50) vs HV (n=18) (Serum MS lipids negative ion mode).

A= no of principal components, R2 predicts goodness of fit, and Q2 is goodness of prediction of the model. Significant p-values (<0.05)]

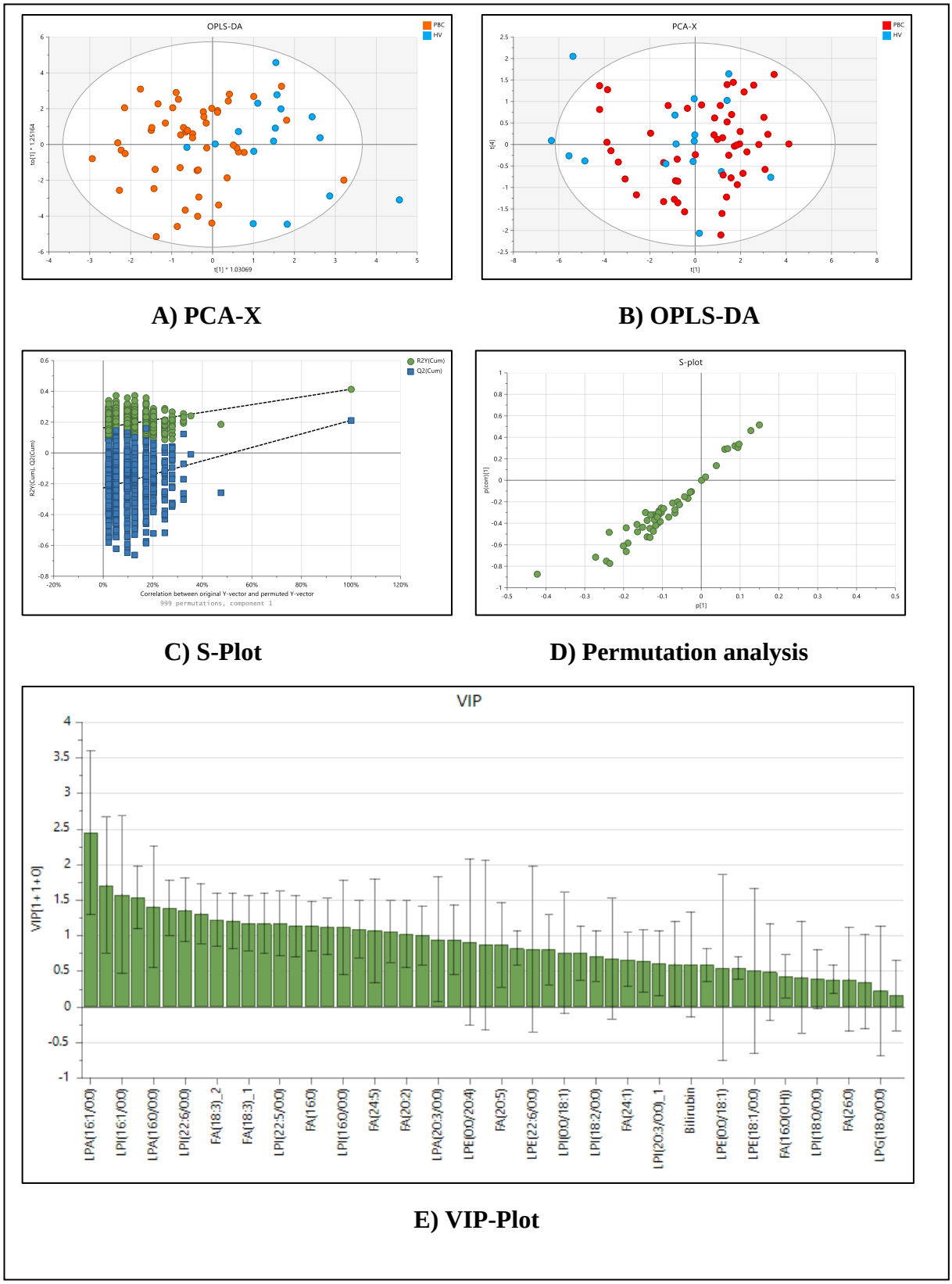


Figure 8: Serum Lipid (MS-negative ion mode) profile model PBC (n=50) vs HV (n=18)

These multivariate models demonstrate discrimination of patients with PBC and HV. (A) Principal components analysis (PCA) scores plot [2-component model $R^2X=0.735$, $Q^2=0.565$]. (B) Orthogonal projection least squares discriminant analysis (OPLS-DA) scores plot [(1+1+0), $R^2X=0.547$, $R^2Y=0.414$, $Q^2=0.211$]. (C) S-line loading plot (D) Permutation analysis (999 permutations) (E) VIP-score plot.

Metabolites with a VIP score of ≥ 1 were used to predict their influence on class separation between the two study groups (Table 15):

LPA(16:1/0:0)	2.45	FA(20:1)	1.18
FA(18:4)	1.71	LPI(22:5/0:0)	1.18
LPI(16:1/0:0)	1.58	FA(18:2)	1.14
FA(16:1)	1.54	FA(16:0)	1.14
LPA(16:0/0:0)	1.41	FA(12:0)	1.14
FA(17:1)	1.40	LPI(16:0/0:0)	1.12
LPI(22:6/0:0)	1.37	LPA(22:6/0:0)	1.10
FA(18:1)	1.32	FA(24:5)	1.07
FA(18:3)_2	1.23	FA(22:5)_1	1.06
FA(14:0)	1.21	FA(20:2)	1.03
FA(18:3)_1	1.18	FA(22:5)_2	1.01

Table 15: Serum MS lipid metabolites with VIP >1, PBC (n=50) vs HV (n=18)

	PBC	HV	p- value Mann- Whitney
LPA(16:1/0:0)	2014 (968.0 – 2808)	327.2 (218.8 - 798.9)	<0.0001
FA(18:4)	1971 (1013 – 3849)	1110 (158.4 – 2003)	0.02
LPI(16:1/0:0)	2801 (1590 – 3592)	1141 (491.6 – 3334)	0.01
FA(16:1)	340126 (116176 – 615828)	144085 (54850 –240246)	0.008
LPA(16:0/0:0)	10906 (7670 – 13506)	4922 (3723 – 5765)	<0.0001
FA(17:1)	24948 (11503 – 40350)	15424 (3670 – 18871)	0.01
LPI(22:6/0:0)	3339 (2505 – 4399)	1954 (1459 – 2568)	<0.0001
FA(18:1)	4397125 (2140594 – 8093413)	2906683 (1006660 – 3818876)	0.08
FA(18:3)_2	19303 (11317 – 31243)	11816 (3781 – 16303)	0.01
FA(14:0)	112366 (65667 – 180031)	83736 (23880 – 140364)	0.08

Table 16: Mass Spectrometry (negative ion mode)- serum lipids in PBC and Healthy Volunteers

Mass spectrometry observed serum lipid metabolites with intensity differences (Median +/- IQR) associated with patients with PBC [n=50] and HV [n=18]. Statistical significance was determined by a non-parametric Mann-Whitney test, comparing ranks. Significant p-values (<0.05) are highlighted in green. Resonances increased in patients with PBC were collection of metabolites of Lysophosphatidylcholine and Fatty acids.

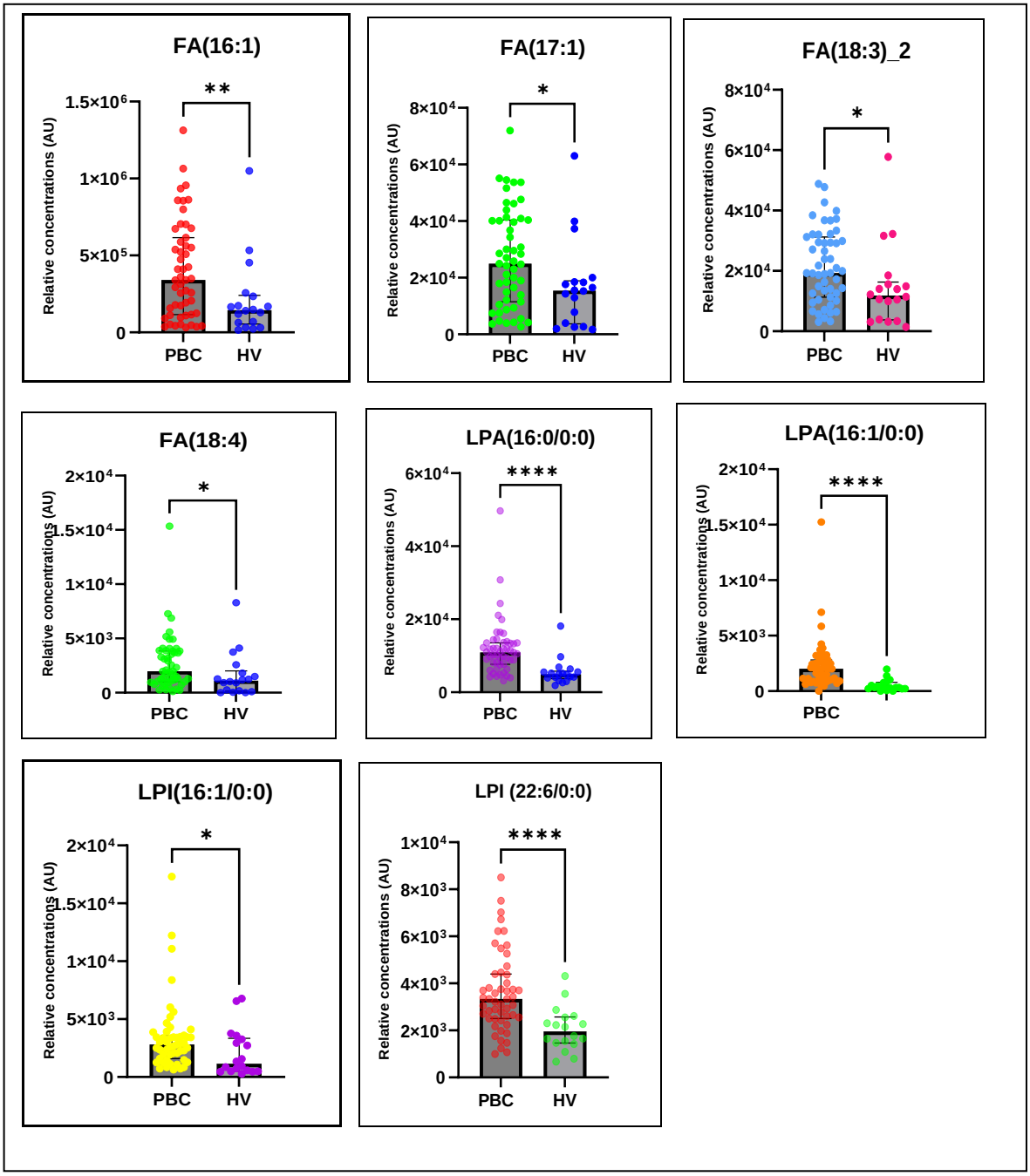


Figure 9: Targeted Metabolomics: Panel to compare lipid metabolites measured (Lysophosphatidylcholine and Fatty acids) in serum in patients with PBC [n=50] and HV [n=18] using mass spectrometry (negative ion mode).

The X-axis shows the relative serum concentrations expressed in Arbitrary Units (AU). p-value: ns= no significant, <0.05*, <0.01**, <0.001***, <0.0001****.

- **Serum MS Lipids (Positive ion mode) PBC vs HV**

A total of 51 patients with PBC and 18 HVs were included in the final analysis. A 2-component PCA model (R^2X=0.673, Q^2=0.499, good model) attained visual discrimination between patients with PBC and HV, in serum lipids on mass spectrometry in positive ion mode. This was confirmed subsequently on a 2-component OPLS DA model (Table 17), with the following statistics: (1+1+0), R^2X=0.282, R^2Y=0.544, Q^2=0.242, CV-ANOVA p=0.001. Permutation testing demonstrated that the model was valid in that the cut-offs for R^2 and Q^2 were lower in the randomly permutated model (999 permutations) (Figure 10). Metabolites with a VIP score of ≥ 1 was used to predict their influence on class separation between the two study groups. Resonance **increased in PBC** patients including metabolite collection of phospholipids-Phosphatidylcholine and Sphingomyelins (Table 18).

				CV-ANOVA p-value
PCA- X Coordinates	A	R^2X	Q^2	
	6	0.673	0.499	0.001

OPLS-DA Coordinates	A	R^2X	R^2Y	Q^2
	1+1+0	0.282	0.544	0.242

Table 17: PCA and OPLS-DA coordinates of PBC (n=51) vs HV (n=18) (Serum MS lipids positive ion mode).

A= no of principal components, R^2 predicts goodness of fit, and Q^2 is the goodness of prediction of the model. Significant p-values (<0.05)]

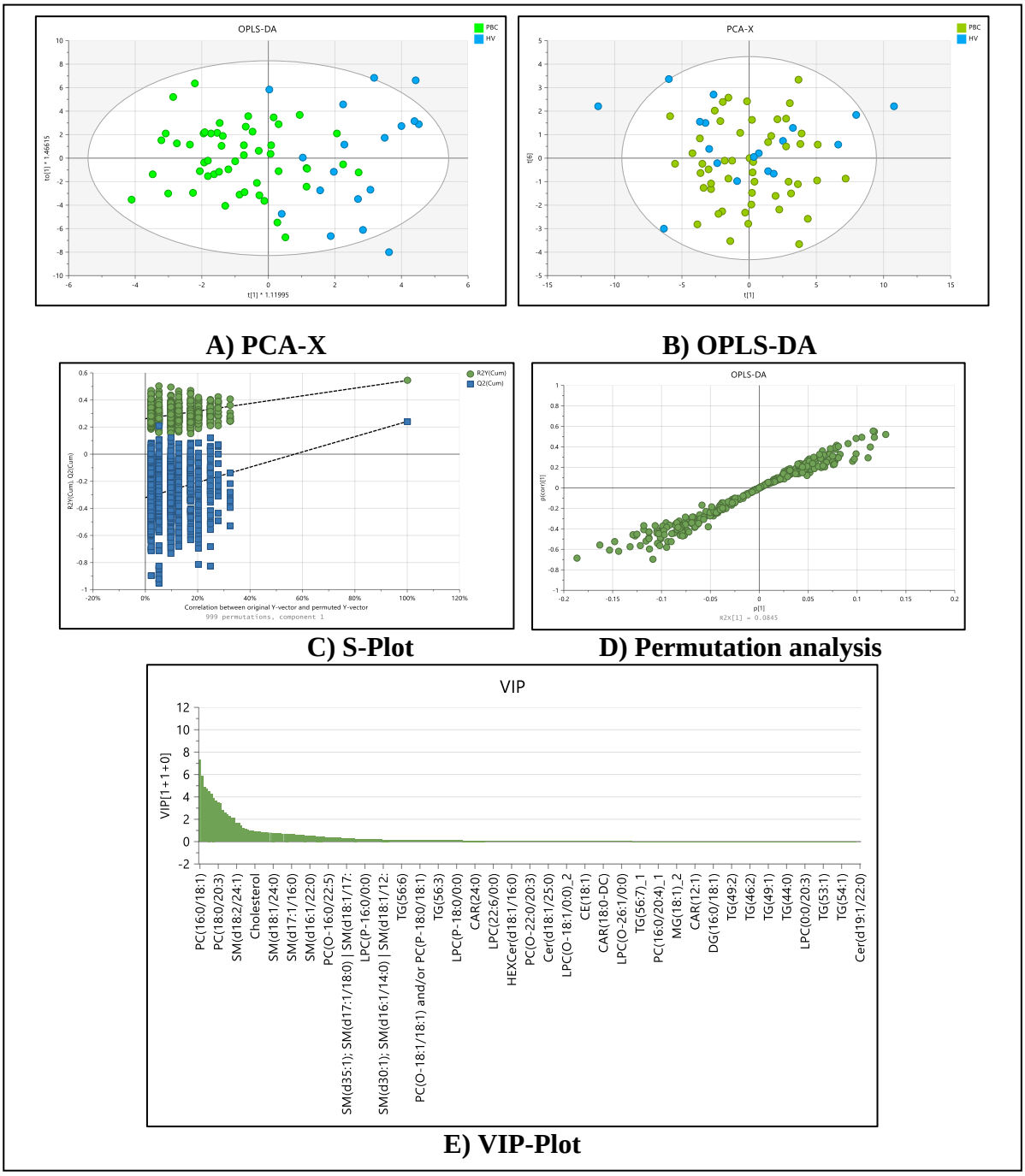


Figure 10: Serum MS Lipid (Positive ion mode) profile model PBC (n=51) vs HV (n=18)

These multivariate models demonstrate discrimination of patients with PBC and HV. (A) Principal components analysis (PCA) scores plot [2-component model R^2X=0.673, Q^2=0.499]. (B) Orthogonal projection least squares discriminant analysis (OPLS-DA) scores plot [(1+1+0), R^2X=0.282, R^2Y=0.544, Q^2=0.242]. (C) S-line loading plot (D) Permutation analysis (E) VIP-scores plot).

	PBC	HV	p-value Mann-Whitney
PC(16:0/18:1)	56535662 (48251476 – 66523816)	46613189 (40203013 – 61685288)	0.01
PC(16:0/20:4)_3	32292075 (27926924 – 37108890)	27485979 (23705914 – 31315880)	0.01
PC(18:0/20:4)	21015895 (17674460 – 25868923)	18084657 (14411564 – 21310237)	0.01
PC(16:0/20:3)	25816761 (21914046 – 29094902)	20283472 (17157749 – 26398319)	0.01
PC(18:0/18:1)	12676803 (9843551 – 15477819)	9401872 (7703169 – 12961843)	0.01
PC(16:0/22:6)	21112785 (16416394 – 26222991)	18687480 (14759906 – 23502468)	0.11
PC(18:0/20:3)	10767700 (9216049 – 13317210)	46613189 (40203013 – 61685288)	<0.0001
PC(18:1/18:2)	19961776 (15513923 – 24034611)	18018383 (14762954 – 21942612)	0.49
SM(d18:1/24:1)	10181906 (9515207 – 11672396)	8179743 (7331015 – 10521775)	0.0005
SM(d18:1/16:0)	26549888 (17736867 – 29461865)	23237036 (22147727 – 26880093)	0.04

Table 18: Mass Spectrometry (positive ion mode)- serum lipids in PBC and Healthy Volunteers

Mass spectrometry observed serum lipid metabolites with intensity differences (Median +/- IQR) associated with patients with PBC [n=51] and HV [n=18]. Statistical significance was determined by a non-parametric Mann-Whitney test, comparing ranks. Significant p-values (<0.05) are highlighted in green. Resonances increased in patients with PBC were collection of metabolites of Phosphatidylcholine and Sphingomyelins.

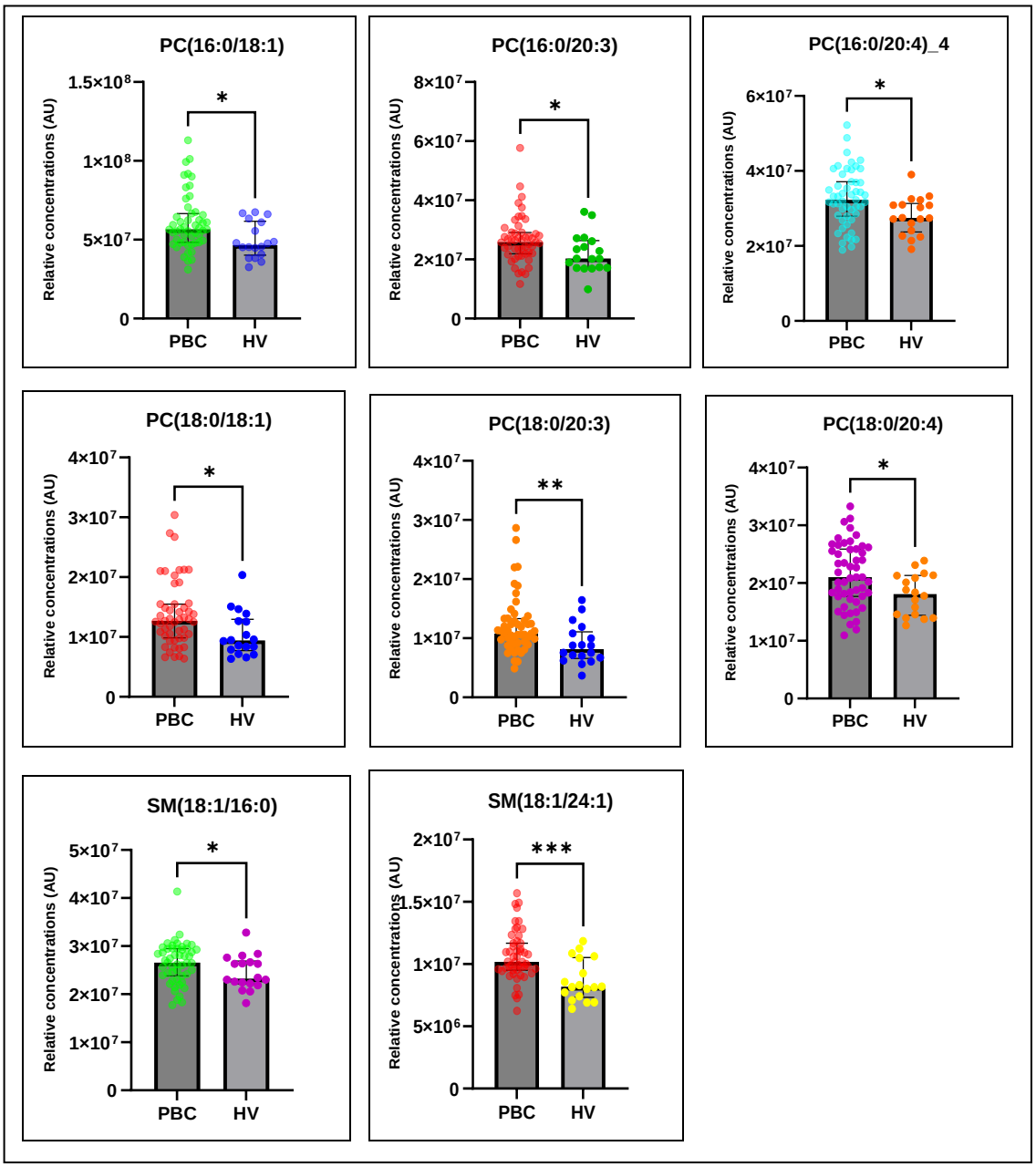


Figure 11: Targeted Metabolomics: Panel to compare lipid metabolites (Phosphatidylcholine and Sphingomyelins) measured in serum in patients with PBC [n=51] and HV [n=18] using mass spectrometry (positive ion mode).

The X-axis shows the relative serum concentrations expressed in Arbitrary Units (AU). p-value: ns= no significant, <0.05*, <0.01**, <0.001***, <0.0001****.

- **Serum NMR BiLISA (PBC vs HV)**

A total of 51 patients with PBC and 18 HVs were included in the final analysis. A 2-component PCA model (R^2X=0.758, Q^2=0.679) attained visual discrimination between patients with PBC and HV, in the serum lipids on NMR. This was confirmed subsequently on a 3-component OPLS DA model (Table 19), with the following statistics: (1+2+0), R^2X=0.588, R^2Y=0.401, Q^2=0.227, CV-ANOVA p=0.01. Permutation testing demonstrated that the model was valid in that cut-offs for R^2 and Q^2 were lower in the randomly permutated model (999 permutations) (Figure 12). Metabolites with a VIP score of ≥ 1 was used to predict their influence on class separation between the two study groups (Table 20). Resonance **increased in PBC** patients included metabolite collection of High-density lipoproteins and Very-low-density lipoproteins (Table 21).

				CV-ANOVA p-value
PCA- X Coordinates	A	R^2X	Q^2	
	3	0.758	0.679	0.01

OPLS-DA	A	R^2X	R^2Y	Q^2
Coordinates	1+2+0	0.588	0.401	0.227

Table 19: PCA and OPLS-DA coordinates of PBC (n=51) vs HV (n=18) (Serum NMR BiLISA).

A= no of principal components, R^2 predicts goodness of fit, and Q^2 is the goodness of prediction of the model. Significant p-values (<0.05)]

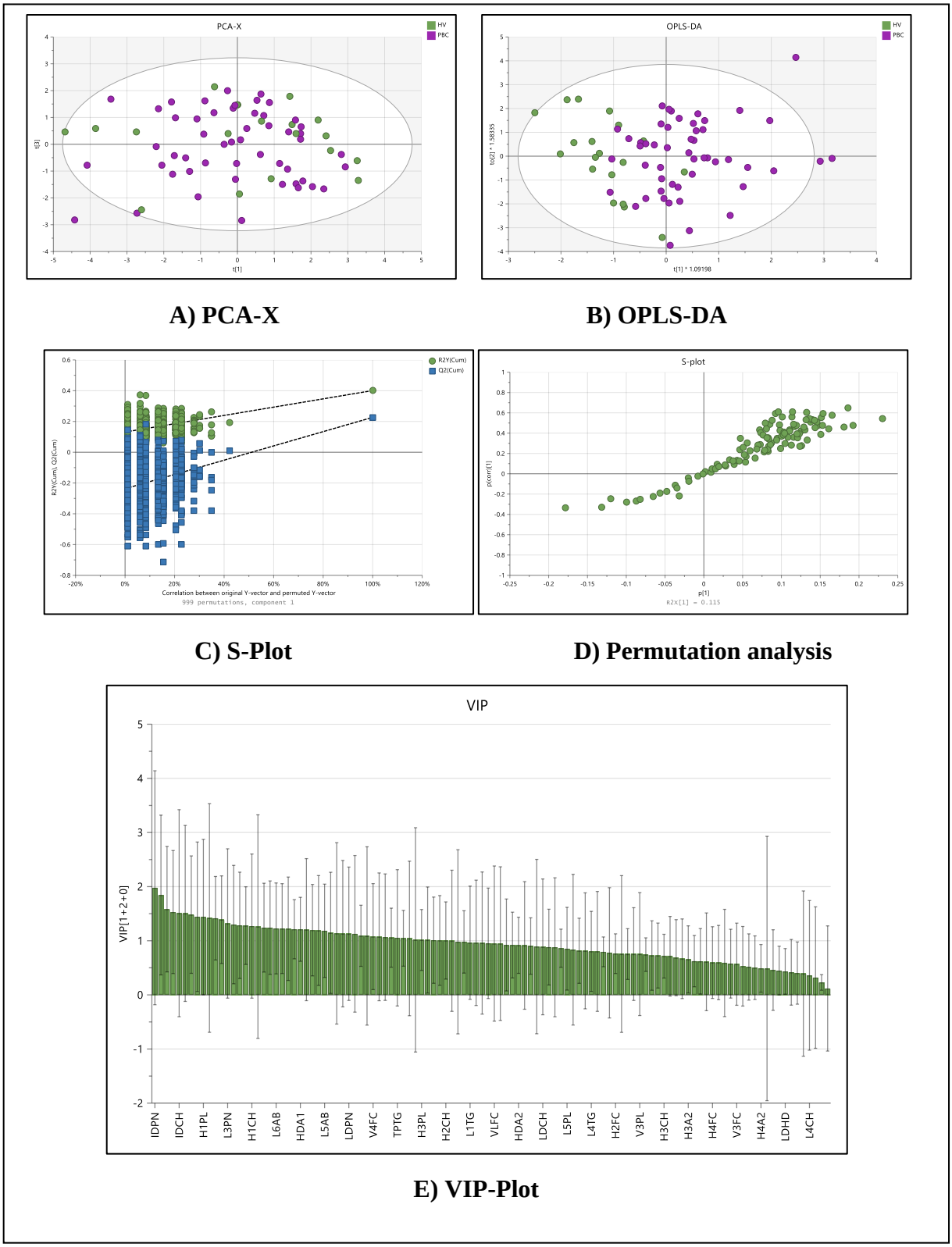


Figure 12: Serum NMR BiLISA profile model PBC (n=51) vs HV (n=18)

These multivariate models demonstrate discrimination of patients with PBC and HV. (A) Principal components analysis (PCA) scores plot 2-component model [R^2X=0.758, Q^2=0.679]. (B) Orthogonal projection least squares discriminant analysis (OPLS-DA) scores plot [(1+2+0), R^2X=0.588, R^2Y=0.401, Q^2=0.227]. (C) S-line loading plot (D) Permutation analysis (E) VIP-scores plot.

Metabolites with a VIP score of ≥ 1 were used to predict their influence on class separation between the two study groups (Table 20):

H1A1	1.50	HDA1	1.21
V4CH	1.48	IDAB	1.21
L1PN	1.44	TPCH	1.20
H1PL	1.44	HDTG	1.19
VLPN	1.42	L5AB	1.18
HDPL	1.42	L1AB	1.15
L2CH	1.39	V4TG	1.14
L3PN	1.32	V4PL	1.13
LDTG	1.30	LDPN	1.13
TBPN	1.28	L1CH	1.13
L6CH	1.28	L6PL	1.09
H1CH	1.27	VLTG	1.09
VLCH	1.26	V4FC	1.08
H2PL	1.24	LDAB	1.07
HDCH	1.24	L1PL	1.07
L6AB	1.23	L2PL	1.06
H2A1	1.22	TPTG	1.05
TPAB	1.22	L2AB	1.04
TPA1	1.21	IDFC	1.04

Table 20: Serum NMR BiLISA metabolites with VIP >1, PBC (n=51) vs HV (n=18)

	PBC (median + IQR)	HV (median + IQR)	Mann- Whitney p-value
HDL	41.96 (32.9 – 56.81)	30.34 (26.37 – 47.93)	0.01
LDL	64.36 (58.22 – 72.52)	67.57 (52.49 – 76.65)	0.64
VLDL	35.78 (25.60 – 52.08)	32.16 (19.56 – 42.30)	0.37

Table 21: NMR BiLISA- serum lipids in PBC and Healthy Volunteers

Total HDL, LDL, and VLDL in serum with intensity differences (Median +/- IQR) associated with patients with PBC [n=51] and HV [n=18]. Statistical significance was determined by a non-parametric Mann-Whitney test, comparing ranks. Significant p-values (<0.05) are highlighted in green.

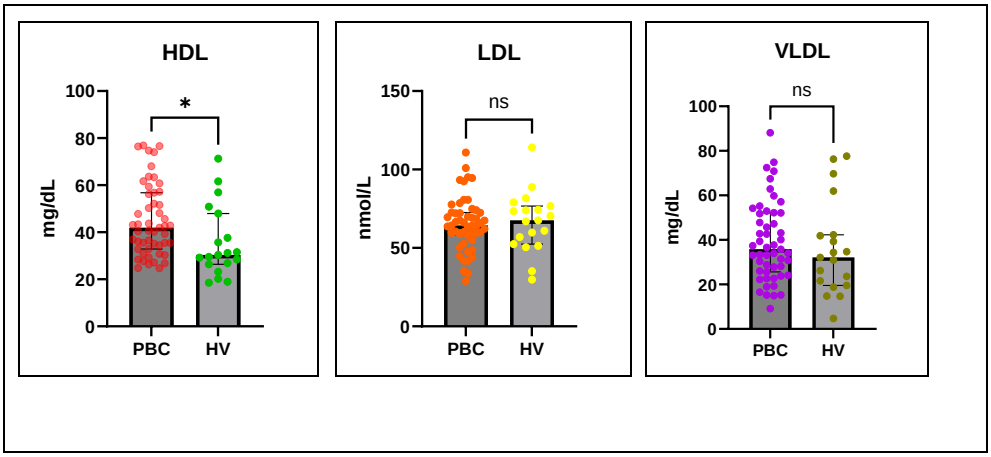


Figure 13: Targeted Metabolomics: Median values compared with p-values for HDL, LDL, and VLDL in serum of PBC vs HV using NMR.

The X-axis shows concentrations of HDL (mg/dl). LDL (nmol/L) and VLDL (mg/dl). p-value: ns= no significant, <0.05*, <0.01**, <0.001***, <0.0001****.

Urine Analysis PBC vs HV:

- **Urine NMR QUANT**

A total of 51 patients with PBC and 20 HVs were included in the final analysis. A 2-component PCA model [R^2X=0.719, R^2Y=0.254] did not show any discrimination between the two groups in urine metabolites on NMR (p=0.54)

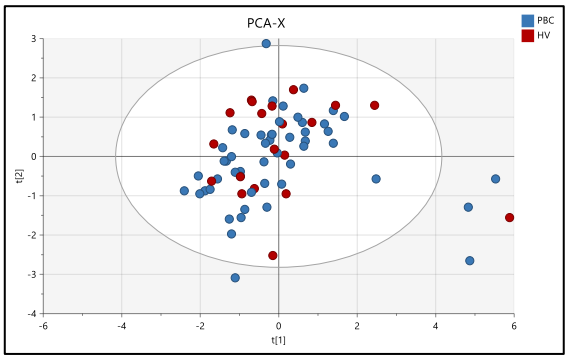


Figure 14: PCA model comparing urine NMR QUANT in PBC (n=51) vs HV (n=20).

- **Urine MS (Negative ion mode)**

A total of 51 patients with PBC and 20 HVs were included in the final analysis. A 3-component PCA model [R^2X=0.46, R^2Y=0.259] did not show any discrimination between the two groups in urine metabolites on mass spectrometry in negative ion mode (p=0.37)

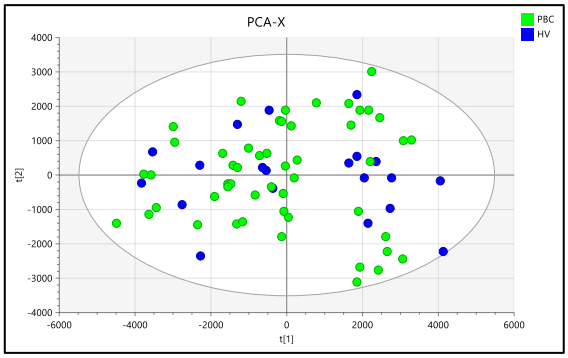


Figure 15: PCA model comparing urine MS (negative ion mode) in PBC (n=51) vs HV (n=20).

- **Urine MS (Positive ion mode)**

A total of 51 patients with PBC and 20 HVs were included in the final analysis. A 2-component PCA model [R^2X=0.491, R^2Y=0.399] did not show any discrimination between the two groups in urine metabolites on mass spectrometry in positive ion mode (p=0.52)

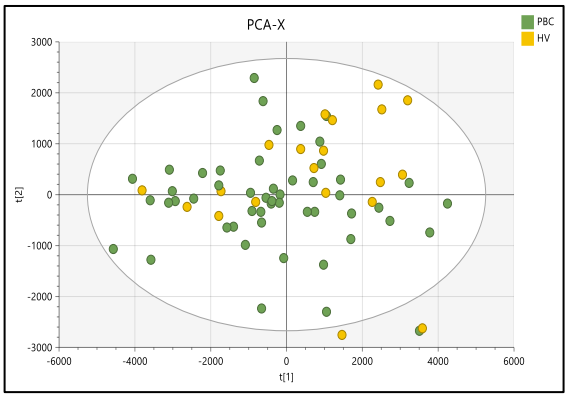


Figure 16: PCA model comparing urine MS (positive ion mode) in PBC (n=51) vs HV (n=20).

Faecal analysis comparing PBC patients with HV

- **<u>Faecal Bile Acid (Negative ion mode) PBC vs HV</u>**

A total of 50 patients with PBC and 19 HVs were included in the final analysis. A 5-component PCA model (R^2X=0.712, Q^2=0.495) attained visual discrimination between patients with PBC and HV. This was confirmed subsequently on a 2-component OPLS DA model, with the following statistics: (1+1+0), R^2X=0.47, R^2Y=0.489, Q^2=0.351, CV-ANOVA p= 8.01948e-06 (Table 22). Permutation testing demonstrated that the model was valid in that the cut-offs for R^2 and Q^2 were lower in the randomly permutated model (999 permutations) (Figure 17). Metabolites with a VIP score of ≥ 1 were used to predict their influence on class separation between the two study groups (Table 23). Resonance **increased in faeces of PBC** patients included metabolites of UDCA (Ursodeoxycholic Acid-3-Sulfate, Glycoursodeoxycholic Acid) and secondary bile acid metabolites (Lithocholic Acid 3-Sulfate, omega Muricholic Acid, beta

Muricholic Acid, 6-Oxolithocholic Acid, Murocholic Acid, Lithocholic Acid, Glycolithocholic Acid and Hyodeoxycholic Acid) (Table 24).

				CV-ANOVA p-value
PCA- X Coordinates	A	R^2X	Q^2	
	5	0.712	0.495	8.01948e-06

OPLS-DA	A	R^2X	R^2 Y	Q^2
Coordinates	1+1+0	0.47	0.489	0.351

Table 22: PCA and OPLS-DA coordinates of PBC (n=50) vs HV (n=19) (Faecal bile acids negative ion mode).

A= no of principal components, R^2 predicts goodness of fit and Q^2 is the goodness of prediction of the model. Significant p-values (<0.05)

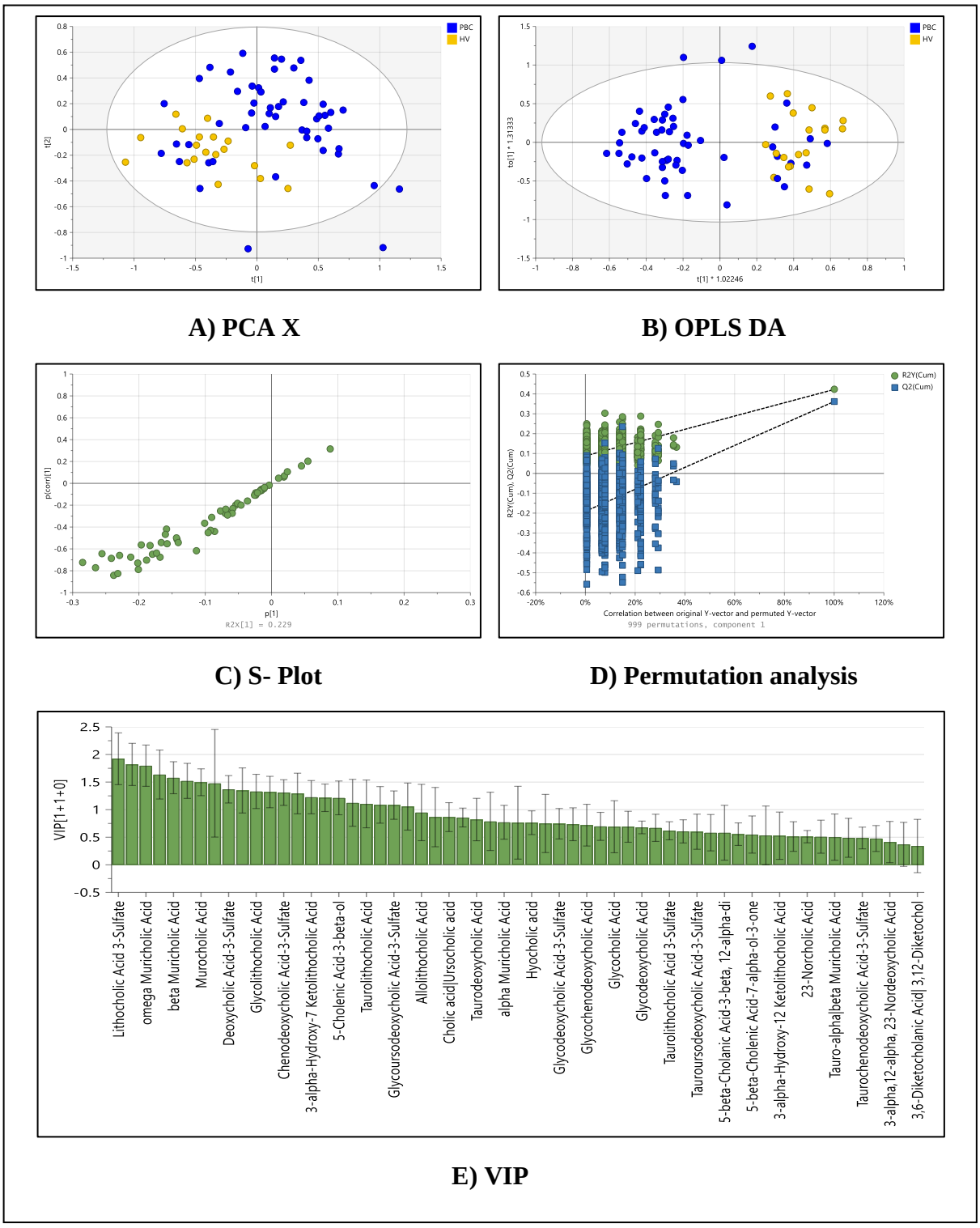


Figure 17: Fecal BA profile models PBC (n=50) vs HV (n=19)

These multivariate models demonstrate discrimination of patients with PBC and HV. (A) Principal components analysis (PCA) scores plot 5-component model [R^2X=0.712, Q^2=0.495]. (B) Orthogonal projection least squares discriminant analysis (OPLS-DA) scores plot [(1+1+0), R^2X=0.47, R^2Y=0.489, Q^2=0.351]. (C) S-line loading plot (D) Permutation analysis (E) VIP-scores plot.

Metabolites with a VIP score of ≥ 1 was used to predict their influence on class separation between the two study groups. (Table 26).

Lithocholic Acid 3-Sulfate	1.88
Ursodeoxycholic Acid-3-Sulfate	1.79
omega Muricholic Acid	1.79
Glycoursodeoxycholic Acid	1.62
beta Muricholic Acid	1.55
6-Oxolithocholic Acid	1.50
Murocholic Acid	1.47
Lithocholic Acid	1.47
Tauroursodeoxycholic Acid	1.39
Deoxycholic Acid-3-Sulfate	1.34
Ursodeoxycholic Acid	1.32
Chenodeoxycholic Acid-3-Sulfate	1.29
5-alpha-Cholanic Acid-3-alpha-ol-6-one	1.28
Glycolithocholic Acid	1.27
5-Cholenic Acid-3-beta-ol	1.21
3-alpha-Hydroxy-7 Ketolithocholic Acid	1.21
Hyodeoxycholic Acid	1.17
Taurolithocholic Acid	1.15
Glycoursodeoxycholic Acid-3-Sulfate	1.09
3-Ketocholanic Acid	1.07
Lithocholenic Acid	1.05
Glycolithocholic Acid-3-Sulfate	1.02

Table 23: Faecal Bile acid metabolites with VIP >1, PBC (n=50) vs HV (n=19)

	PBC median +/- IQR	HV median +/- IQR	p-value Mann Whitney
Lithocholic Acid 3-Sulfate	500381 (30712 – 5765829)	8915 (6247 – 62458)	<0.0001
Ursodeoxycholic Acid-3-Sulfate	253417 (27086 – 4907406)	13334 (6576 – 34182)	<0.0001
omega Muricholic Acid	32226 (5561 – 111990)	1007 (727.5 – 1969)	<0.0001
Glycoursodeoxycholic Acid	90051 (20438 – 320399)	3758 (1640 – 11919)	<0.0001
beta Muricholic Acid	41692 (16302 – 110196)	3932 (3094 – 8832)	<0.0001
6-Oxolithocholic Acid	27650 (11093 – 90502)	5314 (2148 – 7249)	<0.0001
Murocholic Acid	111265 (33121 – 267398)	8583 (5860 – 24716)	<0.0001
Lithocholic Acid	31740469 (15679845 – 42696379)	4796711 (2843537 – 7077689)	<0.0001
Deoxycholic Acid-3-Sulfate	15656 (2818 – 194301)	4716 (1867 – 22330)	0.06
Glycolithocholic Acid	11926 (5865 – 23160)	1975 (691.9 – 3809)	<0.0001
Hyodeoxycholic Acid	97912 (40858 – 247884)	23073 (12703 – 34813)	<0.0001

Table 24: Mass Spectrometry- faecal bile acids in PBC and Healthy Volunteers

Mass spectrometry observed faecal bile acid metabolites with intensity differences (Median +/- IQR) associated with patients with PBC [n=50] and HV [n=19]. Statistical significance was determined by a non-parametric Mann-Whitney test, comparing ranks. Significant p-values (<0.05) are highlighted in green.

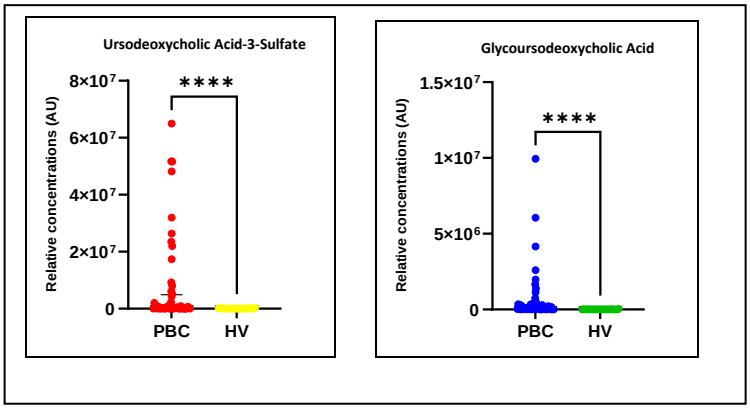


Figure 18: Targeted Metabolomics: Panel to compare individual UDCA metabolites in faeces in patients with PBC [n=50] and HV [n=19].

The X-axis shows the relative faecal concentrations of bile acids expressed in Arbitrary Units (AU). p-value: ns= no significant, <0.05*, <0.01**, <0.001***, <0.0001****.

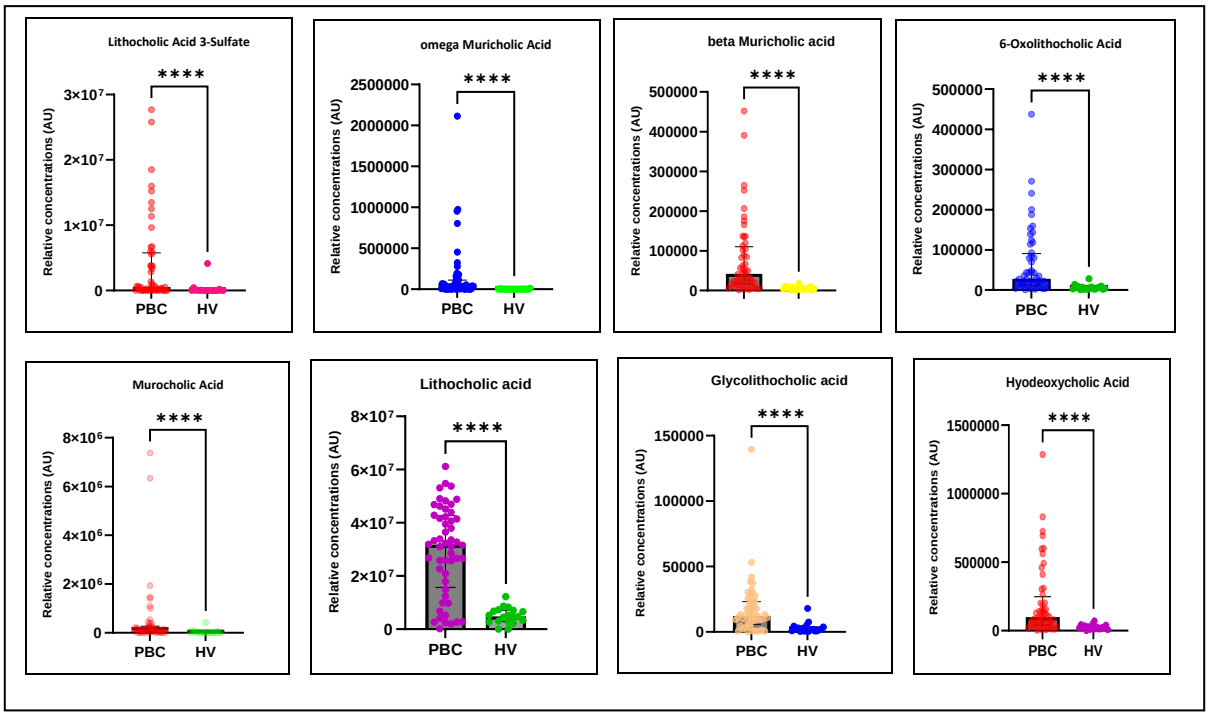


Figure 19: Targeted Metabolomics: Panel to compare individual (other) secondary BA metabolites in faeces in patients with PBC [n=50] and HV [n=19].

The X-axis shows the relative faecal concentrations of bile acids expressed in Arbitrary Units (AU). p-value: ns= no significant, <0.05*, <0.01**, <0.001***, <0.0001****.

2.2.2 Conclusion:

Total BAs were significantly higher in PBC patients in our study. The majority (90%) of our patients were on established UDCA therapy. Hence the exogenous administration of the UDCA altered the overall bile acid pool in our cohort. Our findings are similar to the study by ***Chen et al*** in 65 PBC patients (Comparing UDCA naive patients with HV; and PBC before and after UDCA therapy). In their study, total primary BAs (TCA, GCA and GCDAC proportions) were increased and total secondary BAs (DCA, LCA, TLCA proportions) were reduced in the PBC group (Chen et al., 2020). Similarly, total primary BAs were increased, and secondary BAs decreased in the faeces of the UDCA naive PBC patients. However, compared to their study most patients in our study group were on established UDCA therapy. After UDCA therapy in the Chen et al study, total BAs were still higher in PBC patients. UDCA and its conjugate metabolites dominated the circulating BA pool. Our findings are similar to the findings of this study (Chen et al., 2020). The ratio of secondary to primary BAs was 0.62 in PBC compared to

2.06 in HVs. Though we did not have pre- and post-UDCA, comparative groups, findings are consistent with the Chen et al study where the ratio of Secondary: primary bile acids was lower in the PBC cohort (both pre- and post-UDCA therapy).

Lipid metabolism dysregulation has been associated with cholestatic liver disease. BAs are formed from cholesterol and a feedback mechanism involving the FXR receptors regulated by BAs is in play. Alterations in BAs due to cholestasis and impaired mitochondrial beta-oxidation of FAs affects this homeostasis. Significantly higher levels of cholesterol, TGs, SMs and PCs were found in PBC patients in our study which are consistent with findings from previous studies (Kowdley, 1998, Lang et al., 2001). JM de Oliveira etal in their study of 30 (female) PBC patients on established therapy with UDCA performed metabolomic analysis on urine and serum samples in comparison to 20 (female) healthy controls.(de Oliveira et al., 2024) They found 47 plasma and 56 urine-differentiating metabolites on untargeted analysis. They found that 7 lipids (mainly LPC) were downregulated and 7 (mainly PC) were upregulated in PBC patients (total mean ratio PC vs LPC, FC= 1.76, $p<0.0008$). The result reflects the effect of UDCA administration which promotes LPC reduction by reducing cholesterol saturation in bile. Similar to their study we had seen raised levels of PC in our cohort of PBC patients (majority on UDCA) therapy. LPC is a precursor of LPA which is a pruritogenic in cholestatic liver disease. Reduced levels of LPC are also seen in various inflammatory conditions.

Chen et al in their study demonstrated alterations in gut microbiota in PBC patients pre-UDCA compared to HV; and post-UDCA compared to UDCA-naive PBC patients, with alterations in taurine metabolism related to Bilophila species (Chen et al., 2020). We did not have faecal bacterial analysis in our study.

2.3 Comparison of Metabolomic profile of AMA positive with normal LFTs and Healthy Volunteers:

2.3.1 Summary of result:

A total of 15 patients in AMANL and 18 in the HV group were included in the final analysis. The median age did not show any statistically significant difference between the two study groups (57 years for AMANL and 56 years for HV), (p=0.52). In the AMANL group, 8 (53%) had one or a combination of symptoms of fatigue, pruritus, or cognitive impairment. 4/15 (26%), 6/15 (40%) and 1/15 (6%) had fatigue, pruritus, and cognitive impairment respectively. **No significant differences were found in the metabolomic profiles from serum, urine, and faeces of AMANL patients and HVs** (Table 25). (Figure 20) shows PCA-X graphs of various assays where valid models were achieved, however, none of the models reached statistical discriminatory significance on multivariate analysis (p-values > 0.05).

Overview of analysis:

		A	R^2X	Q^2	p-value CV-ANOVA
Serum	MS Bile acid (NEG)	3	0.625	0.219	1
	MS Lipid (NEG)	3	0.712	0.524	0.36
	MS Lipid (POS)	6	0.778	0.533	1
	NMR BiLISA	4	0.872	0.779	1
Urine	NMR QUANT	Invalid model			
	MS (NEG)	2	0.423	0.224	
	MS (POS)	2	0.525	0.391	1
Faecal	MS BA (NEG)	5	0.698	0.169	1

Table 25: Summary of PCA analysis in AMANL vs HV

PCA-X co-ordinated and corresponding CV-ANOVA p-values for serum, urine, and faecal assays of AMA antibody positive with normal LFT patients [n=15] compared with HVs [n=18]. A= no of principal components, R^2 predicts goodness of fit and Q^2 goodness of prediction of the model. Significant p-values (<0.05).

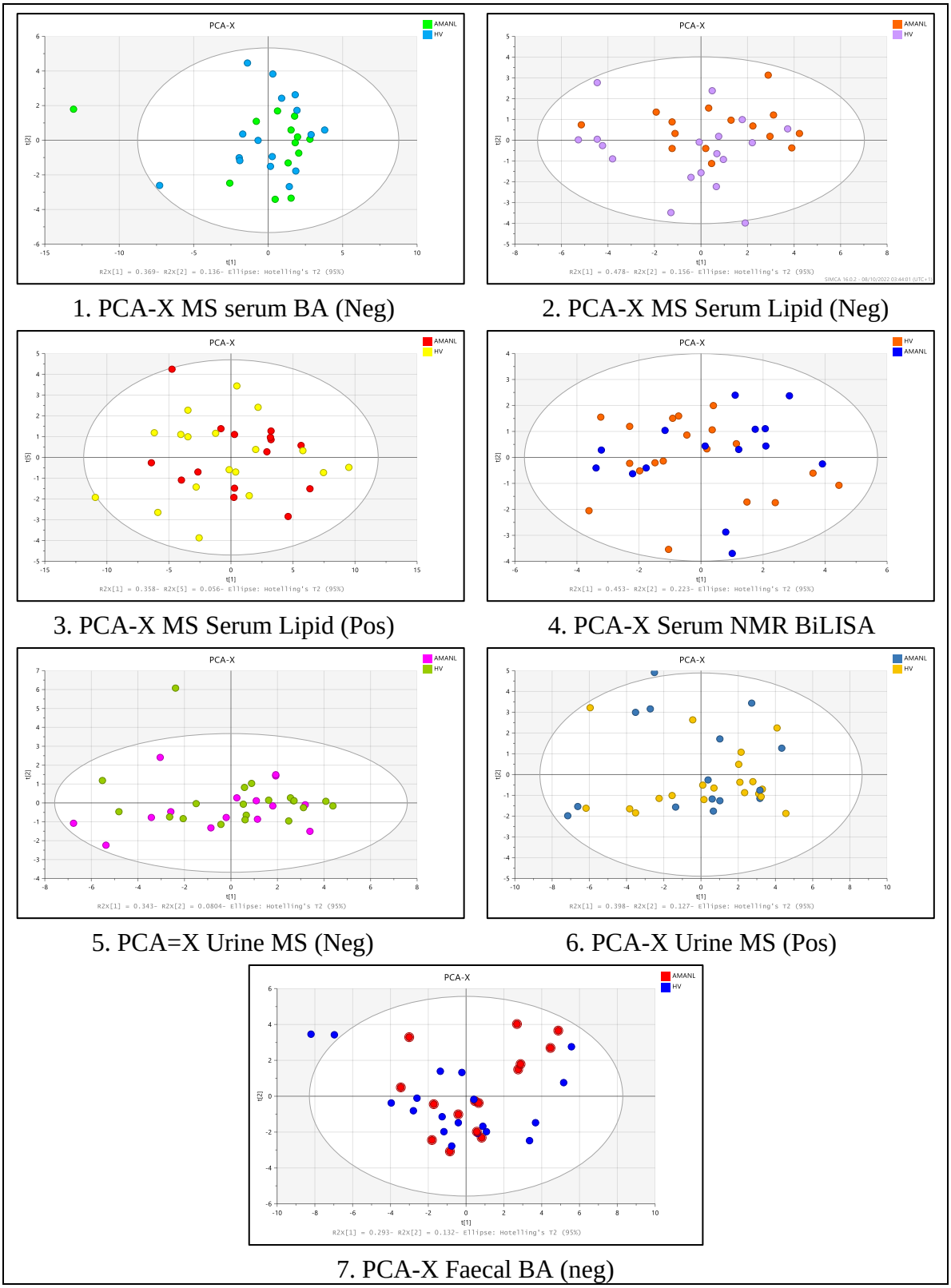


Figure 20: PCA-X, comparing AMANL vs HV

1. MS serum BA (Negative ion mode), 2. MS Serum Lipid (Negative ion mode), 3. MS Serum Lipid (Positive ion mode), 4. Serum NMR BiLISA, 5. Urine MS (Negative ion mode), 6. Urine MS (Positive ion mode), 7. Faecal BAs. AMANL [n=15] compared with HVs [n=18]. No significant discrimination was seen in metabolites.

2.3.2 Conclusion:

Only a few studies in the literature have focused on patients who are AMA-positive but have normal liver chemistry (normal ALP levels). **This study did not find any significant differences** in the urine, serum, or fecal metabolomic profiles of these patients compared to a healthy control group. Therefore, biochemically, AMANL patients behaved similarly to healthy volunteers, except for the presence of AMA.

The study did identify a **significant number of patients experiencing one or more PBC-related symptoms**, including fatigue, pruritus (itching), or cognitive impairment. Specifically, pruritus was reported in 4 patients (26%), fatigue in 6 patients (40%), and cognitive impairment in 1 patient (6%) within this cohort. Additionally, three patients (20%) experienced both pruritus and fatigue. According to existing literature, a large proportion of these patients exhibit some histological changes associated with PBC, and there is a 1 in 6 risk of progression to PBC.

However, our study has some limitations. The sample size is small, and we do not have available histological data. Larger cohort studies are needed to gain a better understanding of the pathophysiology and the natural history of the disease in this subset of patients.

Chapter 3. Results 1: Comparison of Metabolomic Profile of Primary Biliary Cholangitis and AMA Positive with Normal LFTs

3.1 Result of analysis:

51 PBC and 15 AMANL patients were included in the final analysis. There was no difference in the median age, (PBC = 64 years, AMANL = 57 years, p=0.12) between the two groups. PBC patients compared to AMANL had significantly higher median levels of ALP and GGT, markers of cholestasis (109 vs 79, p=0.009) and (54 vs 27, p=0.05) respectively (Table 26). PCA followed by OPLS-DA gave robust differentiating models, which were cross-validated with multivariate CV-ANOVA analysis. **Resonance increased in secondary bile acids in serum and faeces of PBC patients.** The overview of the analysis is summarised in (Table 27).

	PBC (Median / IQR)	AMANL (Median / IQR)	p- value
Age	64 (57-70)	57 (47-67)	0.12
Albumin	45 (43-46)	44 (43-48)	0.73
Bilirubin	7 (6-8)	7 (6-12)	0.78
ALP	109 (78-148)	79 (56-102)	0.009
ALT	26 (15-35)	14 (11-27)	0.07
GGT	54 (29-131)	27 (12-44)	0.05
AST	29 (23-42)	25 (13-29)	0.09

Table 26: Median and IQR compared (with p-value) for age and liver biochemistry.

(Bilirubin, ALP-alkaline phosphates, ALT- alanine aminotransferase, GGT- gamma-glutamyl transferase, AST-aspartate aminotransferase) between PBC and AMANL group.

Analysis overview:

		A	R^2X	Q^2	p-value
Serum	MS Bile acid (NEG)	3	0.756	0.541	0.03
	MS Lipid (NEG)	4	0.713	0.507	0.57
	MS Lipid (POS)	6	0.654	0.479	0.91
	NMR BiLISA	4	0.854	0.762	0.11
Urine	NMR QUANT	2	0.693	0.09	0.87
	MS (NEG)	3	0.468	0.277	1
	MS (POS)	2	0.485	0.403	1
Faecal	MS BA (NEG)	5	0.724	0.49	0.01

Table 27: Summary of PCA analysis in PBC vs AMANL

PCA-X co-ordinates [A= no of principal components with R^2X and Q^2 values] and corresponding multivariate analysis CV-ANOVA p-values for serum, urine, and faecal assays of PBC patients compared with AMA antibody positive with normal LFT patients. R^2 predicts goodness of fit and Q^2 is goodness of prediction of the model. Significant p-values (<0.05) are highlighted in green.

Serum analysis comparing PBC vs AMANL

- **Serum Bile Acid (negative ion mode) PBC vs AMANL**

A total of 40 patients with PBC and 13 AMANL were included in the final analysis. A 3-component PCA model (R^2X=0.756, Q^2=0.541, good model) attained visual discrimination between patients with PBC and HV, in serum bile acids on mass spectrometry. This was confirmed subsequently on a 3-component OPLS DA model, with the following statistics: [(1+2+0), R^2X=0.575, R^2Y=0.597, Q^2=0.262, CV-ANOVA p=0.03] (Table 28). Permutation testing demonstrated that the model was valid in that cut-offs for R^2 and Q^2 were lower in the randomly permutated model (999 permutations) (Figure 21). Metabolites with a VIP score of ≥ 1 were used to predict their influence on class separation between the two study groups (Table 29). **Resonance increased in serum bile acids of PBC patients included metabolites of UDCA** (Glycoursodeoxycholic Acid-3-Sulfate, Glycoursodeoxycholic Acid, Tauroursodeoxycholic Acid, Ursodeoxycholic Acid-3-Sulfate, Ursodeoxycholic Acid,

Tauroursodeoxycholic Acid-3-Sulfate), **and secondary bile acid metabolites** (Taurohyocholic Acid, Murocholic Acid, Lithocholic Acid 3-Sulfate and Glycolithocholic Acid-3-Sulfate) (Table 30).

				CV-ANOVA p-value
PCA- X Coordinates	A	R^2X	Q^2	
	3	0.756	0.541	0.03

OPLS-DA	A	R^2X	R^2Y	Q^2
Coordinates	1+2+0	0.575	0.597	0.262

Table 28: PCA and OPLS-DA coordinates of PBC (n=40) and AMANL (n=13) (Serum bile acids, MS- negative ion mode).

A= no of principal components, R^2 predicts goodness of fit and Q^2 goodness of prediction of the model. Significant p-values (<0.05)]

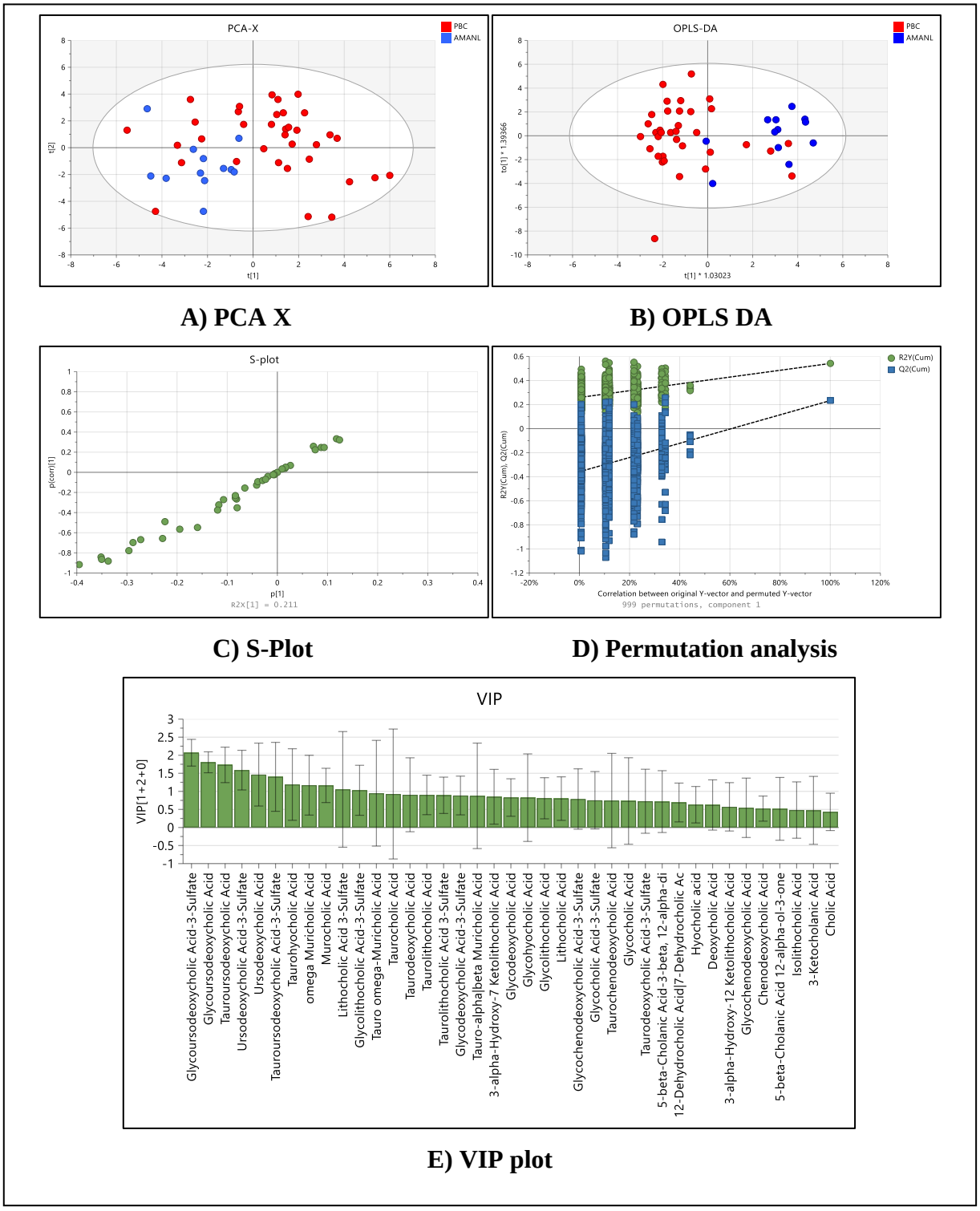


Figure 21: Serum BA profile models PBC (n=40) vs AMANL (n=13)

These multivariate models demonstrate discrimination of patients with PBC and AMANL. (A) Principal components analysis (PCA) scores plot 3-component model [R^2X=0.756, Q2=0.541]. (B) Orthogonal projection least squares discriminant analysis (OPLS-DA) scores plot [(1+2+0), R^2X=0.575, R^2Y=0.597, Q^2=0.262]. (C) S-line loading plot (D) Permutation analysis (E) VIP-scores plot.

Glycoursodeoxycholic Acid-3-Sulfate	2.07
Glycoursodeoxycholic Acid	1.80
Tauroursodeoxycholic Acid	1.73
Ursodeoxycholic Acid-3-Sulfate	1.59
Ursodeoxycholic Acid	1.46
Tauroursodeoxycholic Acid-3-Sulfate	1.40
Taurohyocholic Acid	1.19
Murocholic Acid	1.16
Lithocholic Acid 3-Sulfate	1.05
Glycolithocholic Acid-3-Sulfate	1.03

Table 29: Serum Bile acid metabolites with VIP >1, PBC (n=40) vs AMANL (n=13)

Serum BA	PBC (median + IQR)	AMANL (median + IQR)	p-value Mann-Whitney
Glycoursodeoxycholic Acid-3-Sulfate	178530 (30082 - 332558)	2567 (1233 – 7950)	<0.0001
Glycoursodeoxycholic Acid	475315 (139011 - 1166084)	19296 (5615 – 61765)	<0.0001
Tauroursodeoxycholic Acid	10517 (3640 - 45737)	790.0 (364.6 – 1772)	0.0004
Ursodeoxycholic Acid-3-Sulfate	17308 (5114 - 44957)	852.6 (392.3 – 3498)	<0.0001
Ursodeoxycholic Acid	118301 (17172 – 427461)	3946 (2423 – 7763)	0.003
Tauroursodeoxycholic Acid-3-Sulfate	3494 (2044 – 7213)	1000 (835.3 – 1905)	<0.0001
Murocholic Acid	62290 (16513 – 179113)	13269 (2197 – 27203)	0.001
Lithocholic Acid 3-Sulfate	3153 (2335 – 7417)	2297 (1525 – 2966)	0.01
Glycolithocholic Acid-3-Sulfate	47658 (16473 – 87993)	10483 (4383 – 23828)	0.0009

Table 30: Mass spectrometry -serum bile acids negative ion mode in PBC vs AMANL

Mass spectrometry observed serum bile acid metabolites with intensity differences (Median + IQR) associated in patients with PBC [n=40] and AMANL [n=13]. Statistical significance was determined by a non-parametric Mann Whitney test, comparing ranks. Significant p-values (<0.05) are highlighted in green. Resonances increased in patients with PBC were collection of metabolites of UDCA and secondary BA

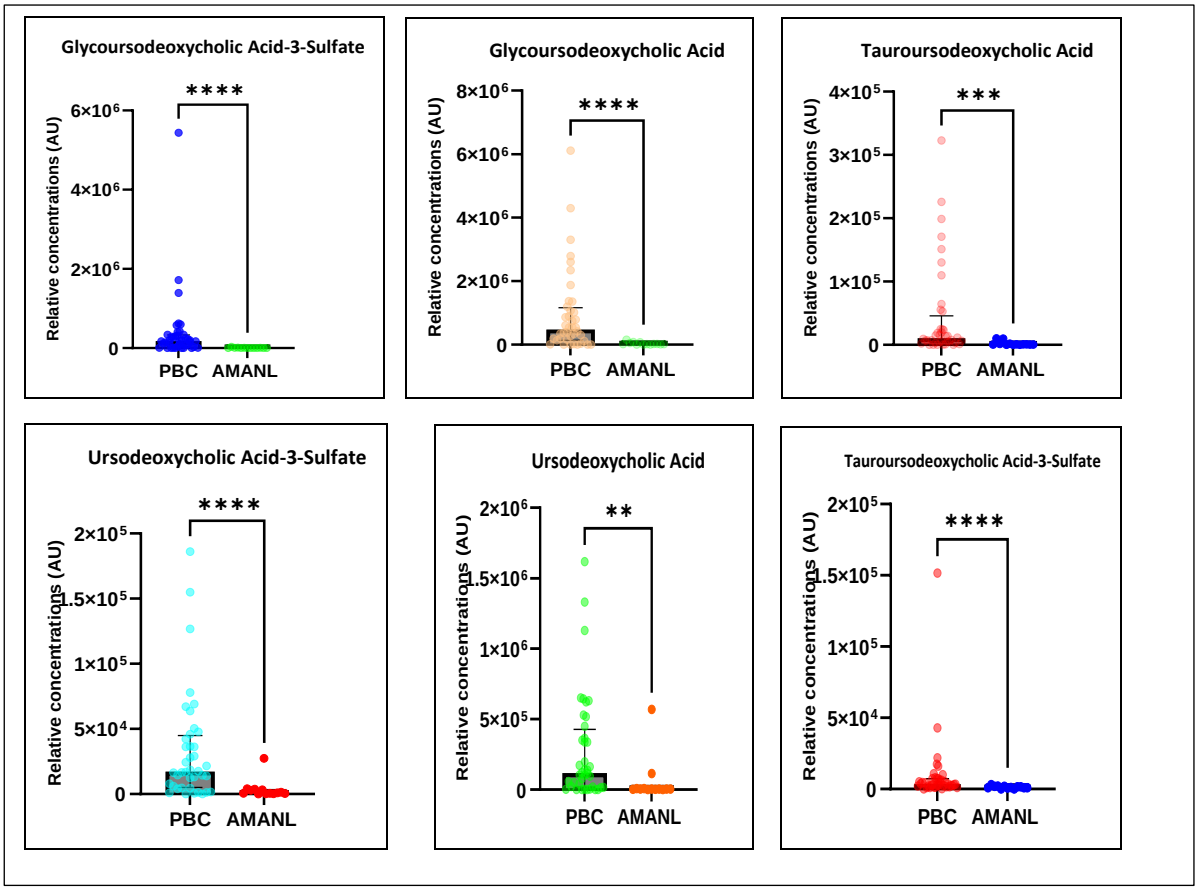


Figure 22: Targeted metabolomics: Panel to compare individual UDCA metabolites measured in the serum of patients with PBC [n=40] and AMANL [n=13].

The X-axis shows the relative serum concentrations expressed in Arbitrary Units (AU). p-value: ns= no significant, <0.05*, <0.01**, <0.001***, <0.0001****.

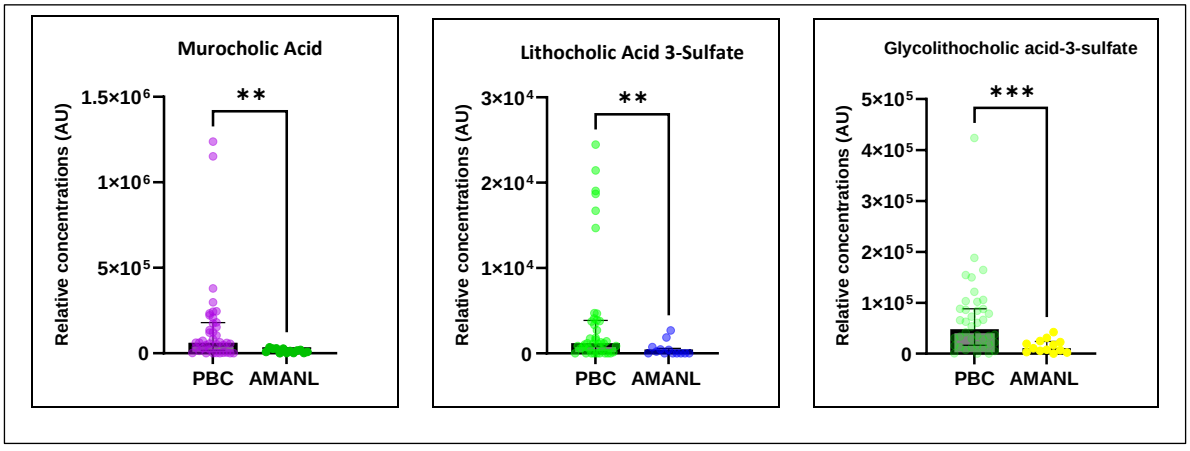


Figure 23: Targeted Metabolomics: Panel to compare individual (other) secondary BA metabolites measured in serum in patients with PBC [n=40] and AMANL [n=13].

The X-axis shows the relative serum concentrations expressed in Arbitrary Units (AU). p-value: ns= no significant, <0.05*, <0.01**, <0.001***, <0.0001****.

- **Serum MS Lipids (negative ion mode) PBC vs AMAL**

A 4-component PCA model [R^2X=0.713, R^2Y=0.507] did not show any discrimination between the two groups in serum lipids on mass spectrometry in negative ion mode (p=0.57)

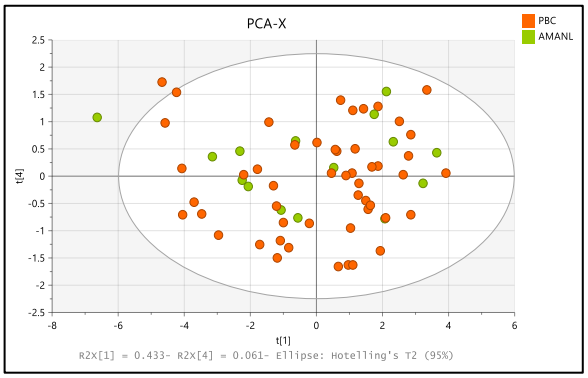


Figure 24: PCA model comparing serum MS lipids (negative ion) in PBC (n=50) and AMANL (n=15).

- **Serum MS Lipids (positive ion mode) PBC vs AMANL**

A 6-component PCA model [R^2X=0.654, R^2Y=0.479] did not show any discrimination between the two groups in serum lipids on mass spectrometry in positive ion mode (p=0.91)

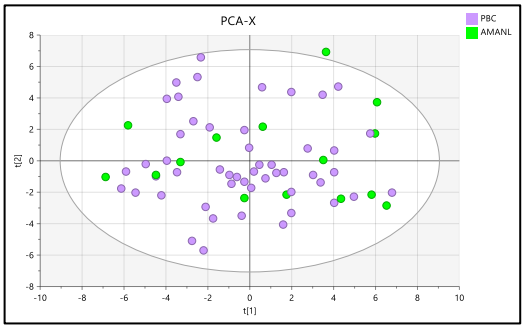


Figure 25: PCA model comparing serum MS lipids (positive ion mode) in PBC (n=51) and AMANL (n=15).

- **Serum NMR BiLISA PBC vs AMANL**

A 4-component PCA model [R^2X=0.854, R^2Y=0.762] did not show any discrimination between the two groups in serum lipids on NMR (p=0.11)

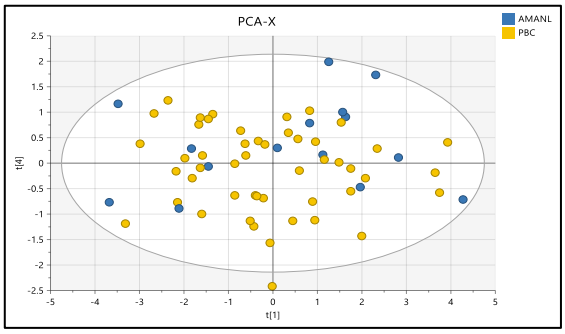


Figure 26: PCA model comparing serum NMR BiLISA in PBC (n=51) and AMANL (n=15).

Urine analysis PBC vs AMANL

- **Urine NMR QUANT PBC vs AMANL**

A 2-component PCA model [R^2X=0.693, R^2Y=0.09] did not show any discrimination between the two groups in urine metabolites on NMR (p=0.87)

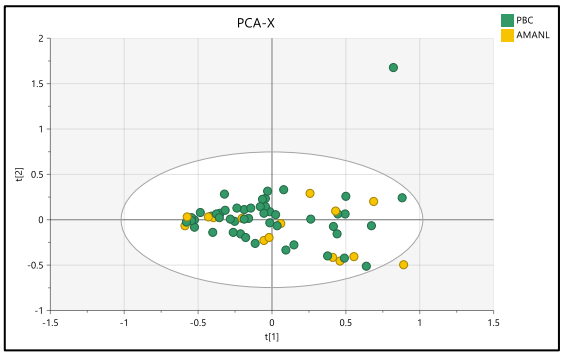


Figure 27: PCA model comparing Urine NMR in PBC (n=51) and AMANL (n=15).

- **<u>Urine MS (negative ion mode) PBC vs AMANL</u>**

A 3-component PCA model [R^2X=0.468, R^2Y=0.277] did not show any discrimination between the two groups in urine metabolites on mass spectrometry in negative ion mode (p=1)

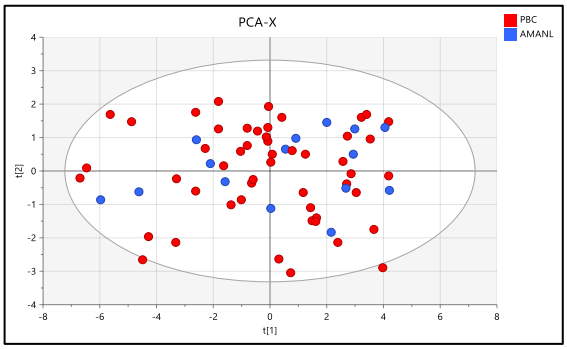


Figure 28: PCA model comparing Urine MS (negative ion mode) in PBC (n=51) and AMANL (n=15).

- **<u>Urine MS (positive ion mode) PBC vs AMANL</u>**

A 2-component PCA model [R^2X=0.485, R^2Y=0.403] did not show any discrimination between the two groups in urine metabolites on mass spectrometry in positive ion mode (p=1)

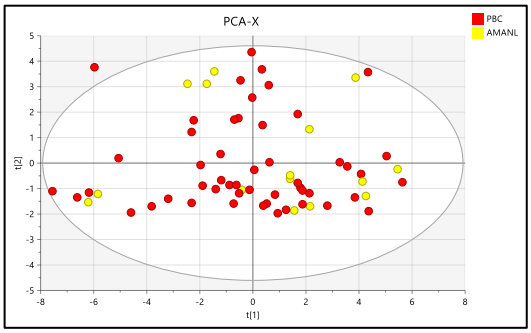


Figure 29: PCA model comparing Urine MS (positive ion mode) in PBC (n=51) and AMANL (n=15).

Faecal Analysis PBC vs AMANL

Faecal Bile Acids (negative ion mode) PBC vs AMANL

A total of 50 patients with PBC and 15 AMANL were included in the final analysis. A 5-component PCA model (R^2X=0.724, Q^2=0.49) attained visual discrimination between patients with PBC and HV, in faecal bile acids on mass spectrometry. This was confirmed subsequently on the 2-component OPLS DA model, with the following statistics: [(1+1+0), R^2X=0.45, R^2Y=0.379, Q^2=0.179, CV-ANOVA p=0.01] (Table 31). Permutation testing demonstrated that the model was valid in that cut-offs for R^2 and Q^2 were lower in the randomly permutated model (999 permutations) (Figure 30). Metabolites with a VIP score of ≥ 1 was used to predict their influence on class separation between the two study groups (Table 32). **Resonance increased in faeces of PBC patients included metabolites of UDCA** (Ursodeoxycholic Acid-3-Sulfate, Glycoursodeoxycholic Acid) **and secondary bile acid metabolites** (Lithocholic Acid 3-Sulfate, omega Muricholic Acid, beta Muricholic Acid, Glycolithocholic Acid, 6-Oxolithocholic Acid, 5-alpha-Cholanic Acid-3-alpha-ol-6-one, Allolithocholic Acid, Lithocholic Acid, and 3-ketocholanic-acid) (Table 33).

				CV-ANOVA p-value
PCA- X Coordinates	A	R^2X	Q^2	
	5	0.724	0.49	0.01

OPLS-DA	A	R^2X	R^2 Y	Q^2
Coordinates	1+1+0	0.45	0.379	0.179

Table 31: PCA and OPLS-DA coordinates of PBC (n=50) and AMANL (n=15) (Faecal bile acids negative ion mode).

A= no of principal components, R^2 predicts goodness of fit, and Q^2 goodness of prediction of the model. Significant p-values (<0.05)

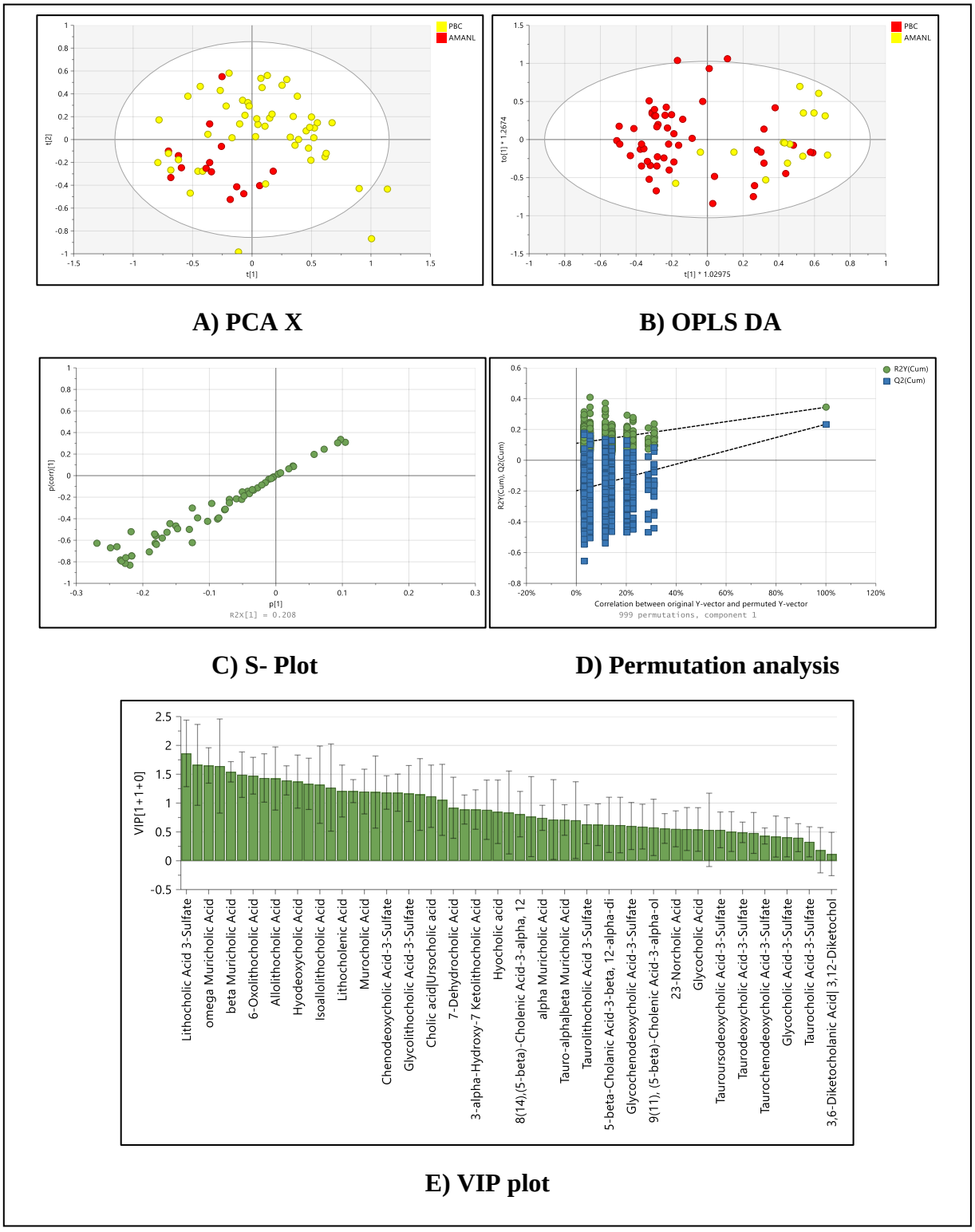


Figure 30: Fecal BA profile models PBC (n=50) and AMANL (n=15).

These multivariate models demonstrate discrimination of patients with PBC and AMANL. (A) Principal components analysis (PCA) scores plot 5-component model [R^2X=0.724, Q^2=0.49]. (B) Orthogonal projection least squares discriminant analysis (OPLS-DA) scores plot [(1+1+0), R^2X=0.45, R^2Y=0.379, Q^2=0.179]. (C) S-line loading plot (D) Permutation analysis (E) VIP-scores plot.

Lithocholic Acid 3-Sulfate	1.84
omega Muricholic Acid	1.64
Ursodeoxycholic Acid-3-Sulfate	1.61
Glycoursodeoxycholic Acid	1.59
beta Muricholic Acid	1.52
Glycolithocholic Acid	1.46
6-Oxolithocholic Acid	1.46
5-alpha-Cholanic Acid-3-alpha-ol-6-one	1.45
Allolithocholic Acid	1.40
Lithocholic Acid	1.37
Hyodeoxycholic Acid	1.34
Deoxycholic Acid-3-Sulfate	1.30
Isoallolithocholic Acid	1.29
Tauroursodeoxycholic Acid	1.28
5-Cholenic Acid-3-beta-ol	1.19
Glycoursodeoxycholic Acid-3-Sulfate	1.18
Lithocholenic Acid	1.18
Murocholic Acid	1.15
Chenodeoxycholic Acid-3-Sulfate	1.15
3-Ketocholanic Acid	1.13
Glycolithocholic Acid-3-Sulfate	1.13
Ursodeoxycholic Acid	1.09
Cholic acid| Ursocholic acid	1.08
5-alpha-Cholanic Acid-3-one	1.05

Table 32: Faecal BA metabolites with VIP >1 PBC (n=50) vs AMANL (n=15)

FECAL BA	PBC (median + IQR)	AMANL (median + IQR)	p-value Mann-Whitney
Lithocholic Acid 3-Sulfate	500381 (30712 – 5765829)	9872 (6932 – 64036)	0.001
omega Muricholic Acid	253417 (27086 – 4907406)	40312 (17170 – 159104)	0.0067
Ursodeoxycholic Acid-3-Sulfate	32226 (5561 – 111990)	1924 (1023 – 4433)	<0.0001
Glycoursodeoxycholic Acid	90051 (20438 – 320399)	5334 (2273 – 12379)	<0.0001
beta Muricholic Acid	41692 (16302 -110196)	5713 (2284 – 11081)	<0.0001
Glycolithocholic Acid	11926 (5865 – 23160)	1975 (691.9 – 3809)	<0.0001
6-Oxolithocholic Acid	27650 (11093 – 90502)	4271 (2652 – 10469)	<0.0001
5-alpha-Cholanic Acid-3-alpha-ol-6-one	7755 (2127 – 21964)	1202 (750.5 – 1697)	<0.0001
Allolithocholic Acid	26899 (9071 – 65221)	5415 (2827 – 22782)	0.0036
Lithocholic Acid	31740469 (15679845 – 42696379)	5760324 (175358 – 15027126)	<0.0001
Hyodeoxycholic Acid	91912 (40858 – 247884)	24878 (10104 – 52181)	<0.0001
Deoxycholic Acid-3-Sulfate	15656 (2818 – 194301)	3939 (1792 – 9523)	0.085
Isoallolithocholic Acid	1698654 (663561 – 2811258)	525048 (338106 – 720948)	0.002

Table 33: Mass Spectrometry- faecal bile acids in PBC and AMANL

Mass spectrometry observed faecal bile acid metabolites with intensity differences (Median + IQR) associated with patients with PBC [n=50] and AMANL [n=15]. Statistical significance was determined by a non-parametric Mann-Whitney test, comparing ranks. Significant p-values (<0.05) are highlighted in green.

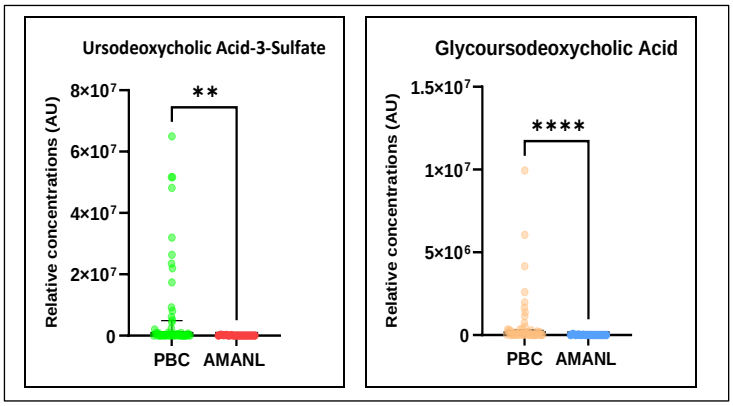


Figure 31: Targeted Metabolomic: Panel to compare individual UDCA metabolites measured in faeces in patients with PBC [n=50] and AMANL [n=15].

X-axis shows the relative faecal concentrations of bile acids expressed in Arbitrary Units (AU). p-value: ns= no significant, <0.05*, <0.01**, <0.001***, <0.0001****.

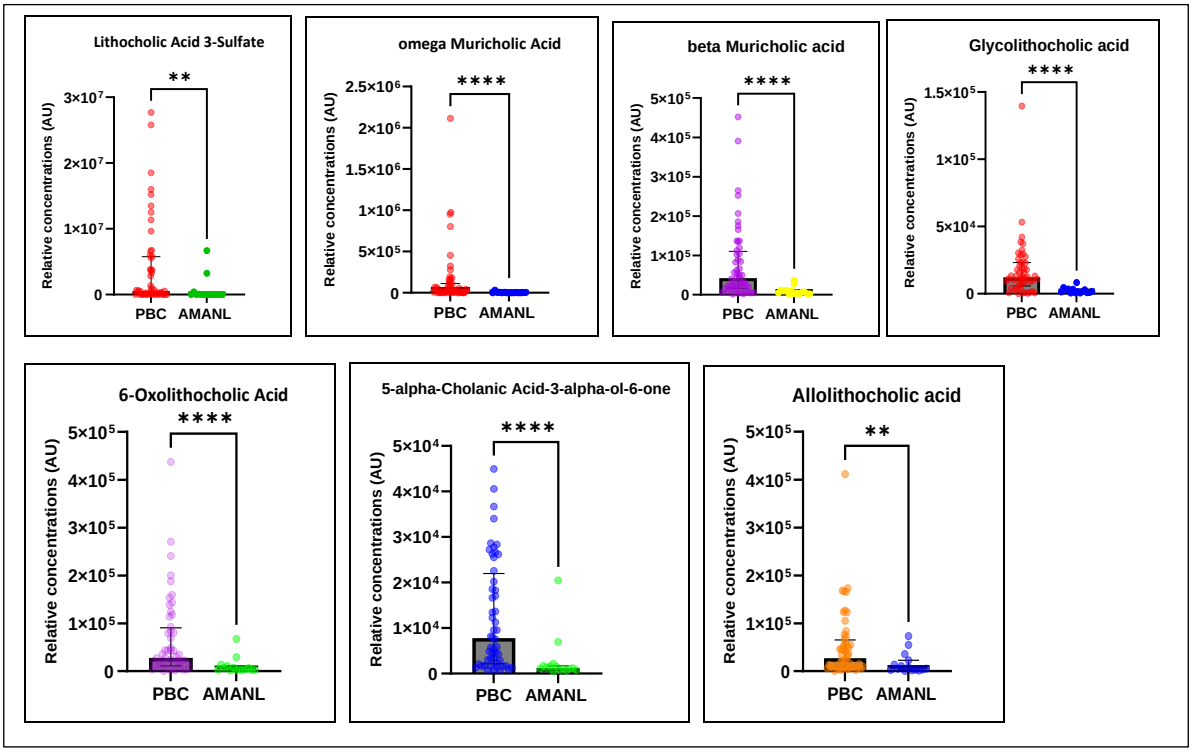


Figure 32: Targeted Metabolomics- Panel to compare individual (other) secondary BA metabolites measured in faeces in patients with PBC [n=50] and AMANL [n=15].

X-axis shows the relative faecal concentrations of bile acids expressed in Arbitrary Units (AU). p-value: ns= no significant, <0.05*, <0.01**, <0.001***, <0.0001****.

3.2 Discussion:

The majority of the patients in this study in the **PBC group were on established UDCA therapy**. This altered the total bile acid pool which was significantly higher in the PBC group, with a major contribution from UDCA and metabolites. The higher levels of the UDCA in PBC patients can be explained by the fact that 49/51 (96.1%) of the PBC patients were on UDCA {median (range) dose = 13.8 (7-16) mg/kg. Increased LCA levels, could in part represent a result of the UDCA therapy. LCA is mainly produced by bacterial 7-dehydroxylation of unabsorbed bile acids in the colon.

Lithocholic acid (LCA) is a secondary bile acid that is produced in the human intestine by deconjugation and α-hydroxylation of a primary bile acid Chenodeoxycholic acid (CDCA) (Guzior and Quinn, 2021, Kakiyama et al., 2014). In cholestasis, LCA is thought to be toxic to hepatocytes. Levels of LCA are increased in liver tissue in chronic cholestasis. In addition, there is an impairment of sulfation of LCA(Fischer et al., 1996). LCA in cholestatic mice models has been shown to impair bile acid transporters and cause obstruction to biliary canaliculi by forming crystal plugs(Yang et al., 2018a, Fickert et al., 2006). High levels of LCA are shown to stimulate oxidative stress, and tissue proliferation and inhibit DNA damage repair, thereby acting as a potential carcinogen(Nguyen et al., 2021). However, more recent reports have shown LCA to exert anti-inflammatory, anti-tumour and anti-ageing effects (Li et al., 2022, Goldberg et al., 2010, Lajczak-McGinley et al., 2020, Arlia-Ciommo et al., 2014). Alterations in the gut microbiota (dysbiosis) have been well-reported in PBC patients.

An in vitro study has demonstrated the antibacterial activity of LCA against Helicobacter pylori (González et al., 2020). The minimal bactericidal concentration (MBC) is defined as the lowest concentration of an antibacterial agent that can kill a specific strain of bacteria. The study found that LCA exhibited antibacterial effects even against clarithromycin and metronidazole-resistant strains of H. pylori, with an MBC of 32 mg/L. Additionally, LCA showed synergistic effects with clarithromycin and levofloxacin, indicated by fractional inhibitory concentration indices (FICI) of 0.53 and 1.0, respectively. Another study testing LCA against six different bacterial strains found significant antibacterial activity in Escherichia coli (MBC = 0.08 mM), Bacillus cereus (MBC = 0.08 mM), and Staphylococcus aureus (MBC = 0.04 mM) (Do Nascimento et al., 2015).

LCA has been shown to have a protective effect against TNF-α-induced injury to the intestinal mucosal barrier. An in vivo study using human Caco-2 epithelial cells derived from colonic tissue demonstrated that LCA prevented changes in membrane permeability when exposed to TNF-α ($p<0.01$). Permeability, measured by transepithelial electrical resistance (TEER),

exhibited a progressive decrease in a dose-dependent manner with LCA treatment. Immunofluorescence analysis revealed that LCA treatment attenuated the TNF-α induced disruption of tight junction proteins. Additionally, LCA treatment led to an upregulation of SIRT1, nuclear factor erythroid 2-related factor 2 (Nrf2), and heme oxygenase-1 (HO-1), counteracting the effects of TNF-α. The TNF-α induced upregulation of NF-κB p-p65 and p-IκB-α was significantly inhibited by LCA ($p<0.01$). SIRT1 is known for its antioxidative and DNA repair functions, while NF-κB regulates pro-inflammatory cytokines. Consequently, the upregulation of SIRT1 and downregulation of NF-κB by LCA exert a protective effect on intestinal mucosal epithelial cells (Yao et al., 2019).

A recent in vitro study using human colonic cancer Caco-2 cells reported that LCA treatment upregulated Vitamin D receptors (VDR). The pretreatment with LCA inhibited the degradation of IκBα induced by IL-1β and prevented the release of NFκ-B. Levels of phosphorylated p65, a marker of NFκ-B activity, were also significantly reduced with LCA pretreatment. Similarly, IL-8 levels showed a significant decrease ($p<0.01$) (Sun et al., 2008).

Sinha et al. utilized metabolomic techniques to analyse stool samples from patients with Ulcerative Colitis (UC) and familial adenomatous polyposis (FAP) with pouches. They found that the levels of secondary bile acids (SBAs), specifically LCA and DCA, were significantly lower in the UC group, which was associated with reduced alpha and beta diversity in this group. Additionally, using DSS-induced colitis mice, they demonstrated that treatment with LCA and DCA alleviated colitis, as indicated by reduced histological inflammation and lower leukocyte infiltration in the colon. Through Luminex multiplex immunoassay, they showed that LCA treatment significantly reduced pro-inflammatory cytokines (CCL5, CXCL10, IL-17A, and TNF-α) in the colonic cells of these experimental colitis mice. These findings support the anti-inflammatory effects of secondary bile acid (LCA) in the colon (Sinha et al., 2020).

Another study reported the protective effects of LCA in gall bladder cancer (GBC). They used 88 pair tissue samples from confirmed GBC and para-cancerous resections (Li et al., 2022). Compared to gallbladder adenoma (GA) LCA levels in bile and serum were significantly lower in GBC ($p<0.001$). Higher levels of glutaminase which inhibits the antiproliferative effect of LCA were seen in GBC and negatively correlated with patient survival ($p<0.001$). Another study reported antineoplastic effects of LCA in pancreatic adenocarcinoma (Schwarcz et al., 2024).

UDCA therapy has a limited effect on the restoration of this gut microbial homeostasis in treated PBC patients(Tang et al., 2018). The intestine being the site of formation of secondary bile acids, will be directly impacted by alterations in gut microbiota.

The relatively high abundance of secondary bile acid LCA metabolites in PBC patients compared to AMANL (3-6-fold), in addition to increased availability of UDCA in the colon, could also reflect a result of alterations in the bile acid metabolism secondary to alteration in the gut microbiota in this group of patients. Though we have not studied the gut microbiota composition in our study patients, these alterations have been shown to be implicated in the pathogenesis of cholestatic liver disease.

Limitations:

1. The study did not include treatment-naive patients with PBC who were followed prospectively. It's possible that ursodeoxycholic acid (UDCA) therapy influenced the overall composition of the bile acid pool.
2. We assumed that the AMANL group did not have liver disease because their liver blood tests were normal. However, some data suggest that there may be histological evidence of PBC in this group.
3. The study did not have microbiome data to analyse the effects of microbiomes on the composition of bile acids in the two study groups.

Chapter 4. Results 2: Comparison of Metabolomic Profile of Primary Biliary Cholangitis with Symptoms to no Symptoms; and UDCA Responders to UDCA Non-responders

4.1 PBC symptoms vs PBC no symptoms:

Comparison of PBC patient with pruritus to PBC patients with no pruritus

4.1.1 Results of analysis: PBC pruritus vs PBC no pruritus:

16 PBC pruritus and 34 PBC no-pruritus patients were included in the final analysis. PBC pruritus compared to PBC non-pruritus patients; there was no statistically significant difference in median age 59 vs 67 years (p=0.06), ALP levels 109 vs 114 (p=0.81), bilirubin levels 7 vs 7 (p=0.73), ALT levels 26 vs 26 (p=0.68) and GGT levels 40 vs 59 (p=0.45) **(Table 34)**. PCA followed by OPLS-DA gave robust differentiating models, which were cross-validated with multivariate CV-ANOVA analysis. The overview of the analysis is summarised in **(Table 35).**

	PBC no pruritus (Median/IQR)	PBC pruritus (Median/IQR)	p- value
Age	67 (61-71)	59 (43-77)	0.06
Albumin	44 (43-46)	46 (43-48)	0.07
Bilirubin	7 (6-8)	7 (5-9)	0.73
ALP	109 (79-144)	114 (69-240)	0.81
ALT	26 (15-33)	26 (13-55)	0.68
GGT	40 (28-119)	59 (30-297)	0.45
AST	28 (22-40)	36 (26-66)	0.15

Table 34: Age and liver biochemistry compared between PBC with pruritus and PBC with no-pruritus.

Median and IQR compared (with p-value) for Bilirubin, ALP- alkaline phosphatase, ALT- alanine aminotransferase, GGT- gamma glutamyl-transferase and AST- aspartate aminotransferase), between PBC pruritus and PBC no pruritus group.

Overview of analysis:

		A	R^2X	Q^2	p-value
Serum	MS Bile acid (NEG)	4	0.826	0.562	0.73
	MS Lipid (NEG)	4	0.727	0.517	1
	MS Lipid (POS)	7	0.695	0.453	1
	NMR BiLISA	4	0.864	0.746	1
Urine	NMR QUANT	2	0.559	0.005	0.48
	MS (NEG)	3	0.482	0.266	1
	MS (POS)	5	0.674	0.361	0.85
Faecal	MS BA (NEG)	7	0.806	0.492	0.01

Table 35: Summary of PCA-X co-ordinates and corresponding CV-ANOVA p-values for serum, urine, and faecal assays of PBC patients with pruritus (n=14) compared to those with no-pruritus (n=36).

R^2 predicts goodness of fit and Q^2 is goodness of prediction of the model. Significant p-values (<0.05) are highlighted in green.

Serum Analysis PBC pruritus vs PBC no pruritus:

- **Serum Bile Acid (Negative ion mode)**

A 4-component PCA model [R^2X=0.826, R^2Y=0.562] did not show any discrimination between the PBC pruritus and PBC no-pruritus groups in serum bile acids on mass spectrometry (p=0.73)

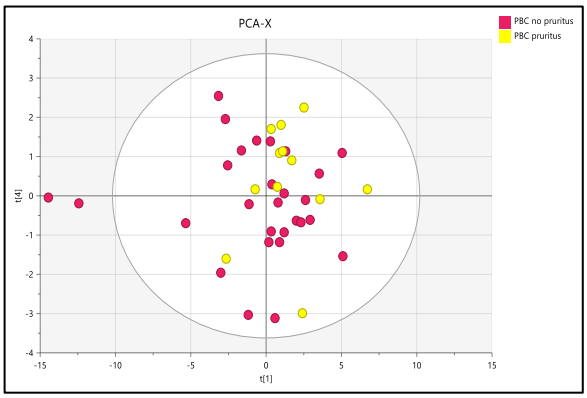


Figure 33: PCA model comparing serum bile acids in in PBC pruritus (n=14) vs PBC no pruritus (n=36).

Serum MS Lipids (Negative ion mode)

A 4-component PCA model [R^2X=0.727, R^2Y=0.517] did not show any discrimination in serum lipids between the PBC pruritus and PBC no-pruritus groups on mass spectrometry in negative ion mode (p=1)

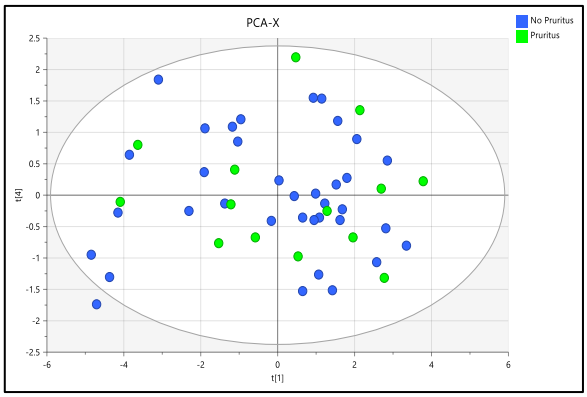


Figure 34: PCA model comparing serum lipids (MS negative ion mode) in in PBC pruritus (n=14) vs PBC no pruritus (n=36).

Serum MS Lipids (Positive ion mode)

A 7-component PCA model [R^2X=0.695, R^2Y=0.453] did not show any discrimination in serum lipids between the PBC pruritus and PBC no-pruritus groups on mass spectrometry in positive ion mode (p=1)

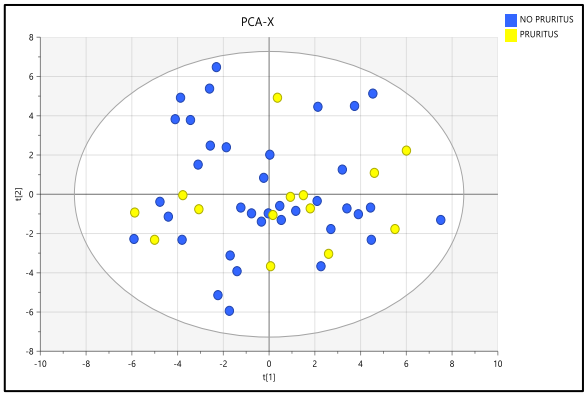


Figure 35: PCA model comparing serum lipids (MS positive ion mode) in in PBC pruritus (n=14) vs PBC no pruritus (n=36).

- **Serum NMR BiLISA**

A 4-component PCA model [R^2X=0.864, R^2Y=0.746] did not show any discrimination in serum lipids between the PBC pruritus and PBC no-pruritus groups on NMR (p=1)

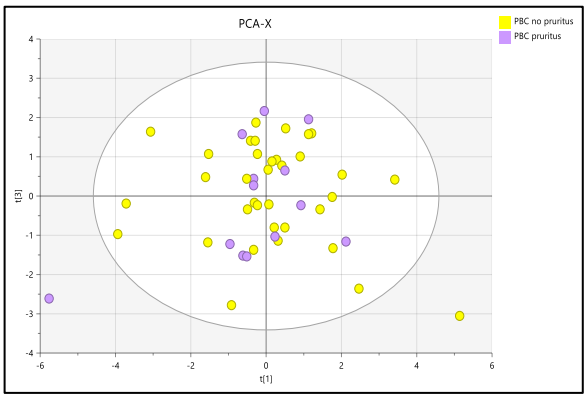


Figure 36: PCA model comparing serum lipids (NMR) in PBC pruritus (n=14) vs PBC no pruritus (n=36).

Urine analysis PBC pruritus vs PBC no pruritus:

- **Urine NMR QUANT**

A 2-component PCA model [R^2X=0.559, R^2Y=0.005] did not show any discrimination in urine metabolites between the PBC pruritus and PBC no-pruritus groups on NMR (p=0.48)

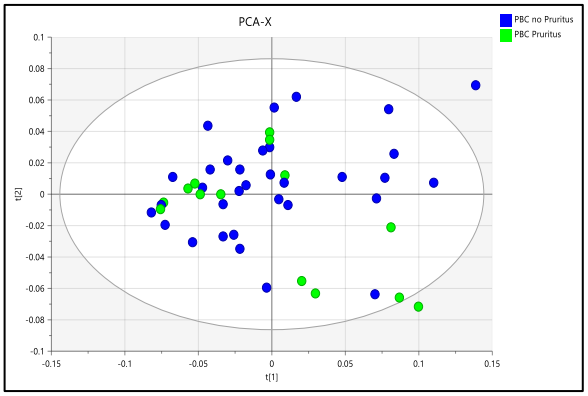


Figure 37: PCA model comparing Urine (NMR) in in PBC pruritus (n=14) vs PBC no pruritus (n=36).

- **Urine MS (Negative ion mode)**

A 3-component PCA model [R^2X=0.482, Q^2=0.266] did not show any discrimination in urine metabolites between the PBC pruritus and PBC no-pruritus groups on mass spectrometry in negative ion mode (p=1)

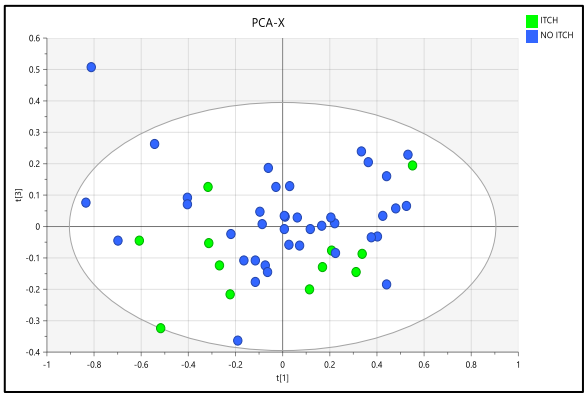


Figure 38: PCA model comparing Urine MS (Negative ion mode) in PBC pruritus (n=14) vs PBC no pruritus (n=36).

- **Urine MS (Positive ion mode)**

A 5-component PCA model [R^2X=0.674, Q^2=0.361] did not show any discrimination in urine metabolites between the PBC pruritus and PBC no-pruritus groups on mass spectrometry in positive ion mode (p=0.85)

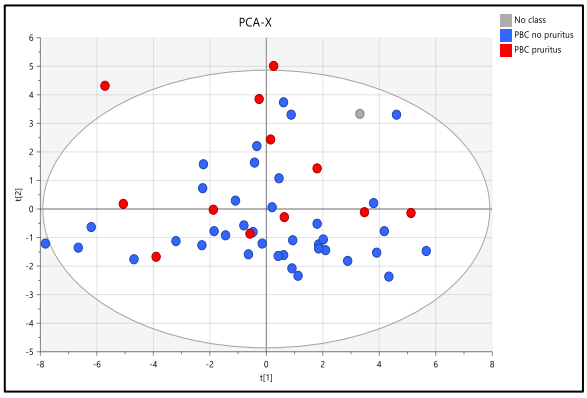


Figure 39: PCA model comparing Urine MS (Positive ion mode) in PBC pruritus (n=14) vs PBC no pruritus (n=36).

Faecal analysis PBC pruritus vs PBC no pruritus:

- **Faecal Bile Acid (Negative ion mode):**

A 7-component PCA model (R^2X=0.806, Q^2=0.492) attained visual discrimination between patients with PBC pruritus (n=14) and PBC no pruritus (n=36). This was confirmed subsequently on a 3-component OPLS DA model, with the following statistics: [(1+2+0), R^2X=0.479, R^2Y=0.662, Q^2=0.28, CV-ANOVA p=0.01] (Table 36). Permutation testing demonstrated that the model was valid in that cut-offs for R^2 and Q^2 were lower in the randomly permutated model (999 permutations) (Figure 40). Metabolites with a VIP score of ≥ 1 was used to predict their influence on class separation between the two study groups (Table 37). **Resonance increased in faeces of PBC patients with pruritus** included metabolites of secondary bile acids- Hyocholic acid, Glycohyocholic Acid, Hyodeoxycholic Acid, alpha Muricholic Acid, beta Muricholic Acid, omega Muricholic Acid and Taurolithocholic Acid (Table 38).

				CV-ANOVA p-value
PCA- X Coordinates	A	R^2X	Q^2	
	7	0.806	0.492	0.01

OPLS-DA Coordinates	A	R^2X	R^2Y	Q^2
	1+2+0	0.479	0.662	0.28

Table 36: PCA and OPLS-DA co-ordinates comparing Faecal Bile Acid in PBC pruritus (n=14) vs PBC no pruritus (n=36).

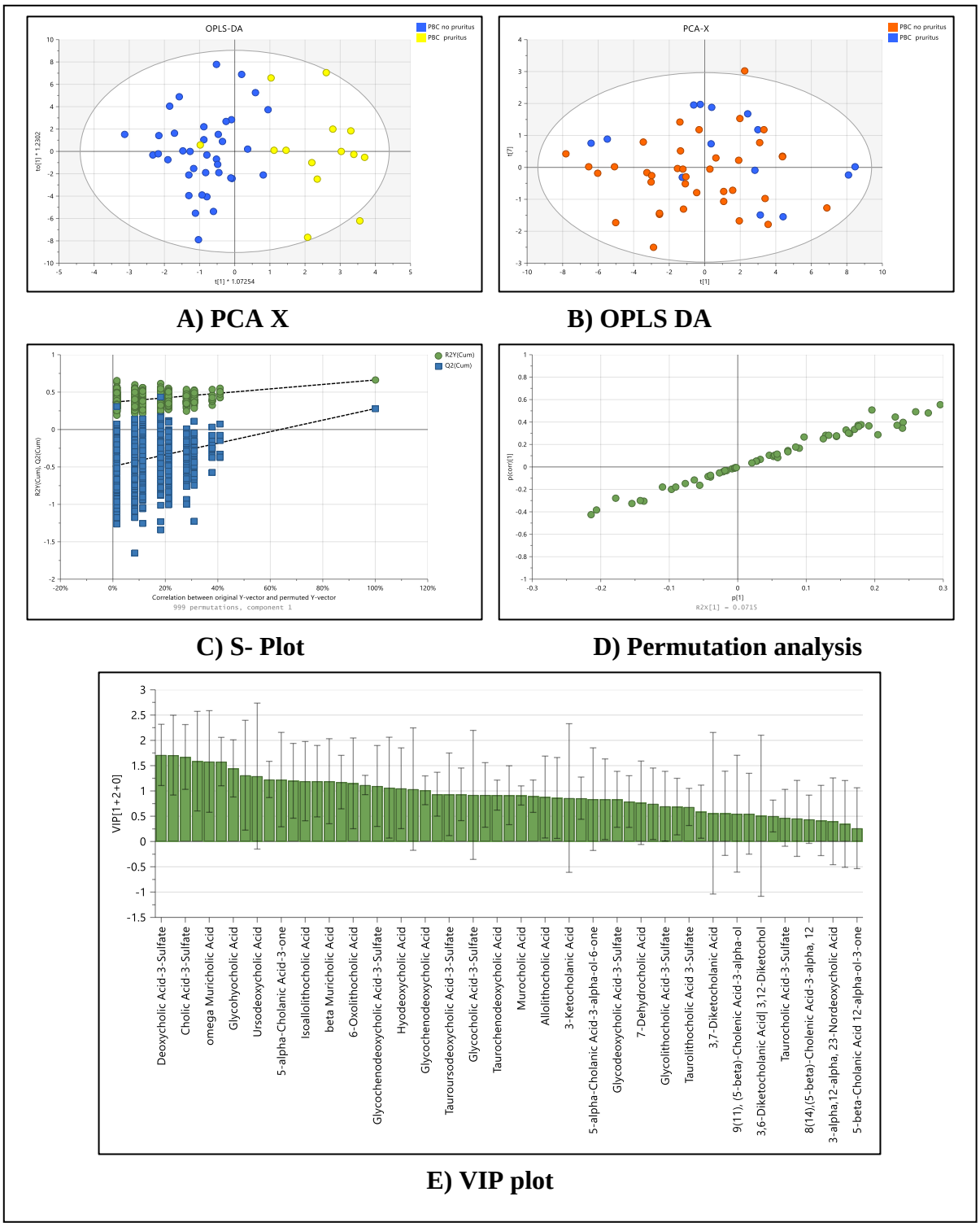


Figure 40: Fecal BA profile models PBC pruritus (n=14) vs PBC no pruritus (n=36).

These multivariate models demonstrate discrimination of patients with PBC pruritus and PBC no-pruritus. (A) Principal components analysis (PCA) scores plot [7-component model R^2X=0.806, Q^2=0.492]. (B) Orthogonal projection least squares discriminant analysis (OPLS-DA) scores plot [(1+2+0), R^2X=0.479, R^2Y=0.662, Q^2= 0.28]. (C) S-line loading plot (D) Permutation analysis (E) VIP-scores plot

Deoxycholic Acid-3-Sulfate	1.71
Hyocholic acid	1.67
Cholic Acid-3-Sulfate	1.59
Chenodeoxycholic Acid-3-Sulfate	1.58
omega Muricholic Acid	1.58
Ursodeoxycholic Acid-3-Sulfate	1.45
Glycohyocholic Acid	1.31
23-Norcholic Acid	1.29
Ursodeoxycholic Acid	1.23
Lithocholic Acid 3-Sulfate	1.22
5-alpha-Cholanic Acid-3-one	1.20
Isoallolithocholic Acid	1.19
Taurohyocholic Acid	1.19
beta Muricholic Acid	1.17
alpha Muricholic Acid	1.15
Glycoursodeoxycholic Acid	1.10
Glycochenodeoxycholic Acid-3-Sulfate	1.06
Taurodeoxycholic Acid-3-Sulfate	1.05
Hyodeoxycholic Acid	1.04
Taurolithocholic Acid	1.01
Glycochenodeoxycholic Acid	1.71

Table 37: Faecal BA metabolites with VIP >1, PBC pruritus (n=14) vs PBC no pruritus (n=36).

		PBC pruritus Median (IQR)	PBC no pruritus Median (IQR)	p-value Mann-Whitney
Deoxycholic Acid-3-Sulfate	1.71	158083 (6690 - 83537)	11203 (2426 - 111426)	0.14
Hyocholic acid	1.67	20023 (2417 - 56998)	2453 (1092 - 7757)	0.01
Cholic Acid-3-Sulfate	1.59	23556 (1126 - 135817)	1605 (1009 - 7150)	0.07
Chenodeoxycholic Acid-3-Sulfate	1.58	36234 (3108 - 1371463)	6435 (2552 - 40789)	0.14
omega Muricholic Acid	1.58	102982 (9064 - 578001)	25382 (1993 - 62390)	0.009
Ursodeoxycholic Acid-3-Sulfate	1.45	4139328 (82811 - 29667423)	253417 (24432 - 1252695)	0.18
Glycohyocholic Acid	1.31	879.7 (632.0 - 1104)	334.0 (113.7 - 514.9)	0.001
23-Norcholic Acid	1.29	6903 (4599 – 13312)	3635 (1513 - 9324)	0.06
Ursodeoxycholic Acid	1.23	803821 (293518 - 1519915)	1095708 (316802 – 4865123)	0.44
Lithocholic Acid 3-Sulfate	1.22	903.0 (549.0 – 2312)	863.4 (573.8 - 1366)	0.60
5-alpha-Cholanic Acid-3-one	1.20	12737 (3161 - 24244)	11964 (7542 - 43379)	0.34
Isoallolithocholic Acid	1.19	110087 (13506 – 430181)	150240 (54897 – 547191)	0.43
Taurohyocholic Acid	1.19	919.4 (468.3 - 2053)	569.7 (315.6 - 864.5)	0.09
beta Muricholic Acid	1.17	115736 (25085 – 115736)	9690 (1430 – 83014)	0.01
alpha Muricholic Acid	1.15	3788 (1546 – 5701)	1696 (855.8 – 5652)	0.007
Glycoursodeoxycholic Acid	1.10	61386 (25094 – 304763)	91471 (15691 – 413618)	0.76
Glycochenodeoxycholic Acid-3-Sulfate	1.06	818.0 (545.1 – 2232)	637.6 (465.5 - 917.0)	0.15
Taurodeoxycholic Acid-3-Sulfate	1.05	765.4 (316.5 – 1144)	530.0 (164.7 - 744.4)	0.12
Hyodeoxycholic Acid	1.04	195302 (68233 – 701185)	81738 (36710 – 151666)	0.02
Taurolithocholic Acid	1.01	17476 (3463 – 57711)	2782 (1579 – 10612)	0.01
Glycochenodeoxycholic Acid	1.71	29491 (6456 – 44625)	40849 (13977 – 75854)	0.20

Table 38: Mass spectrometry Faecal bile acids in PBC with pruritus and PBC with no pruritus.

Mass spectrometry observed serum lipid metabolites with intensity differences (Median +/- IQR) associated with patients with PBC pruritus (N=14), and PBC no-pruritus (N=36). Statistical significance was determined by a non-parametric Mann-Whitney test, comparing ranks. Significant p-values (<0.05) are highlighted in green. Resonances increased in patients with PBC with pruritus were metabolites of Hyocholic acid and muricholic acid.

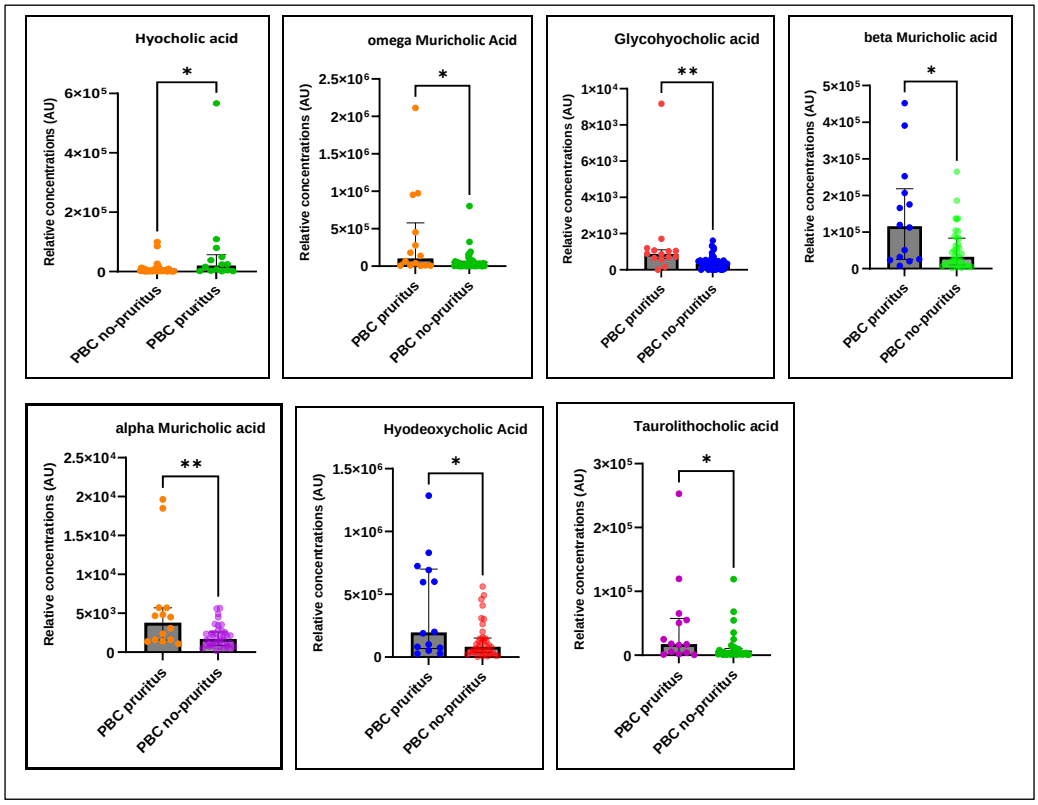


Figure 41: Targeted Metabolomics: Panel to compare BA metabolites measured in faeces in patients with PBC pruritus (N=14) compared to PBC no pruritus (N=36).

The X-axis shows the relative serum concentrations expressed in Arbitrary Units (AU). p-value: ns= no significant, <0.05*, <0.01**, <0.001***, <0.0001****.

Comparison of PBC patients with Fatigue to PBC patients without Fatigue

4.1.2 Results of analysis: PBC fatigue vs PBC no fatigue:

Overview of analysis:

		A	R^2X	Q^2	p-value
Serum	MS Bile acid (NEG)	4	0.826	0.562	0.82
	MS Lipid (NEG)	4	0.727	0.517	1
	MS Lipid	7	0.695	0.453	1
	NMR BiLISA	4	0.86	0.793	1
Urine	NMR QUANT	2	0.685	0.29	0.99
	MS (NEG)	5	0.428	0.266	0.11
	MS (POS)	5	0.674	0.361	0.28
Faecal	MS BA (NEG)	5	0.733	0.473	1

Table 39: Summary of PCA-X co-ordinated and corresponding CV-ANOVA p-values for serum, urine, and faecal assays of PBC patients with fatigue (n=19) compared to those with no fatigue (n=32).

R^2 predicts goodness of fit and Q^2 goodness of prediction of the model.

Serum analysis PBC fatigue vs PBC no fatigue:

- **Serum Bile Acid (Negative ion mode)**

A 4-component PCA model [R^2X=0.862, Q^2=0.562] did not show any discrimination in serum bile acids between the PBC fatigue and PBC no-fatigue groups on mass spectrometry (p=0.82).

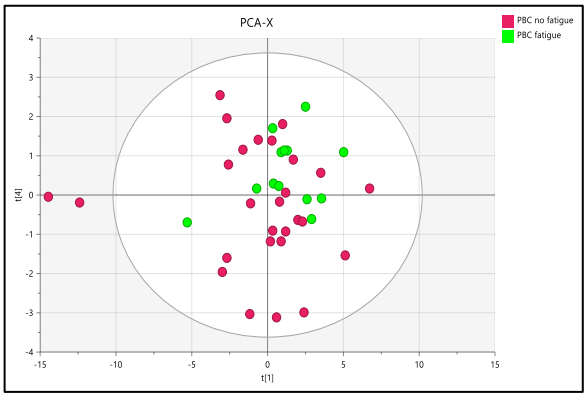


Figure 42: PCA model comparing Serum MS BAs negative ion mode in PBC fatigue (n=19) vs PBC no fatigue (n=32).

- **Serum MS Lipids (Negative ion mode)**

A 4-component PCA model [R^2X=0.727, Q^2=0.517] did not show any discrimination in serum lipids between the PBC fatigue and PBC no-fatigue groups on mass spectrometry in negative ion mode (p=1)

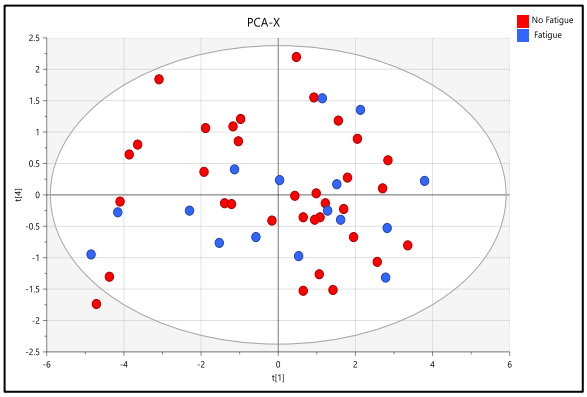


Figure 43: PCA model comparing Serum MS Lipids negative ion mode in PBC fatigue (n=19) vs PBC no fatigue (n=32).

- **Serum MS Lipids (Positive ion mode)**

A 7-component PCA model [R^2X=0.695, Q^2=0.453] did not show any discrimination in serum lipids between the PBC fatigue and PBC no-fatigue groups on mass spectrometry in positive ion mode (p=1)

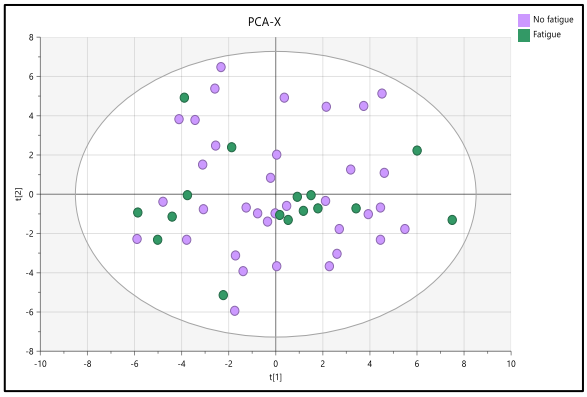


Figure 44: PCA model comparing Serum MS Lipids positive ion mode in PBC fatigue (n=19) vs PBC no fatigue (n=32).

- **Serum NMR BiLISA**

A 4-component PCA model [R^2X=0.86, Q^2=0.793] did not show any discrimination in serum lipids between the PBC fatigue and PBC no-fatigue groups on NMR (p=1)

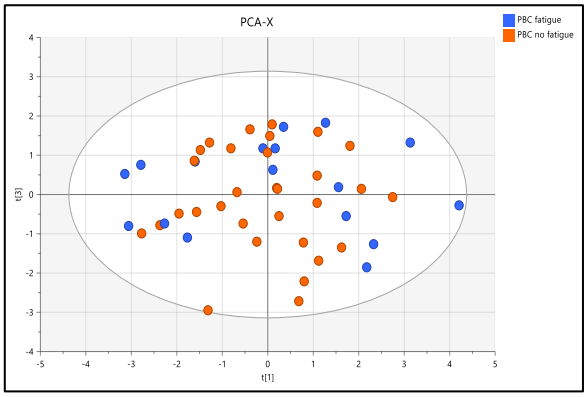


Figure 45: PCA model comparing Serum NMR BiLISA in PBC fatigue (n=19) vs PBC no fatigue (n=32).

Urine analysis PBC fatigue vs PBC no fatigue:

- **Urine NMR QUANT**

A 2-component PCA model [R^2X=0.685, Q^2=0.29] did not show any discrimination in urine metabolites between the PBC fatigue and PBC no-fatigue groups on NMR (p=0.99)

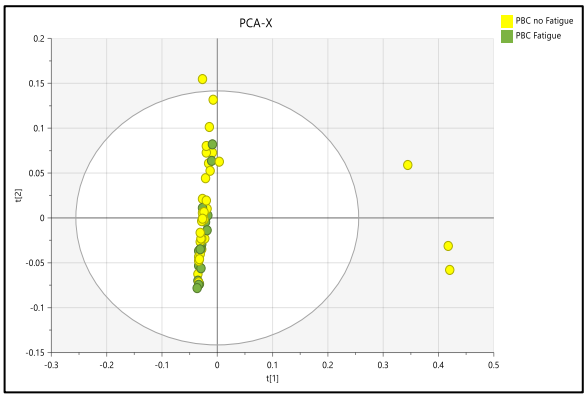


Figure 46: PCA model comparing Urine NMR in PBC fatigue (n=19) vs PBC no fatigue (n=32).

- **Urine MS (Negative ion mode)**

A 3-component PCA model [R^2X=0.482, Q^2=0.266] did not show any discrimination in urine metabolites between the PBC fatigue and PBC no-fatigue groups on mass spectrometry in negative ion mode (p=0.11)

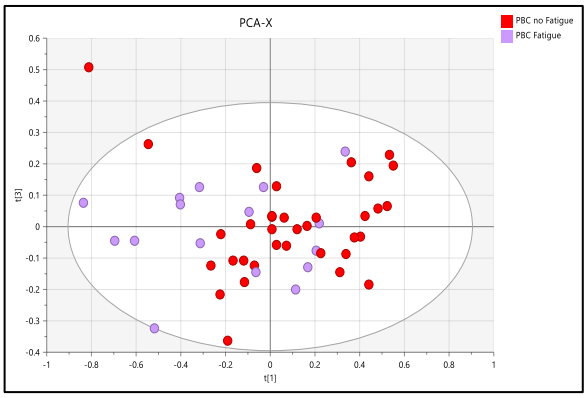


Figure 47: PCA model comparing Urine MS Lipids negative ion mode in PBC fatigue (n=19) vs PBC no fatigue (n=32).

- **Urine MS (Positive ion mode)**

A 5-component PCA model [R^2X=0.674, Q^2=0.361] did not show any discrimination in urine metabolites between the PBC fatigue and PBC no-fatigue groups on mass spectrometry in positive ion mode (p=0.28)

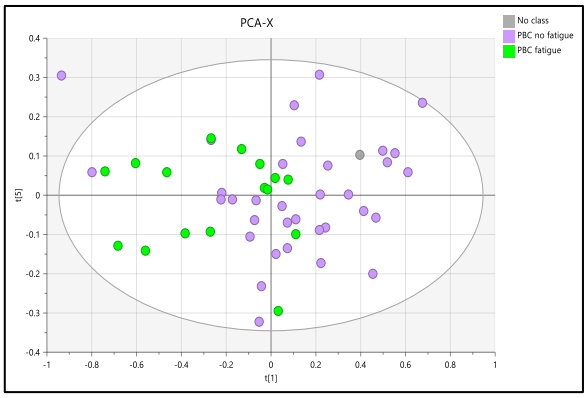


Figure 48: PCA model comparing Urine MS Lipids positive ion mode in PBC fatigue (n=19) vs PBC no fatigue (n=32).

Faecal Analysis PBC fatigue vs PBC no fatigue:

- **Faecal Bile Acid (Negative ion mode)**

A 5-component PCA model [R^2X=0.733, Q^2=0.473] did not show any discrimination in faecal bile acids between the PBC fatigue and PBC no-fatigue groups on mass spectrometry (p=1)

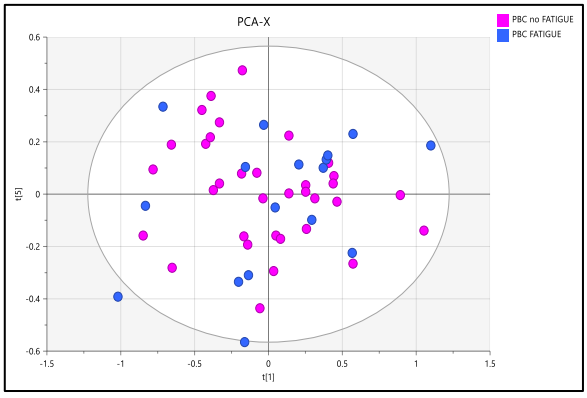


Figure 49: PCA model comparing Faecal BAs in PBC fatigue (n=19) vs PBC no fatigue (n=32).

Comparison of PBC patients with cognitive impairment to PBC patients with no cognitive impairment

4.1.3 Results of analysis: PBC cognitive impairment vs PBC no cognitive impairment:

Overview of analysis:

		A	R^2X	Q^2	p-value
Serum	MS Bile acid (NEG)	4	0.826	0.562	1
	MS Lipid (NEG)	4	0.727	0.517	0.38
	MS Lipid (POS)	8	0.729	0.475	1
	NMR BiLISA	4	0.863	0.765	1
Urine	NMR QUANT	2	0.685	0.29	1
	MS (NEG)	3	0.482	0.266	1
	MS (POS)	4	0.628	0.363	1
Faecal	MS BA (NEG)	7	0.806	0.492	0.95

Table 40: Summary of PCA-X co-ordinated and corresponding CV-ANOVA p-values for serum, urine, and faecal assays of PBC patients with cognitive impairment (n=8) compared to those with no cognitive impairment (n=41).

R^2 predicts goodness of fit and Q^2 goodness of prediction of the model.

Serum Analysis PBC cognitive impairment vs PBC no cognitive impairment:

- **Serum Bile Acid (Negative ion mode)**

A 4-component PCA model [R^2X=0.826, Q^2=0.562] did not show any discrimination in serum bile acids between the PBC cognitive impairment and PBC no-cognitive impairment groups on mass spectrometry (p=1)

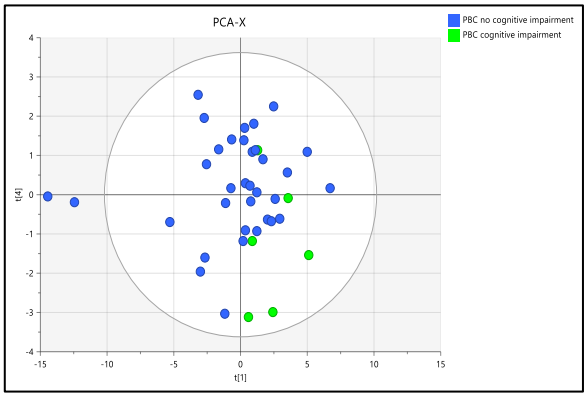


Figure 50: PCA model comparing Serum BAs negative ion mode in PBC cognitive impairment (n=8) vs PBC no cognitive impairment (n=41).

- **Serum MS Lipids (Negative ion mode)**

A 4-component PCA model [R^2X=0.727, Q^2=0.517] did not show any discrimination on serum lipids between the PBC cognitive impairment and PBC no-cognitive impairment groups on mass spectrometry in negative ion mode (p=0.38)

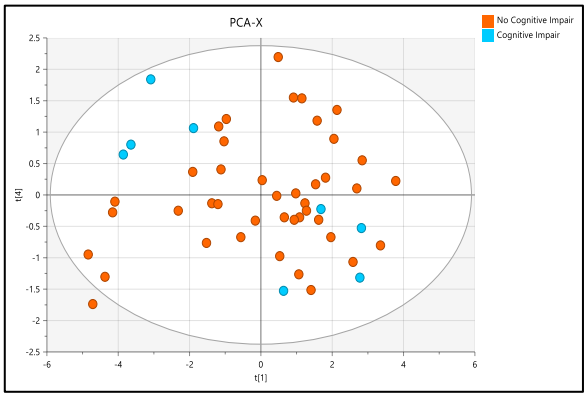


Figure 51: PCA model comparing Serum MS lipids negative ion mode in PBC cognitive impairment (n=8) vs PBC no cognitive impairment (n=41).

- **Serum MS Lipids (Positive ion mode)**

A, 8-component PCA model [R^2X=0.729, Q^2=0.475] did not show any discrimination in serum lipids between the PBC cognitive impairment and PBC no-cognitive impairment groups on mass spectrometry in positive ion mode (p=1)

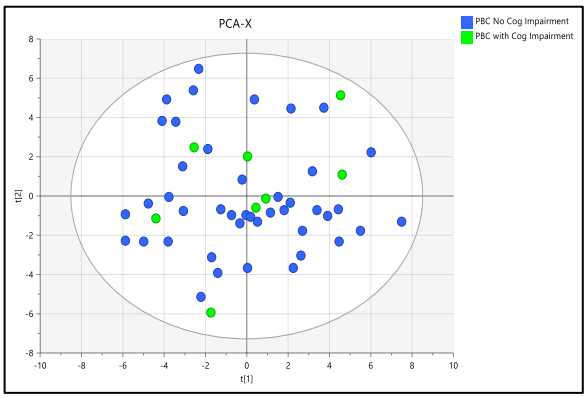


Figure 52: PCA model comparing Serum MS Lipids positive ion mode in PBC cognitive impairment (n=8) vs PBC no cognitive impairment (n=41).

- **Serum NMR BiLISA**

A 4-component PCA model [R^2X=0.863, Q^2=0.765] did not show any discrimination in serum lipids between the PBC cognitive impairment and PBC no-cognitive impairment groups on NMR (p=1)

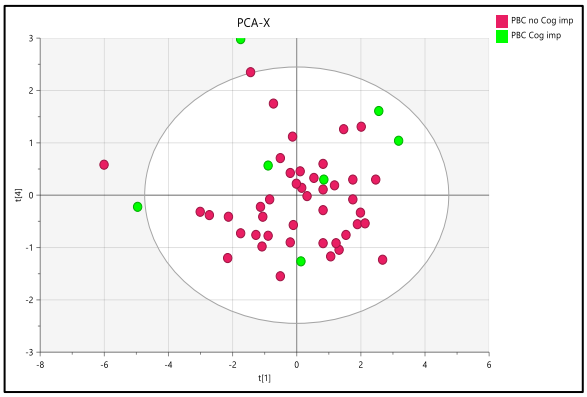


Figure 53: PCA model comparing Serum NMR BiLISA in PBC cognitive impairment (n=8) vs PBC no cognitive impairment (n=41).

Urine analysis PBC cognitive impairment vs PBC no cognitive impairment:

- **Urine NMR QUANT**

A 2-component PCA model [R^2X=0.685, Q^2=0.29] did not show any discrimination in urine metabolites between the PBC cognitive impairment and PBC no-cognitive impairment groups on NMR (p=1)

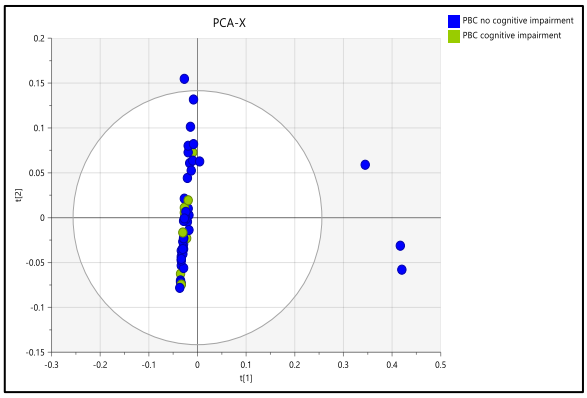


Figure 54: PCA model comparing Urine NMR QUANT in PBC cognitive impairment (n=8) vs PBC no cognitive impairment (n=41).

- **Urine MS (Negative ion mode)**

A 3-component PCA model [R^2X=0.482, Q^2=0.266] did not show any discrimination in urine metabolite between the PBC cognitive impairment and PBC no-cognitive impairment groups on mass spectrometry in negative ion mode (p=1)

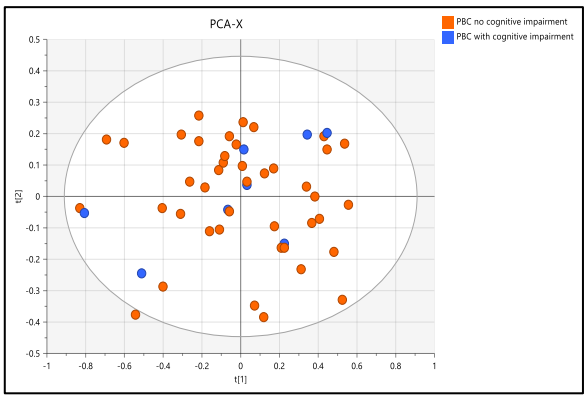


Figure 55: PCA model comparing Urine MS negative ion mode in PBC cognitive impairment (n=8) vs PBC no cognitive impairment (n=41).

- **Urine MS (Positive ion mode)**

A 4-component PCA model [R^2X=0.628, Q^2=0.363] did not show any discrimination in urine metabolites between the PBC cognitive impairment and PBC no-cognitive impairment groups on mass spectrometry in positive ion mode (p=1)

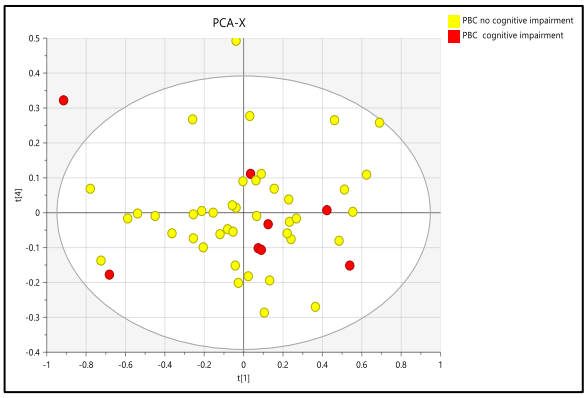


Figure 56: PCA model comparing Urine MS positive ion mode in PBC cognitive impairment (n=8) vs PBC no cognitive impairment (n=41).

Faecal analysis PBC cognitive impairment vs PBC no cognitive impairment:

- **Faecal Bile Acid (Negative ion mode)**

A 7-component PCA model [R^2X=0.806, Q^2=0.492] did not show any discrimination in faecal bile acids between the PBC cognitive impairment and PBC no-cognitive impairment groups on mass spectrometry (p=0.95)

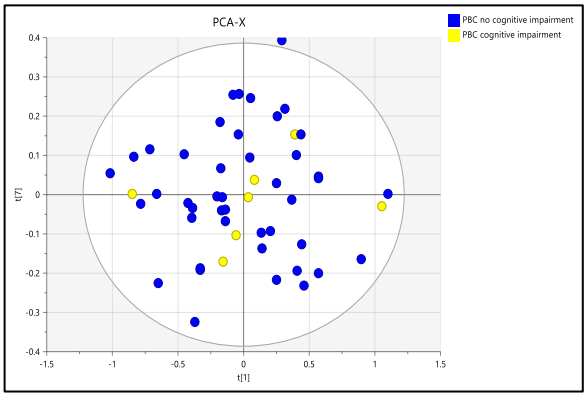


Figure 57: PCA and OPLS-DA model comparing faecal bile Acid in PBC cognitive impairment (n=8) vs PBC no cognitive impairment (n=41).

4.2 PBC UDCA responder vs PBC UDCA non-responder

4.2.1 Results of analysis:

		A	R^2X	Q^2	p-value
Serum	MS Bile acid (NEG)	4	0.838	0.66	0.16
	MS Lipid (NEG)	5	0.776	0.489	1
	MS Lipid (POS)	7	0.709	0.426	1
	NMR BiLISA	4	0.869	0.757	1
Urine	NMR QUANT	2	0.741	0.345	1
	MS (NEG)	3	0.486	0.261	1
	MS (POS)	3	0.587	0.391	0.88
Faecal	MS BA (NEG)	5	0.743	0.487	1

Table 41: Summary of PCA-X co-ordinated and corresponding CV-ANOVA p-values for serum, urine, and faecal assays of PBC patients with PBC UDCA responder (n=42) vs PBC UDCA non-responder (n=7) (based on Toronto Criterion).

R^2 predicts goodness of fit and Q^2 goodness of prediction of the model.

Serum Analysis UDCA responder vs UDCA non-responder

- **Serum Bile Acid (Negative ion mode)**

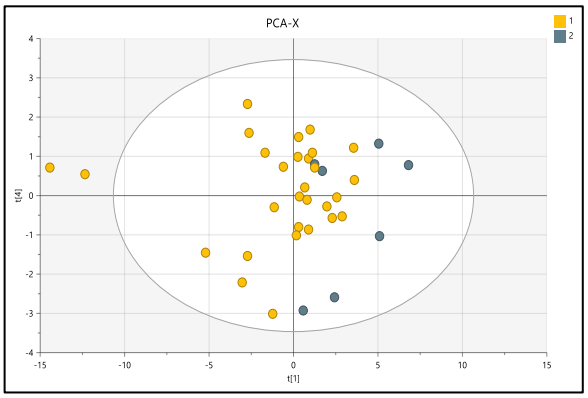


Figure 58: PCA model comparing Serum Bile Acid in PBC UDCA responder (n=42) vs PBC UDCA non-responder (n=7).

A 4-component PCA model [R^2X=0.838, Q^2=0.66] did not show any discrimination in serum bile acids between the PBC UDCA responders and PBC UCA non-responder groups on mass spectrometry (p=0.16)

- **Serum MS Lipids (Negative ion mode)**

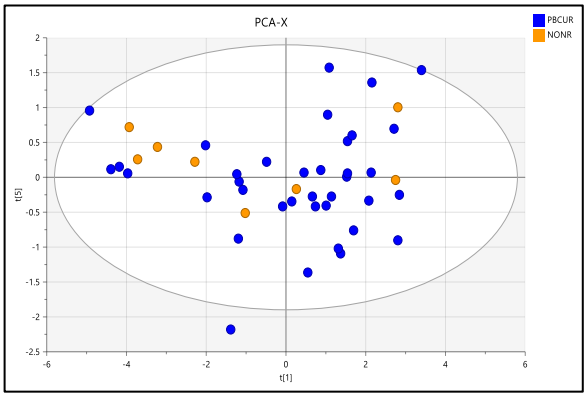


Figure 59: PCA model comparing MS Lipids negative ion mode in PBC UDCA responder (n=42) vs PBC UDCA non-responder (n=7).

A 5-component PCA model [R^2X=0.776, Q^2=0.489] did not show any discrimination in serum lipids between the PBC UDCA responders and PBC UCA non-responder groups on mass spectrometry in negative ion mode (p=1)

- **Serum MS Lipids (Positive ion mode)**

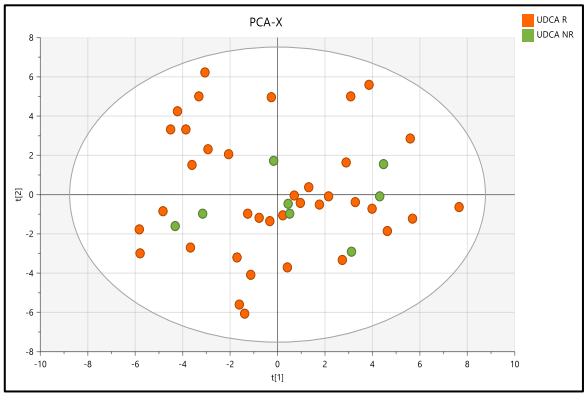


Figure 60: PCA model comparing MS Lipids positive ion mode in PBC UDCA responder (n=42) vs PBC UDCA non-responder (n=7).

A 7-component PCA model [R^2X=0.709, Q^2=0.426] did not show any discrimination in serum lipids between the PBC UDCA responders and PBC UCA non-responder groups on mass spectrometry in positive ion mode (p=1)

- **Serum NMR BiLISA**

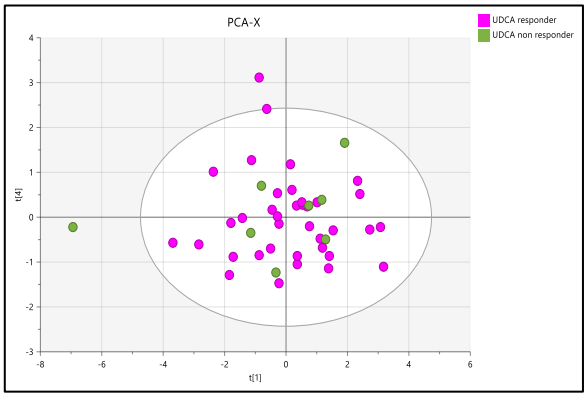


Figure 61: PCA model comparing Serum NMR BiLISA in PBC UDCA responder (n=42) vs PBC UDCA non-responder (n=7).

A 4-component PCA model [R^2X=0.869, Q^2=0.757] did not show any discrimination in serum lipids between the PBC UDCA responders and PBC UCA non-responder groups on NMR (p=1)

Urine Analysis UDCA responder vs non responder:

- **Urine NMR QUANT**

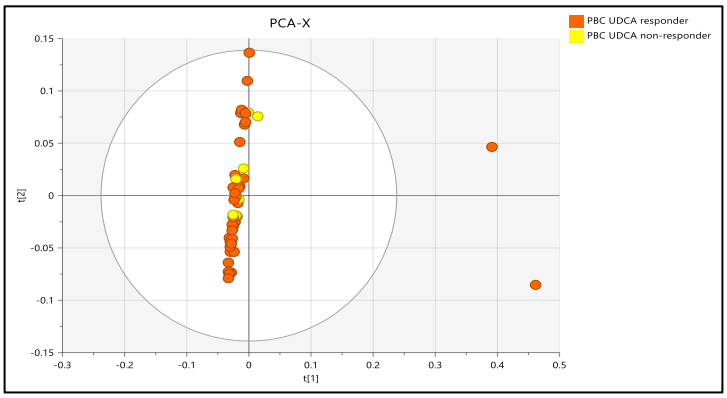


Figure 62: PCA model comparing Urine NMR QUANT in PBC UDCA responder (n=42) vs PBC UDCA non-responder (n=7).

A 2-component PCA model [R^2X=0.741, Q^2=0.345] did not show any discrimination in urine metabolites between the PBC UDCA responders and PBC UCA non-responder groups on NMR (p=1)

- **Urine MS (Negative ion mode)**

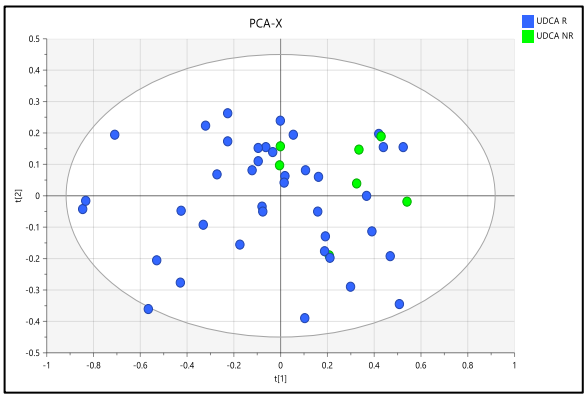


Figure 63: PCA model comparing Urine MS negative ion mode in PBC UDCA responder (n=42) vs PBC UDCA non-responder (n=7).

A 3-component PCA model [R^2X=0.486, Q^2=0.261] did not show any discrimination in urine metabolites between the PBC UDCA responders and PBC UCA non-responder groups on mass spectrometry in negative ion mode (p=1)

- **Urine MS (Positive ion mode)**

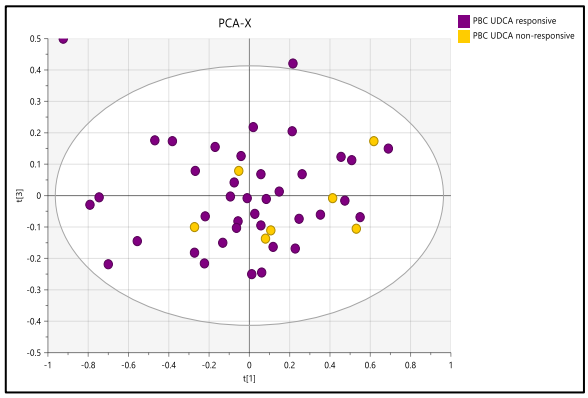


Figure 64: PCA model comparing Urine MS positive ion mode in PBC UDCA responder (n=42) vs PBC UDCA non-responder (n=7).

A 3-component PCA model [R^2X=0.587, Q^2=0.391] did not show any discrimination in urine metabolites between the PBC UDCA responders and PBC UCA non-responder groups on mass spectrometry in positive ion mode (p=0.88)

Faecal analysis UDCA responder vs non responder:

- **Faecal Bile Acid (Negative ion mode)**

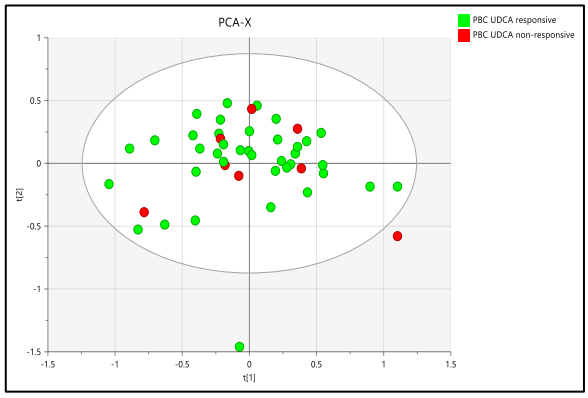


Figure 65: PCA model comparing faecal bile acid in PBC UDCA responder (n=42) vs PBC UDCA non-responder (n=7).

A 5-component PCA model [R^2X=0.743, Q^2=0.487] did not show any discrimination in faecal bile acids between the PBC UDCA responders and PBC UCA non-responder groups on mass spectrometry (p=1)

4.3 Hyocholic acid metabolites

Hyocholic acid metabolites in PBC pruritus compared to PBC no-pruritus, PSC, AMANL and HVs.

Serum Hyocholic acid levels were differentially higher in the PBC pruritus group.

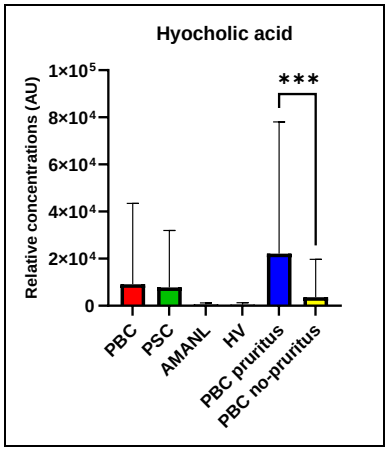


Figure 66: Hyocholic acid levels (median + IQR) in serum comparing PBC, PSC, AMANL, PBC pruritus and PBC no pruritus groups.

Levels of Hyocholic acid and its Glyco- and Tauro-conjugates were significantly raised in the serum of Cholestasis patients (PBC and PSC) when compared to healthy individuals. Median (and IQR), when compared to HV (300.6 (0 - 649.2)), were significantly raised in PBC (561.5 (74.15 – 2543), p=0.003) and PSC (1080 (72.39 - 3981), p=0.0003). There was no statistically significant difference in the serum levels of Hyocholic acid and its metabolites between the two cholestatic groups, PBC (561.5 (74.15 – 2543)) compared to PSC (1080 (72.39 - 3981)), (p=0.25). In the subset of patients with PBC-pruritus (2036 (396.1- 7671)) compared to those with PBC no-pruritus (395.2 (0 -1299)) levels were significantly higher (p=0.0003). Interestingly, hydroxylated bile acid levels were significantly raised when PBC pruritus patients were compared to HV (p=0.0001) and not raised when PBC non-pruritus patients were compared to HVs (p=0.12). A similar pattern was observed when AMANL patients who biochemically behave as HVs were compared to PBC pruritus (p=0.0001) and PBC non-pruritus (0.70). In contrast, in PSC patients' levels were not raised compared to PBC-pruritus (p=0.12) and raised when compared to PBC no-pruritus (p=0.01). This implies that in a subset of PBC pruritus patients, the levels were high enough to match the levels in cholestatic controls when taken together with PBC non-pruritus patients where levels were otherwise significantly lower. Group-wise comparison of serum Hyocholic acid levels (median + IQR) along with p-values is summarized in (Table 42).

	Median (IQR)		Median (IQR)	p-value
PBC pruritus	2036 (396.1- 7671)	PBC no-pruritus	395.2 (0 -1299)	0.0003
PBC pruritus	2036 (396.1- 7671)	HV	300.6 (0 - 649.2)	0.12
PBC no-pruritus	395.2 (0 -1299)	HV	300.6 (0 - 649.2)	0.0001
PBC pruritus	2036 (396.1- 7671)	AMANL	439 (103.9 – 766.9)	0.0001
PBC no-pruritus	395.2 (0 -1299)	AMANL	439 (103.9 – 766.9)	0.70
PBC	561.5 (74.15 – 2543)	PSC	1080 (72.39 - 3981)	0.25
PBC	561.5 (74.15 – 2543)	HV	300.6 (0 - 649.2)	0.003
PSC	1080 (72.39 - 3981)	HV	300.6 (0 - 649.2)	0.0003
PSC	1080 (72.39 - 3981)	PBC pruritus	2036 (396.1- 7671)	0.12
PSC	1080 (72.39 - 3981)	PBC no-pruritus	395.2 (0 -1299)	0.01

Table 42: Median and IQR compared (with p-value) for serum Hyocholic acid (in PBC (all), PSC, AMANL, HV, PBC pruritus and PBC no pruritus groups).

Significant p-values (<0.05) are highlighted in green.

Faecal Hyocholic acid levels were higher in the PBC pruritus group.

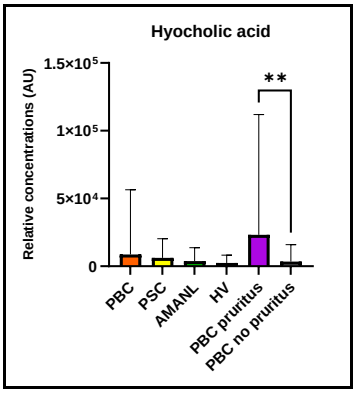


Figure 67: Hyocholic acid levels (median + IQR) in Faeces comparing PBC, PSC, AMANL, PBC pruritus and PBC no pruritus groups.

Group-wise comparison of Faecal Hyocholic acid levels (median + IQR) along with p-values is summarized in (Table 43 and Figure 67).

	Median (IQR)		Median (IQR)	p-value
PBC pruritus	1122 (670 – 7486)	PBC no-pruritus	611.8 (331.8 – 1406)	0.001
PBC	747.7 (393.9 – 1831)	PSC	754.9 (237.9- 2901)	0.85
PBC	747.7 (393.9 – 1831)	HV	617.9 (194 – 1937)	0.16
PSC	754.9 (237.9- 2901)	HV	617.9 (194 – 1937)	0.39
PSC	754.9 (237.9- 2901)	PBC pruritus	1122 (670 – 7486)	0.06
PSC	754.9 (237.9- 2901)	PBC no-pruritus	611.8 (331.8 – 1406)	0.55

Table 43: Median and IQR compared (with p-value) for faecal Hyocholic acid (in PBC (all), PSC, AMANL, HV, PBC pruritus and PBC no pruritus groups).

Significant p-values (<0.05) are highlighted in green.

4.4 Discussion:

Symptoms of PBC are common and can be quite incapacitating, with a negative impact on the quality of life. The pathophysiology of PBC symptoms is poorly understood. There are very few treatment options available and may not be effective for everyone.

This study explored the metabolic profile of serum, urine, and faeces of symptomatic PBC patients compared with those who are asymptomatic for fatigue, pruritus, and cognitive impairment, three of the commonest PBC symptoms. **The study did not find any distinguishing metabolic signatures between fatigue and cognitive impairment groups compared to no-fatigue and no-cognitive impairment groups.**

Bile acids have been related to the pathogenesis of cholestatic pruritus 'Bile-salt theory'. Studies have reported alterations in total and individually separate bile acid compositions in the serum of PBC patients who have pruritus compared to those with no pruritus. Glycolated primary bile acids GCA and GCDCA are raised in the serum of PBC patients with pruritus and a correlation with the severity of itch has been observed (Hegade et al., 2019b). There are other studies, however, where no correlation has been seen between bile acid concentrations and the severity of cholestatic itch. We did not observe any (overall) changes in the serum bile acid profile of

our study groups. This may indicate that the pathogenesis of cholestatic pruritus is a very complex interplay and not a direct result of alterations in serum bile acids.

Seven patients experiencing pruritus were on rifampicin, while one was on cholestyramine at the time of recruitment for the study. PCA analysis comparing the two groups—those on rifampicin versus those not on rifampicin—failed to produce a valid model ($Q^2 = -0.09$, indicating an invalid model). The relatively small number of patients in the pruritus group may limit the ability to identify any meaningful differences within subgroups, if they exist. It is also known that antibiotics, including rifampicin, can alter the composition of the gut microbiome, which, in turn, can impact bile acid concentrations (Séraphin et al., 2023, Yang et al., 2021). Our study lacks data on gut micro-bacteria.

Hyocholic acid (HCA) is a bile acid that is found in abundance in pig bile but is typically a minor component in human bile. HCA is present in very low concentrations (1-3%) in human serum and trace quantities in urine and faeces (Spinelli et al., 2016, Zheng et al., 2021a). However, they are increased in cholestasis. HCA is a secondary bile acid that is produced in the human body by the gut microbiota. HCA is synthesized from CDCA by the action of the 7α-hydroxysteroid dehydrogenase (7α-HSDH) enzyme. This reaction is reversible, and HCA can also be converted back to CDCA by the same enzyme. The expression of 7α-HSDH is tissue-specific and is found primarily in the liver and intestine. HDCA undergoes glucuronidation using UDP-glucuronosyltransferase in human liver(Sacquet et al., 1983, Marschall et al., 1987).

The role of HCA in health and disease in humans is still not fully understood. Some studies have suggested that HCA may have anti-inflammatory and anti-obesity effects, while others have linked it to the development of metabolic disorders such as insulin resistance and non-alcoholic fatty liver disease. HCA has been suggested as a marker of metabolic disorder, with levels being low in diabetes and obesity (Zheng et al., 2021b). HCA has been shown to impact glucose metabolism by its inhibitory action on FXR(Zheng et al., 2021a).

The present study found significantly raised levels of HCA in the serum of cholestatic PBC (all) and PSC patients which showed discriminating differentiation from HVs and AMANL patients. PBC pruritus patients compared to those with no pruritus' had significantly raised Hyocholic acid metabolites. The differentiation was retained when PBC pruritus patients were compared with HV and AMANL. A study by Trottier et al, showed similar raised HCA levels in PSC when compared to controls (Trottier et al., 2012). In the same study, HCA levels did not show any discrimination between PBC and the control group. Similar trends were observed in the comparison of the PBC no-pruritus group and HVs, in this study. However, in Trottier et al study no reference to symptoms was made.

Zheng etal in their study, comprising five human studies, investigated the association of human HCA to the development of the metabolic disorder (Zheng et al., 2021c). Study 1- nested case-control cross-sectional study included 1107 (585 healthy lean (HL), 419 healthy obese (HO), and 103 Overweight/ Obese diabetic (OD)). Fasting serum samples were taken. HCA species (median (first quartile, third quartile)) were significantly decreased in HO (29.13(20.85, 46.92)) and OD (19.53(11, 34.4)) compared to HL (41.83(32.12, 56.11)) [Two-sided Kruskal-Walli's test $p<0.05$]. Spearman co-relation showed an inverse relationship between HCA species and BMI, fasting and post-prandial glucose levels, insulin resistance, and insulin levels ($p<0.05$). Further levels of individual HCA species (HCA, HDCA, GHCA, and GHDCA) were highest in HL and lowest in OD.

Study 2- cross-sectional design included 91 subjects (26 HC, 30 with pre-diabetes, and 35 with newly diagnosed diabetes (drug naive)). Fasting serum and fecal samples were taken. Patients with pre-diabetes and diabetes compared to HCs showed significantly lower levels of total and individual HCA species levels in both serum and faeces. Further inverse correlation was seen between total and individual fecal and serum HCA species, and fasting and post-prandial blood glucose levels.

Study 3- The gastric bypass surgery intervention group included 38 individuals who were obese, diabetic, and underwent Roux-en-Y gastric bypass (RYGB) surgery for weight loss. Fasting serum samples were taken at baseline, 1,3,6, and 12 months. They found a significant increase in total and individual HCA species concentrations from the first month (FC = 2.30-3.85). This was associated with improvement in BMI, HbA1c, fasting, and post-prandial glucose levels and decreased insulin resistance. At 24 months follow-up diabetes recurred in 12 and remained in remission in 24, with higher pre-op HCA specie levels being associated with a higher probability of being in remission ([OR, 95%CI= 0.74, 0.58-0.95]).

Study 4- 10-year longitudinal study included 132 subjects who were metabolically healthy (MH) from a Shanghai diabetes study. At 10 years 46 remained MH and 86 became metabolically unhealthy (MU). Fasting serum samples were taken. Significantly lower concentrations of total HCA concentration median (first quartile, third quartile), 11.04(4.24, 17.46) in MU compared to 15.10(6.10, 31.02) in MH [Two-sided Mann-Whitney U test $p<0.05$] were seen. Similar trends were seen in individual HCA species. A significant association was seen between total HCA levels and future MU [OR (95% CI)) 0.89 (0.86, 0.93)].

Study 5- A 5-year longitudinal study included 207 MH subjects from the Beijing physical examination center [Validation cohort]. At 5 years 117 remained MH and 90 became MU. Fasting serum samples were taken. The study validated the findings of study 4, with

significantly lower concentrations of total and individual HCA concentration in MU compared to MH. In summary, the study suggested that obesity and diabetes (metabolic disorders) are associated with lower serum levels of HCA species. HCA levels may be predictors of future metabolic disease development. Baseline HCA species levels may predict long-term remission of diabetes post gastric bypass surgery.

The same group reported a distinct TGR5 and FXR signaling mechanism underlying the effect of HCA on glucose hemostasis. To better understand this, they first treated pigs with an FXR agonist (GW4064) which significantly decreased serum HCA levels with an associated increase in serum glucose (30%) and a decrease in serum GLP-1 (69%). They then administered HCA species which resulted in a decrease in blood glucose and an increase in GLP-1 indicating independent regulation of glucose homeostasis by HCA. Next HCA and HDCA treatment were given to two diabetic mouse models, the db/db model and the high-fat diet [express mutations in the leptin receptor gene] and streptozotocin (HFD+STZ) [mimicking the partial loss of islet cells] induced diabetic model. Comparison was made with vehicle control and TUDCA (known to regulate glucose tolerance). HCA species treated group showed significantly higher serum levels of GLP-1 and insulin in both the mouse models. BA receptors TGR5 and intestinal FXR are expressed in enteroendocrine L cells of the colon and terminal ileum. Effects of six HCA species and six non-HCA BAs (at 3 different concentrations 5, 25, and 50microM) on GLP-1 secretion and proglucagon gene expression were measured using the enteroendocrine L cell lines. HCA species upregulated proglucagon transcription and GLP-1 secretion significantly more compared to other BAs, and more at higher doses of 50 compared to 25microM.BA-induced TGR5 activation-related GLP-1 secretion is mediated by intracellular cAMP accumulation. A recombinant small heterodimer partner (SHP) is an FXR gene expression protein. In vitro experiments showed high expression of SHP induced by CDCA (FXR agonist) which was inhibited when the cells were co-treated with HCA species, indicating HCA species to be FXR antagonists. Further, an in vitro test was conducted in shTGR5 and shFXR cells. It was found that compared to controls, HCA-induced GLP-1 secretion was significantly decreased in TGR5 knockdown cells HCA species treatments showed no difference between shFXR cells and control cells. In summary, it was suggested that HCA promotes proglucagon transcription and GLP-1 secretion by simultaneous FXR antagonism and TGR5 agonism.

A study from a French group of 205 pre-diabetic patients from IT-DIAB study investigated the link between BA metabolism and glucose homeostasis. 69 (33.7%) developed diabetes in a median 56.9 months follow-up. They found that total HCA species negatively correlated (Spearman's correlation coefficient) with BMI (-0.20), homeostasis model assessment for

insulin resistance (HOMA-IR) [-0.16], and fatty liver index (FLI) [-0.15]. In contrast, plasma C4 levels showed a positive correlation with BMI (0.32), HOMA-IR (0.34), and FLI (0.35). Findings suggested a potential pathophysiological link between 6α-hydroxylation and glucose metabolism warranting further investigation (Chávez-Talavera et al., 2020).

HCA species (HCA and HDCA) were first isolated from pig bile in 1923(Windaus and Bohne, 1923). Various routes for the formation of HCA species have been suggested. In vitro study on isolated human liver microsomes fortified with NADPH demonstrated HDCA production from hydroxylation of TLCA at 6α position (Trülzsch et al., 1974). Later studies found this 6α hydroxylation to be related to CYP3A4 present in human liver microsomes (Araya and Wikvall, 1999). Deo and Bandiera etal, demonstrated in vitro synthesis of HCA from CDCA via 6α hydroxylation mediated by CYP3A4 (Deo and Bandiera, 2008). Studies have also implicated the role of gut microbiota in the formation of HCA species. For example, HDAC-1 (an unknown) gram-positive rod isolated from rat intestinal flora, using an unidentified growth factor produced by a Ruminococcin productus strain was shown to transform β-muricholic acid into HDCA (Eyssen et al., 1999).

A single-centre prospective study reported a corelation between Increased glycine-amidated HCA and early sustained weight loss following sleeve gastrectomy surgery (Kindel et al., 2018). The study recruited 31 morbidly obese patients who underwent SG. 28 patients completed 6 and 12-week follow up. Both fasting and postprandial HCA levels (microM) compared to pre-op were increased post-SG, at 6 weeks (0.027 ± 0.038 vs 0.061 ± 0.046, $p<0.05$) and 12 weeks (0.027 ± 0.038 vs 0.052 ± 0.052). Increased post-meal HCA AUC (microM*min, mean ± SD) was significantly correlated to BMI loss at both 6 (AUC= 1.9 ± 2.0 vs 4.3 ± 3.0, Pearson's correlation 0.379, $p = 0.005$) and 12 weeks (AUC= 1.9 ± 2.0 vs 3.8 ± 2.6, Pearson's correlation 0.507, $p = 0.006$).

Limitations:

The sample size of the pruritus group with PBC is small, and the study lacked data on the severity of the itch. Half of the patients were being treated with second or third-line agents for pruritus management, indicating moderate to severe itching, and one patient even underwent a liver transplant for intractable itch. Additionally, the study did not provide data on the gut microbiome.

In summary, this study demonstrates significantly high levels of HCA specific to the PBC pruritus group, raising the question of whether HCA could serve as a useful therapeutic target

for treating pruritus in patients with PBC. Further studies are needed to determine if elevated HCA levels are the cause of pruritus or are simply a result of more complex metabolic changes in this subset of patients. Validation in larger cohorts will be necessary.

Chapter 5. Results 3: Comparison of Metabolomic Profile Of Primary Biliary Cholangitis And Primary Sclerosing Cholangitis

5.1 Results of analysis:

A total of 51 patients with PBC and 21 PSC were included in the final analysis. There was a statistically significant difference in the median age (64 years for PBC and 52 years for PSC) between the two groups (p=0.0007). Markers of cholestasis were higher in PSC compared to PBC (median), Bilirubin (12 vs 7, p=0.0001), ALP (302 vs 109, p=0.0001), and GGT (240 vs 54, p=0.01) (Table 44). Significant discrimination was seen in the two groups on serum lipids, urine metabolites, and faecal BAs overview of which is presented in (Table 45).

	PBC (Median / IQR)	PSC (Median / IQR)	p- value
Age	64 (43-80)	52 (20-78)	0.0007
Albumin	45 (43-46)	43 (40-45)	0.07
Bilirubin	7 (6-8)	12 (9-24)	<0.0001
ALP	109 (78-148)	302 (147-456)	<0.0001
ALT	26 (15-35)	48 (25-104)	0.001
GGT	54 (29-131)	240 (44-426)	0.01
AST	29 (23-42)	31 (23-84)	0.48

Table 44: Age and Baseline liver chemistry compared between PBC and PSC group.

Median and IQR (with p value) for Bilirubin, ALP- alkaline phosphatase, ALT- alanine aminotransferase, GGT- gamma glutamyl transferase, AST- aspartate aminotransferase. Significant p-values (<0.05) highlighted in green.

Overview of Analysis:

		A	R^2X	Q^2	p-value
Serum	MS Bile acid (NEG)	4	0.817	0.688	0.36
	MS Lipid (NEG)	5	0.758	0.527	0.81
	MS Lipid (POS)	7	0.701	0.526	0.001
	NMR BiLISA	4	0.861	0.782	0.27
Urine	NMR QUANT	2	0.573	0.229	0.002
	MS (NEG)	2	0.39	0.276	0.0006
	MS (POS)	2	0.661	0.419	0.003
Faecal	MS BA (NEG)	6	0.784	0.589	7.33527e-06

Table 45: Summary of PCA analysis in PBC (n=51) vs PSC (n=21).

PCA-X coordinates [A= no of principal components with R^2X and Q^2 values] and corresponding multivariate analysis CV-ANOVA p-values for serum, urine, and faecal assays of PBC patients compared with PSC patients. R^2 predicts goodness of fit and Q^2 goodness of prediction of the model. Significant p-values (<0.05) are highlighted in green.

Serum analysis PBC vs PSC:

- **Serum Bile Acid (negative ion mode)**

A 4-component PCA model [R^2X=0.817, Q^2=0.688] did not show any discrimination in serum bile acids between the PBC and PSC groups on mass spectrometry (p=0.36)

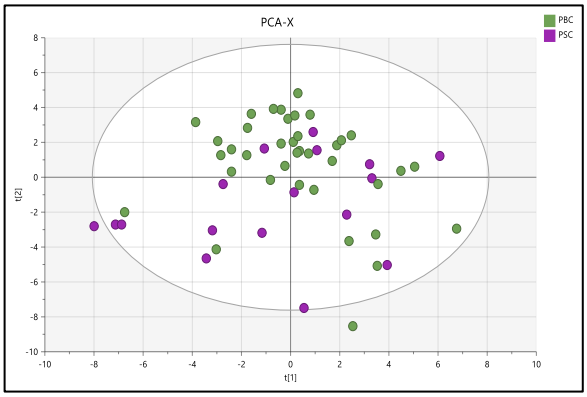


Figure 68: PCA and OPLS-DA model comparing serum bile acid in PBC (n=51) vs PSC (n=21).

- **Serum Lipids (negative ion mode) assay**

A 5-component PCA model [R^2X=0.758, Q^2=0.527] did not show any discrimination in serum lipids between the PBC and PSC groups on mass spectrometry in negative ion mode (p=0.81)

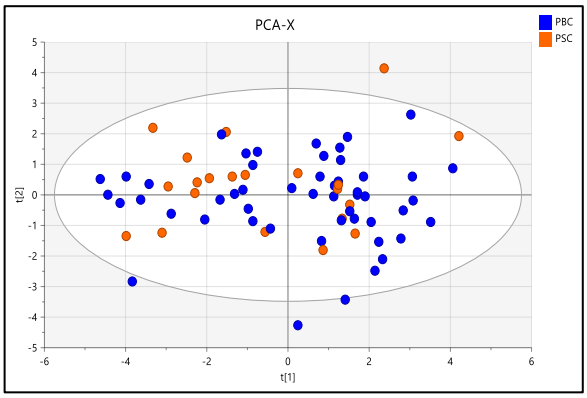


Figure 69: PCA and OPLS-DA model comparing serum lipids negative ion mode in PBC (n=51) vs PSC (n=21).

- **Serum Lipids (positive mode) assay**

A 7-component PCA model (R^2X=0.701, Q^2=0.526) attained visual discrimination between patients with PBC and PSC, in serum lipids on mass spectrometry in positive ion mode. This was confirmed subsequently on a 3-component OPLS DA model, with the following statistic (1+2+0), R^2X=0.399, R^2Y=0.627, Q^2=0.27, CV-ANOVA p=0.001 (Table 46). Permutation testing demonstrated that the model was valid in that cut-offs for R^2 and Q^2 were lower in the randomly permutated model (999 permutations) (Figure 70). Metabolites with a VIP score of $\geq$ 1 was used to predict their influence on class separation between the two study groups (Table 47). Resonances **increased in patients with PBC** were collection of triglycerides, sphingomyelins, ceramides, and carnitine (Table 48).

				CV-ANOVA p-value
PCA- X Coordinates	A	R^2X	Q^2	
	7	0.701	0.526	0.001

OPLS-DA Coordinates	A	R^2X	R^2Y	Q^2
	1+2+0	0.399	0.627	0.27

Table 46: PCA and OPLS-DA coordinates of Serum MS lipid positive ion mode PBC (n=51) vs PSC (n=21).

A= no of principal components, R^2 predicts goodness of fit, and Q^2 goodness of prediction of the model. Significant p-values (<0.05)

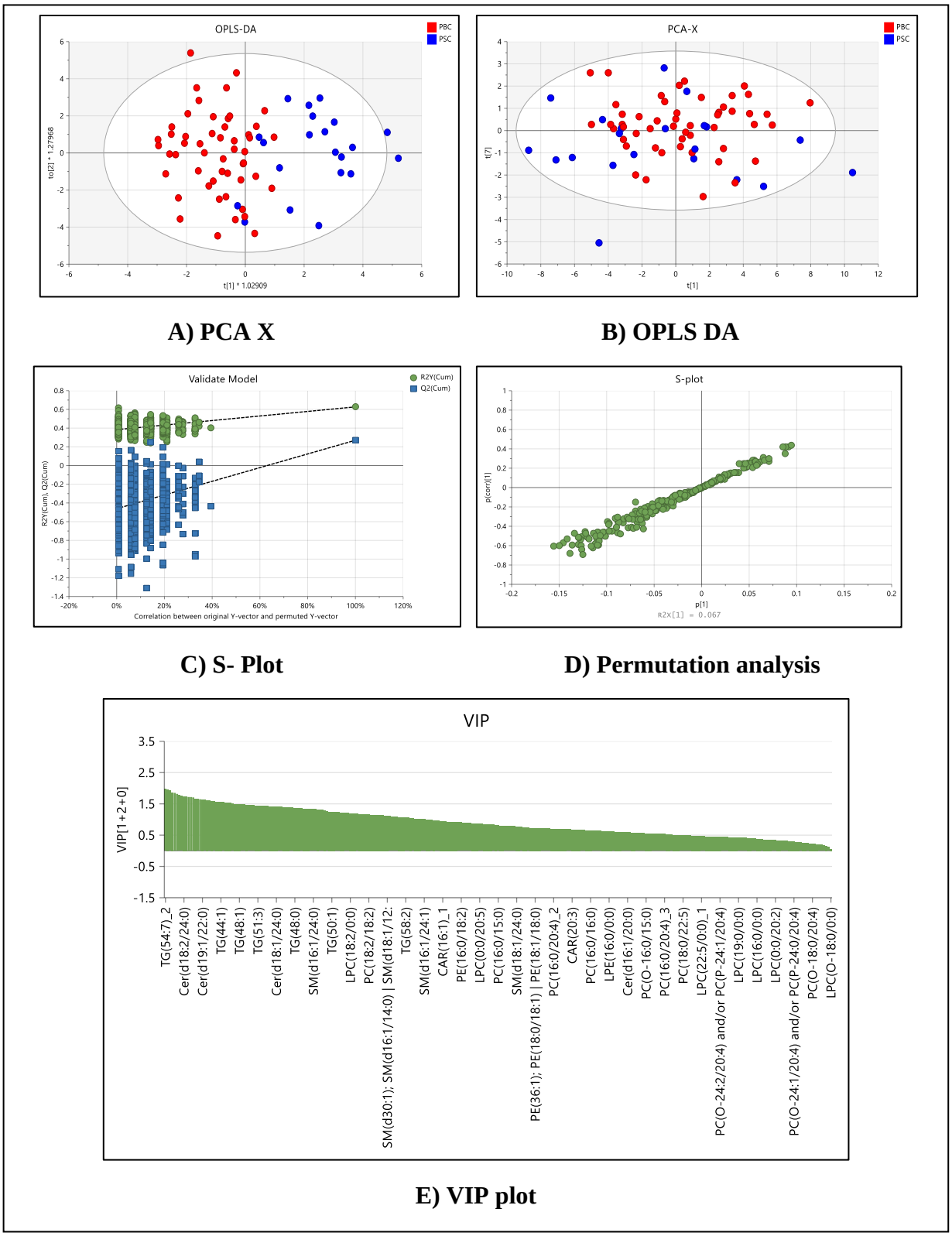


Figure 70: Serum Lipids profile models PBC (n=51) vs PSC (n=21)

These multivariate models demonstrate discrimination of patients with PBC and PSC. (A) Principal components analysis (PCA) scores plot [7-component model R^2X=0.701, Q2=0.526]. (B) Orthogonal projection least squares discriminant analysis (OPLS-DA) scores plot [(1+2+0), R^2X=0.399, R^2Y=0.627, Q^2=0.27]. (C) S-line loading plot (D) Permutation analysis (E) VIP-scores plot

TG(54:7)_2	1.97	Cer(d41:1); Cer(d18:1/23:0) |Cer(d17:1/24:0)	1.66
Cer(t18:0/24:1)	1.97	Cer(d18:1/25:0)	1.65
CE(20:5)	1.93	Cer(d19:1/22:0)	1.63
Cer(d19:1/24:0)	1.86	Cer(d16:1/24:0)	1.62
TG(58:9)	1.85	TG(52:6)_2	1.61
TG(44:2)	1.82	SM(d18:2/18:0)	1.60
TG(54:6)_2	1.77	SM(d35:1); SM(d17:1/18:0) |SM(d18:1/17:0)	1.59
SM(d18:2/23:0)	1.76	Cer(d18:1/26:1)	1.58
Cer(d18:2/24:0)	1.74	TG(56:7)_1	1.57
TG(56:7)_2	1.73	TG(56:5)_2	1.56
TG(58:7)_2	1.72	TG(44:1)	1.56
TG(56:7)_3	1.72	SM(d18:2/14:0)	1.55
TG(44:0)	1.71	TG(54:5)_2	1.54
TG(46:0)	1.67	TG(46:1)	1.54
SM(d18:2/16:0)_2	1.38	SM(d32:1); SM(d16:1/16:0) |SM(d18:1/14:0)	1.34
CAR(8:0)	1.38	DG(32:1)	1.34
TG(48:0)	1.37	TG(48:3)	1.33
SM(d16:1/22:0)	1.37	SM(d16:1/24:0)	1.33
CAR(10:0)	1.36	TG(51:4)	1.33
TG(50:2)	1.36	SM(d18:2/24:0)	1.32
CE(22:6)	1.34	TG(53:5)	1.31
		PC(18:1/22:6)	1.30
TG(52:2)	1.24	PC(18:0/22:6)	1.15
TG(55:4)	1.23	Cer(d18:2/22:0)	1.15
PE(O-18:1/22:6) and/or PE(P-18:0/22:6)	1.30	SM(d17:1/24:1)	1.15
CAR(12:1)	1.18	TG(54:2)	1.15
TG(52:6)_1	1.17	LPC(0:0/18:3)	1.14
TG(52:5)	1.17	DG(16:0/18:1)	1.14
LPC(0:0/18:2)	1.17	TG(56:4)	1.13
PC(18:2/18:2)	1.16	TG(53:4)	1.43

TG(56:6)	1.49	TG(51:2)	1.42
TG(49:2)	1.49	TG(48:2)	1.42
TG(48:1)	1.49	Cer(d18:1/24:0)	1.42
TG(53:2)	1.48	DG(36:4)_2; DG(18:2/18:2) |DG(18:1/18:3)	1.41
PE(16:0/20:4)	1.47	TG(53:3)	1.40
SM(d18:2/16:0)_1	1.47	TG(50:3)	1.39
SM(d19:1/18:0)	1.46	SM(d17:1/16:0)	1.39
TG(49:3)	1.45	SM(d18:1/23:0)	1.39
TG(53:1)	1.45	PE(O-18:1/22:6) and/or PE(P-18:0/22:6)	1.30
SM(d18:1/20:1)	1.45	TG(52:3)	1.26
TG(51:3)	1.44	TG(48:4)	1.25
PE(18:0/20:4)	1.44	TG(50:1)	1.25
TG(49:1)	1.43	SM(d19:1/16:0)	1.24
PC(16:0/20:5)	1.43	TG(52:2)	1.24
PE(O-18:1/22:6) and/or PE(P-18:0/22:6)	1.30	TG(55:4)	1.23
TG(52:3)	1.26	PE(O-18:1/22:6) and/or PE(P-18:0/22:6)	1.30
TG(48:4)	1.25	TG(52:3)	1.26
TG(50:1)	1.25	TG(48:4)	1.25
SM(d19:1/16:0)	1.24	TG(50:1)	1.25
TG(52:2)	1.24	SM(d19:1/16:0)	1.24
TG(55:4)	1.23	DG(36:2); DG(18:1/18:1) | DG(18:0/18:2)	1.05
LPC(18:3/0:0)_1	1.11	CAR(16:1)_2	1.05
TG(56:5)_1	1.08	CAR(16:2)	1.03
SM(d18:1/18:0)	1.08	CAR(14:0-OH)	1.03
CAR(12:0-OH)	1.08	DG(36:4)_1; DG(18:2/18:2) | DG(18:1/18:3)	1.02
CAR(14:2)	1.08	CAR(10:0-OH)	1.01
CAR(14:1)	1.07	TG(54:4)	1.01
CAR(10:1)	1.07	SM(d16:1/24:1)	1.01
TG(58:2)	1.06	TG(54:3)	1.00

Table 47: Serum lipid metabolites with VIP >1 PBC (n=51) vs PSC (n=21).

	PBC median (IQR)	PSC median (IQR)	Mann-Whitney P value
Diacyl Glycerol	27671 (13184 – 50287)	25188 (11060 – 40164)	0.24
Triglycerides	24561 (8493 – 110508)	19109 (5667 – 19109)	0.01
Sphingomyelins	1206806 (422930 – 2507685)	850377 (323590 – 1839904)	0.02
Carnitines	58068 (20006 – 136371)	46175 (19105 – 89454)	0.02
Ceramides	25106 (14870 – 75432)	17455 (8928 – 58379)	0.0008
Phosphatidylcholine	5403079 (545831 – 7747298)	4929328 (737415 – 7032645)	0.19
Glycerophospholipids	271316 (172427 – 408617)	193682 (126491 – 281143)	0.0002
Cholesterol Ester	17062 (12552 – 21589)	10611 (8903 – 15154)	<0.0001

Table 48: Mass spectrometry- serum lipids in PBC vs PSC

Mass spectrometry observed serum bile acid metabolites with associated intensity differences (Median + IQR) in patients with PBC [n=51] and PSC [n=21]. Statistical significance was determined by a non-parametric Mann-Whitney test, comparing ranks. Significant p-values (<0.05) are highlighted in green. Resonances increased in patients with PBC were collection of triglycerides, sphingomyelins, ceramides, and carnitine.

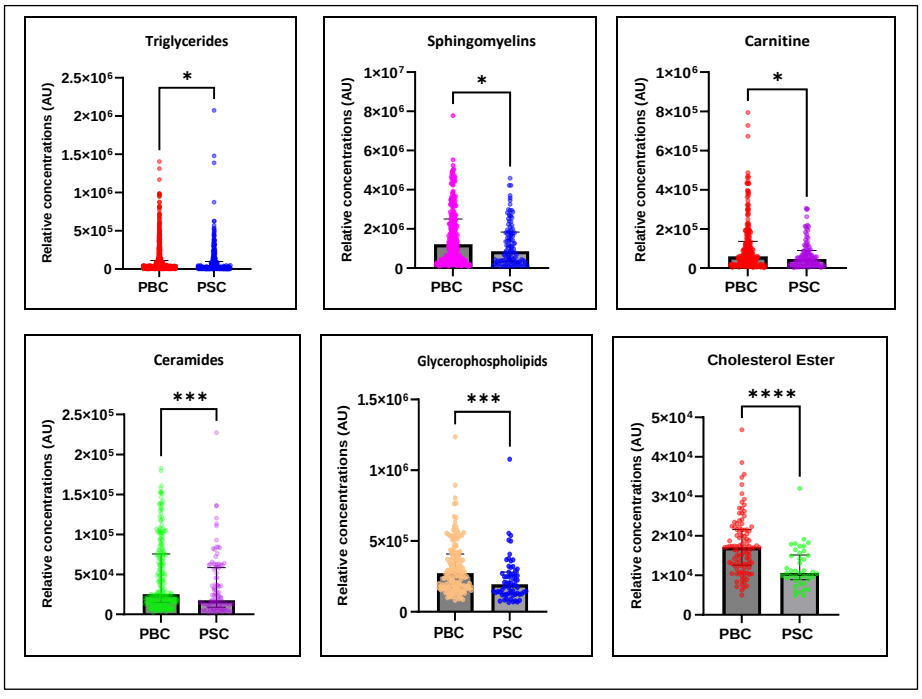


Figure 71: Targeted metabolomics: Panel to compare individual lipid metabolites measured in serum in patients with PBC [n=51] and PSC [n=21].

The X-axis shows the relative serum concentrations expressed in Arbitrary Units (AU). p-value: ns= no significant, <0.05*, <0.01**, <0.001***, <0.0001****.

- **Serum NMR BiLISA**

A 4-component PCA model [R^2X=0.861, Q^2=0.782] did not show any discrimination in serum lipids between the PBC and PSC groups on NMR (p=0.27)

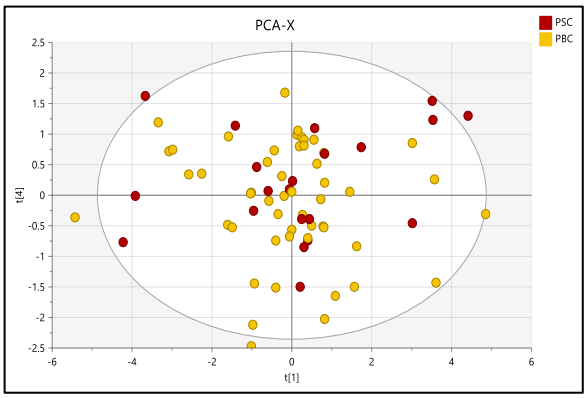


Figure 72: PCA and OPLS-DA model comparing serum NMR BiLISA in PBC (n=51) vs PSC (n=21).

Urine analysis PBC vs PSC:

- **Urine NMR QUANT**

A 2-component PCA model (R^2X=0.573, Q^2=0.229) attained visual discrimination between patients with PBC and PSC, in urine metabolites on NMR. This was confirmed subsequently on a 2-component OPLS DA model, with the following statistic (1+1+0), R^2X=0.545, R^2Y=0.375, Q^2=0.234, CV-ANOVA p=0.002 (Table 49). Permutation testing demonstrated that the model was valid in that cut-offs for R^2 and Q^2 were lower in the randomly permutated model (999 permutations) (Figure 73). Metabolites with a VIP score of ≥ 1 was used to predict their influence on class separation between the two study groups (Table 50). Resonances increased in patients with PSC were creatinine and glycine (Table 51).

				CV-ANOVA p-value
PCA- X Coordinates	A	R^2X	Q^2	
	2	0.573	0.229	0.002

OPLS-DA	A	R^2X	R^2 Y	Q^2
Coordinates	1+1+0	0.545	0.375	0.234

Table 49: PCA and OPLS-DA co-ordinates of Urine NMR PBC (n=51) vs PSC (n=21).

A= no of principal components, R^2 predicts goodness of fit and Q^2 goodness of prediction of the model. Significant p-values (<0.05)]

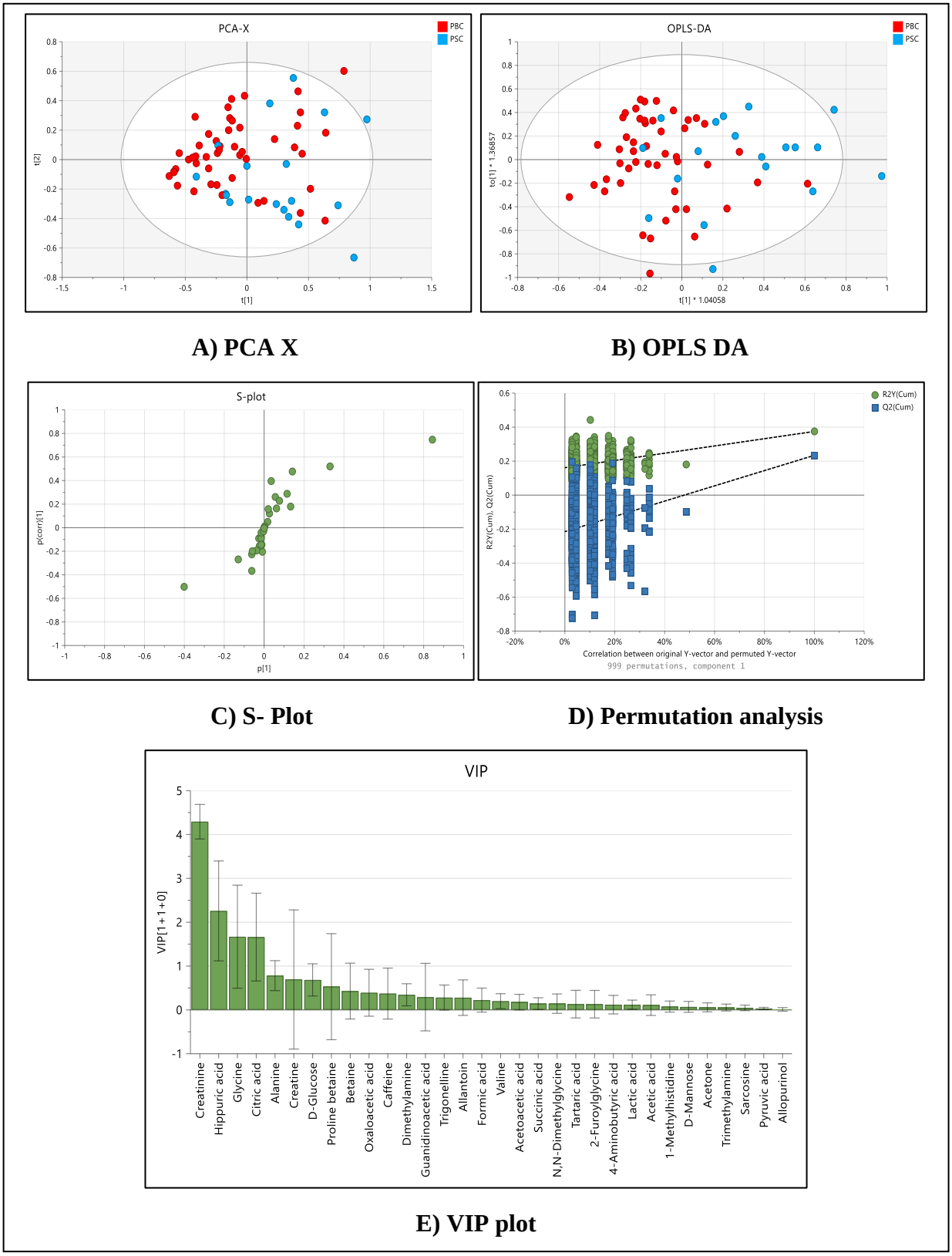


Figure 73: Urine NMR QUANT profile models PBC (n=51) vs PSC (n=21)

These multivariate models demonstrate discrimination of patients with PBC and PSC. (A) Principal components analysis (PCA) scores plot [2-component model R^2X=0.573, Q2=0.229]. (B) Orthogonal projection least squares discriminant analysis (OPLS-DA) scores plot [(1+1+0), R^2X=0.545, R^2Y=0.375, Q^2=0.234]. (C) S-line loading plot (D) Permutation analysis (E) VIP-scores plot.

Creatinine	4.29
Hippuric acid	2.26
Glycine	1.67
Citric acid	1.66

Table 50: Urine NMR metabolites with VIP >1 (PBC (n=51) vs PSC (n=21))

	PBC median (IQR)	PSC median (IQR)	Mann-Whitney P value
Creatinine	4.750 (3.225 - 9.175)	9.450 (6.350 - 13.00)	0.0004
Hippuric acid	1.400 (0.09250 - 2.500)	0.000 (0.000 - 2.050)	0.07
Glycine	0.6150 (0.3175 - 0.8100)	1.000 (0.6750 - 1.525)	0.001
Citric acid	1.550 (0.7300 - 2.175)	1.700 (1.225 - 3.175)	0.21

Table 51: NMR- Urine metabolites in PBC vs PSC

NMR observed urine metabolites with intensity differences (Median +/- IQR) associated with patients with PBC [n=51] and PSC [n=21]. Statistical significance was determined by a non-parametric Mann-Whitney test, comparing ranks. Significant p-values (<0.05) are highlighted in green. Resonances increased in patients with PSC were creatinine and glycine.

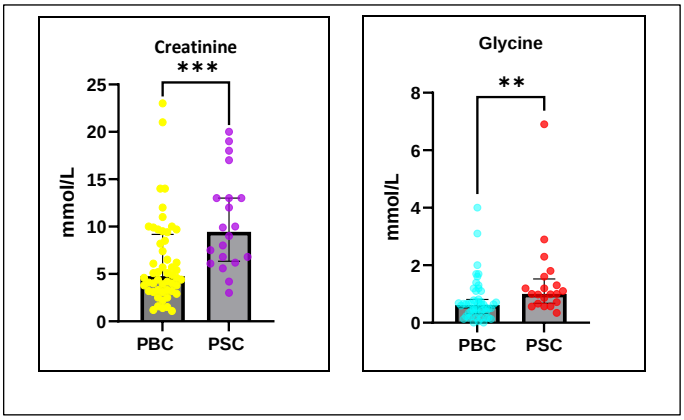


Figure 74: Targeted metabolomics: Panel to compare urine metabolites in patients with PBC [n=51] and PSC [n=21].

The X-axis shows the urine concentrations expressed in mmol/L. p-value: ns= no significant, <0.05*, <0.01**, <0.001***, <0.0001****.

- **Urine MS (negative ion mode) assay**

A 2-component PCA model (R^2X=0.39, Q^2=0.276) attained visual discrimination between patients with PBC and PSC, in urine metabolites on mass spectrometry in negative ion mode. This was confirmed subsequently on a 2-component OPLS DA model, with the following statistics: (1+1+0), R2X=0.386, R2Y=0.443, Q2=0.248, CV-ANOVA p=0.0006 (Table 52). Permutation testing demonstrated that the model was valid in that cut-offs for R^2 and Q^2 were lower in the randomly permutated model (999 permutations) (Figure 75). Metabolites with a VIP score of ≥ 1 was used to predict their influence on class separation between the two study groups (Table 53). Resonances **increased in patients with PSC** were 3-Hydroxyhippurate, Quinate, 1-7-dimethylurate, P-cresol sulphate, P-cresol glucuronide, Cis-aconitate, Cholate, N-acetyl glutamine, 9. Pregnanediol-3-glucuronide and Adenosine 3,5-cyclic monophosphate (Table 54).

				CV-ANOVA p-value
PCA- X Coordinates	A	R^2X	Q^2	
	2	0.39	0.276	0.0006

OPLS-DA	A	R^2X	R^2 Y	Q^2
Coordinates	1+1+0	0.386	0.443	0.248

Table 52: PCA and OLS-DA coordinates of Urine MS negative ion mode PBC (n=51) vs PSC (n=21).

A= no of principal components, R^2 predicts goodness of fit, and Q^2 goodness of prediction of the model. Significant p-values (<0.05)

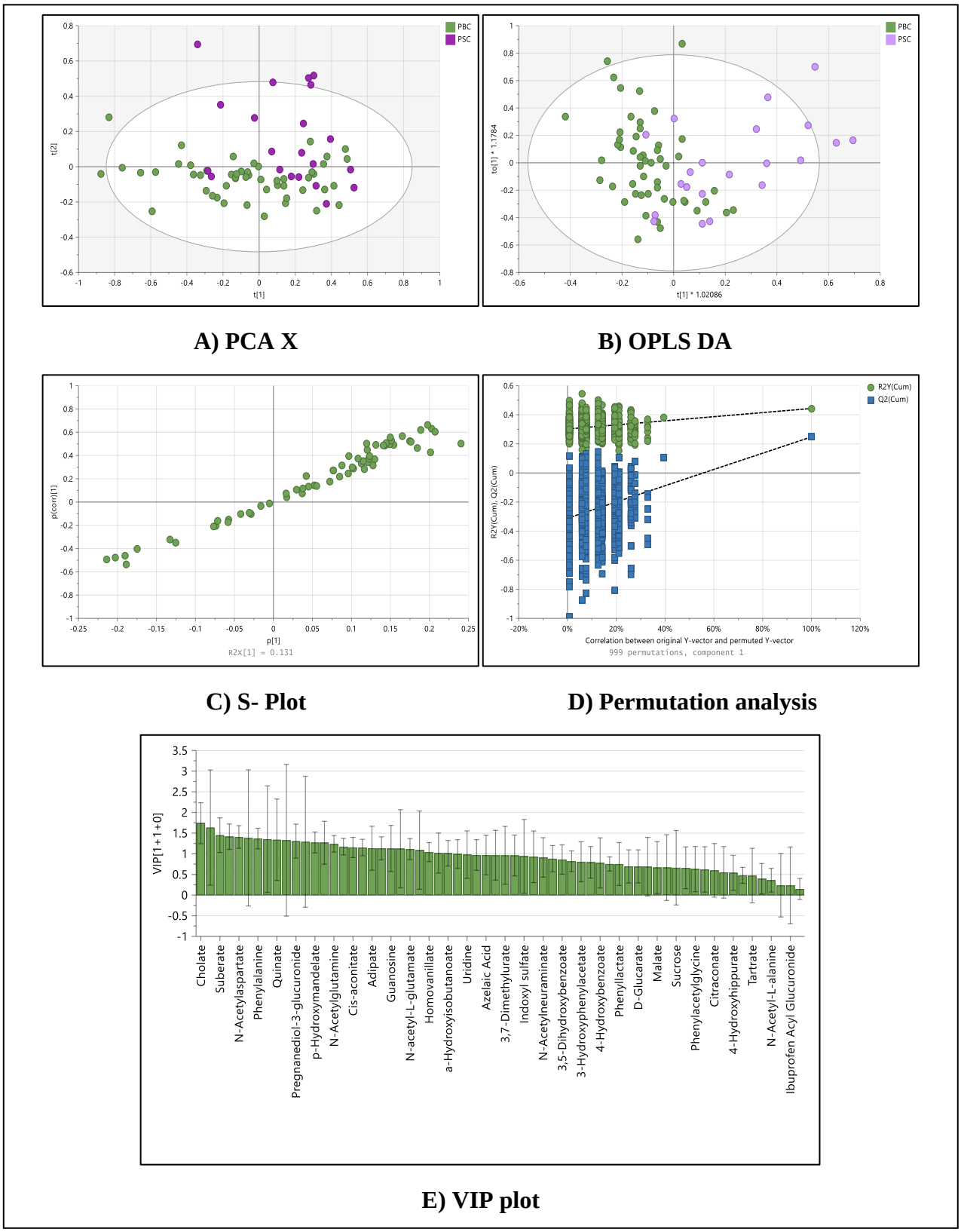


Figure75: Urine MS profile models PBC (n=51) vs PSC (n=21).

These multivariate models demonstrate discrimination of patients with PBC and PSC. (A) Principal components analysis (PCA) scores plot [2-component model $R^2X=0.39$, $Q^2=0.276$]. (B) Orthogonal projection least squares discriminant analysis (OPLS-DA) scores plot [(1+1+0), $R^2X=0.386$, $R^2Y=0.443$, $Q^2=0.248$]. (C) S-line loading plot (D) Permutation analysis (E) VIP-scores plot.

Cholate	1.74
3-Hydroxyhippurate	1.63
Suberate	1.45
Adenosine 3',5'-cyclic monophosphate	1.41
N-Acetylaspartate	1.41
p-Cresol sulfate	1.39
Phenylalanine	1.37
Dehydroepiandrosterone Sulfate	1.35
Quinate	1.34
p-Cresol glucuronide	1.33
Pregnanediol-3-glucuronide	1.30
Indoxyl glucuronide	1.29
p-Hydroxymandelate	1.27
1,2,3-Propanetricarboxylate	1.26
N-Acetylglutamine	1.24
2-Hydroxyphenylacetate	1.17
Cis-aconitate	1.15
2-Hydroxy-2-methylbutanoate	1.15
2,6-Dihydroxybenzoate	1.14
Guanosine	1.13
Adipate	1.13
1,3,7-Trimethylurate	1.12
N-acetyl-L-glutamate	1.11
1,7-Dimethylurate	1.09
Homovanillate	1.04
Xanthosine	1.02
a-Hydroxyisobutanoate	1.02

Table 53: Urine MS (negative ion mode) metabolites with VIP >1 (PBC (n=51) vs PSC (n=21)

		PBC median (IQR)	PSC median (IQR)	Mann-Whitney p-value
Cholate	1.74	15527 (4448 – 48947)	44182 (13151 – 177968)	0.004
3-Hydroxyhippurate	1.63	2230084 (1020923 - 4110062)	1488995 (270748 - 3103468)	0.08
Suberate	1.45	253920 (148865 – 494392)	627606 (426330 – 993920)	0.0002
Adenosine 3',5'-cyclic monophosphate	1.41	285389 (198772 - 401496)	552557 (318767 - 799605)	0.0002
N-Acetylaspartate	1.41	596711 (406212 – 1161558)	1405803 (842806 – 2177038)	0.004
p-Cresol sulfate	1.39	1984392 (989834 – 2885252)	1438409 (503617 - 3179823)	0.27
Phenylalanine	1.37	105614 (53317 – 157215)	181229 (101718 – 281533)	0.0002
p-Cresol glucuronide	1.33	1295716 (710879 - 2332999)	831289 (398550 - 2394187)	0.31
Pregnanediol-3-glucuronide	1.30	207025 (96694 - 442524)	514695 (234768 - 991247)	0.009
Indoxyl glucuronide	1.29	219750 (141223 – 335336)	137367 (60356 – 218815)	0.01
1,2,3-Propanetricarboxylate	1.26	85322 (31346 – 246924)	44182 (13151 – 177968)	0.27
2-Hydroxyphenylacetate	1.17	41149 (25287 – 58742)	64790 (42980 – 97475)	0.001
Cis-aconitate	1.15	1385653 (987865 - 1985104)	2140268 (1581823 - 2729001)	0.0008
Guanosine	1.13	18722 (10370 – 30198)	27473 (22736 – 38100)	0.01
Adipate	1.13	63418 (40868 – 118816)	129234 (78178 – 175116)	0.001
N-acetyl-L-glutamate	1.11	433554 (267382 - 723159)	778700 (442612 - 1257528)	0.001
1,7-Dimethylurate	1.09	3337332 (1918122 - 4569703)	3165373(1804637 - 4002119)	0.27
Xanthosine	1.02	232205 (161545 – 373231)	330020 (266729 – 637417)	0.002

Table 54: Mass spectrometry (negative ion mode) -urine metabolites in PBC and PSC

Mass spectrometry observed urine metabolites with associated intensity differences (Median + IQR) in patients with PBC [n=51] and PSC [n=21]. Statistical significance was determined by a non-parametric Mann-Whitney test, comparing ranks. Significant p-values (<0.05) are highlighted in green.

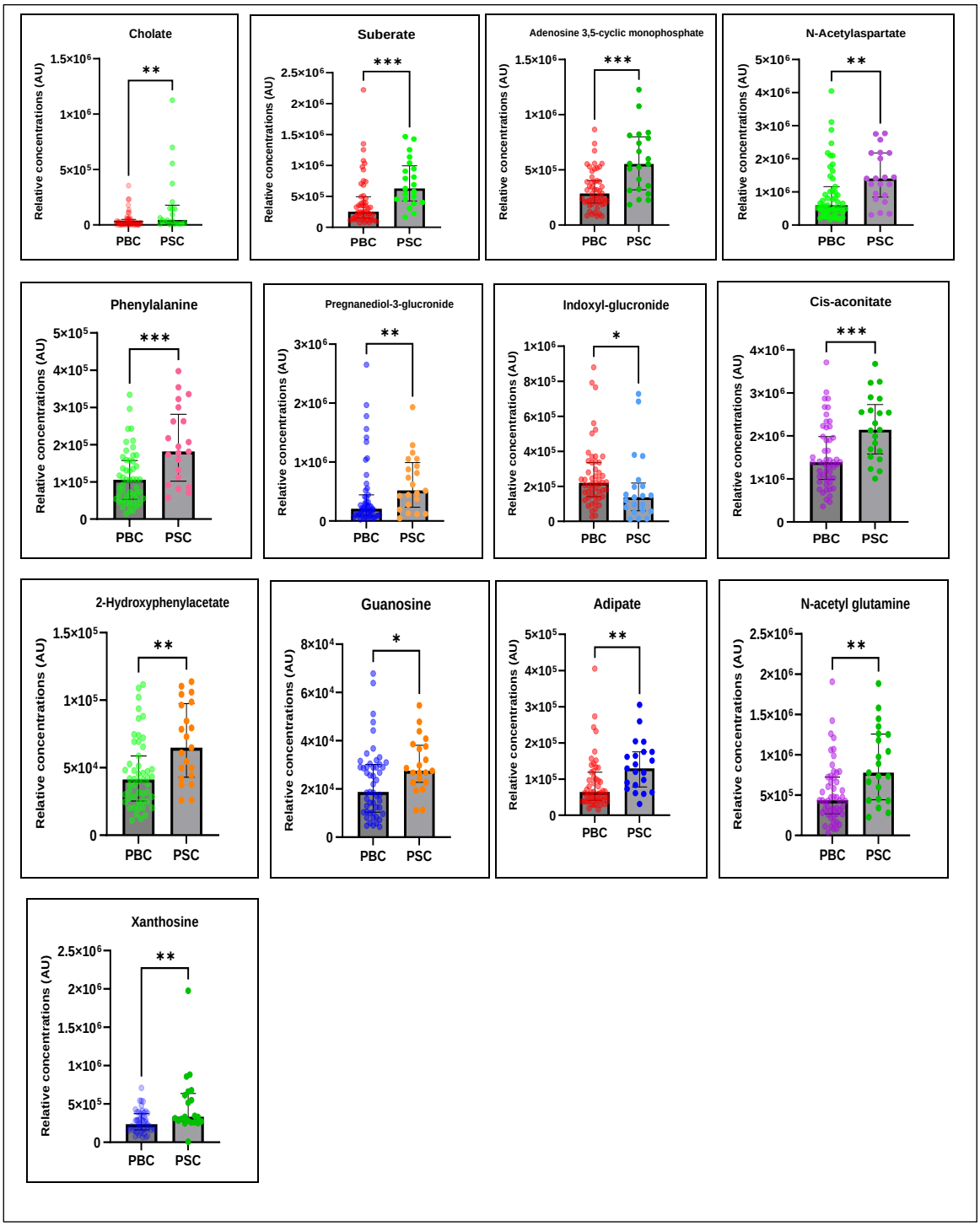


Figure 76: Targeted metabolomics: panel to compare individual metabolites measured in urine in patients with PBC [n=51] and PSC [n=21].

The X-axis shows the relative serum concentrations expressed in Arbitrary Units (AU). p-value: ns= no significant, <0.05*, <0.01**, <0.001***, <0.0001****.

- **Urine MS (positive ion mode)**

A 2-component PCA model (R^2X=0.661, Q^2=0.419) attained visual discrimination between patients with PBC and PSC, in urine metabolites on mass spectrometry in positive ion mode. This was confirmed subsequently on a 2-component OPLS DA model, with the following statistics: (1+1+0), R^2X=0.443, R^2Y=0.396, Q2=0.296, CV-ANOVA p=0.003 (Table 55). Permutation testing demonstrated that the model was valid in that cut-offs for R^2 and Q^2 were lower in the randomly permutated model (999 permutations) (Figure 77). Metabolites with a VIP score of ≥ 1 was used to predict their influence on class separation between the two study groups (Table 56). Resonances **increased in patients with PSC** were Tryptamine, Tryptophan, Indole-3-acetic-acid-O-glucuronide, Tyramine, Hypoxanthine, N2-Dimethylguanosine, Propionylcarnitine, Kynurenate, 5'-Methylthioadenosine, 5-Hydroxyindoleacetate, Butyrylcarnitine and Prolylhydroxyproline (Table 57).

				CV-ANOVA p-value
PCA- X Coordinates	A	R^2X	Q^2	
	5	0.661	0.419	0.003

	A	R^2X	R^2Y	Q^2
OPLS-DA Coordinates	1+1+0	0.443	0.396	0.296

Table 55: PCA and OPLS-DA coordinates of Urine MS positive negative ion mode PBC (n=51) vs PSC (n=21).

A= no of principal components, R^2 predicts goodness of fit, and Q^2 goodness of prediction of the model. Significant p-values (<0.05)

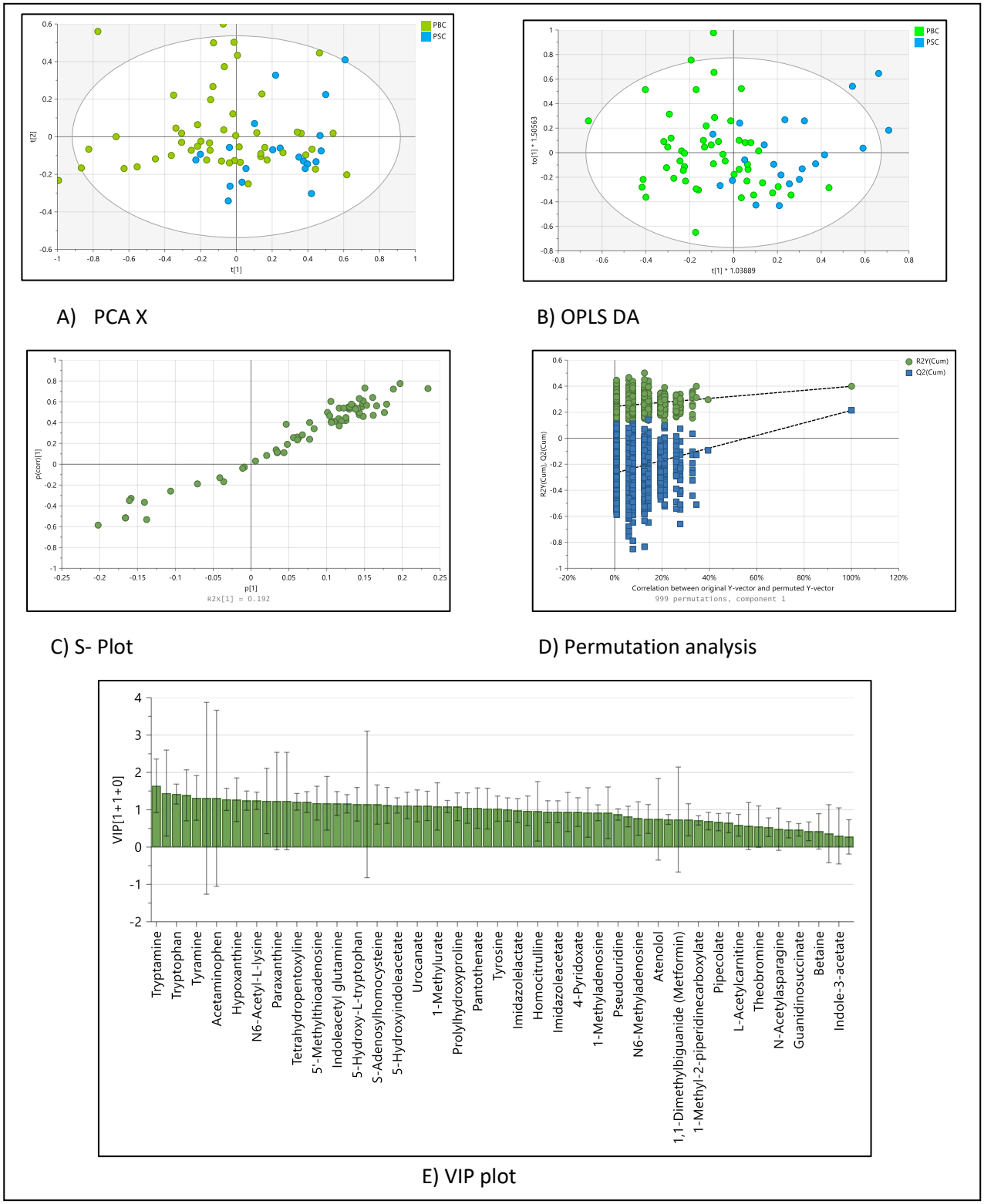


Figure 77: Urine MS profile models PBC (n=51) vs PSC (n=21)

These multivariate models demonstrate discrimination of patients with PBC and PSC. (A) Principal components analysis (PCA) scores plot [2-component model $R^2X=0.661$, $Q^2=0.419$]. (B) Orthogonal projection least squares discriminant analysis (OPLS-DA) scores plot [(1+1+0), $R^2X=0.443$, $R^2Y=0.396$, $Q^2=0.296$]. (C) S-line loading plot (D) Permutation analysis (E) VIP-scores plot.

Tryptamine	1.63
Tryptophan	1.42
Indole-3-acetic-acid-O-glucuronide	1.38
Tyramine	1.31
Hypoxanthine	1.25
N2-N2-Dimethylguanosine	1.24
N6-Acetyl-L-lysine	1.24
Paraxanthine	1.23
Propionylcarnitine	1.23
Kynurenate	1.20
5'-Methylthioadenosine	1.17
2-Octenoylcarnitine	1.16
4-Guanidinobutanoate	1.15
5-Hydroxy-L-tryptophan	1.15
S-Adenosylhomocysteine	1.14
Kynurenine	1.13
5-Hydroxyindoleacetate	1.11
Urocanate	1.10
Butyrylcarnitine	1.08
Prolylhydroxyproline	1.08
Pantothenate	1.04
Tyrosine	1.03
N4-Acetylcytidine	1.02
Xanthurenate	1.01

Table 56: Urine MS (pos) metabolites with VIP >1 (PBC (n=51) vs PSC (n=21)

	PBC **(median + IQR)**	**PSC** **(median + IQR)**	**p-value** **Mann-Whitney**
Tryptamine	37075 (14522 – 58518)	54724 (39535 – 128396)	0.002
Tryptophan	237753 (141371 – 357368)	604321 (354588 – 712242)	<0.0001
Indole-3-acetic-acid-O-glucuronide	148133 (74010 – 327008)	340928 (187123 – 496155)	0.001
Tyramine	31066 (21407 – 52942)	70280 (36674 – 90046)	<0.0001
Hypoxanthine	865463 (397136 – 1465989)	1462902 (867609 – 2240851)	0.005
N2-N2-Dimethylguanosine	459097 (330437 -783258)	715193 (489603 - 1136370)	0.001
Propionylcarnitine	129820 (39397 – 239434)	340492 (87704 – 620873)	0.006
Kynurenate	457150 (260749 -705749)	875080 (468515-1174227)	0.001
5'-Methylthioadenosine	20737 (11058-34983)	36300 (21111-36300)	0.004
5-Hydroxyindoleacetate	131279 (91835-181389)	226282 (157643-266266)	0.0001
Butyrylcarnitine	1397876 (849729 -2016438)	2624534 (1351483 - 3486548)	0.003
Prolylhydroxyproline	3717080 (2365465 -5611653)	5968576 (4204241 - 7648843)	0.001

Table 57: Mass spectrometry (positive ion mode)- urine metabolites in PBC [n=51] and PSC [n=21].

Mass spectrometry observed urine metabolites with associated intensity differences (Median + IQR) in patients with PBC [n=51] and PSC [n=21]. Statistical significance was determined by a non-parametric Mann-Whitney test, comparing ranks. Significant p-values (<0.05) are highlighted in green.

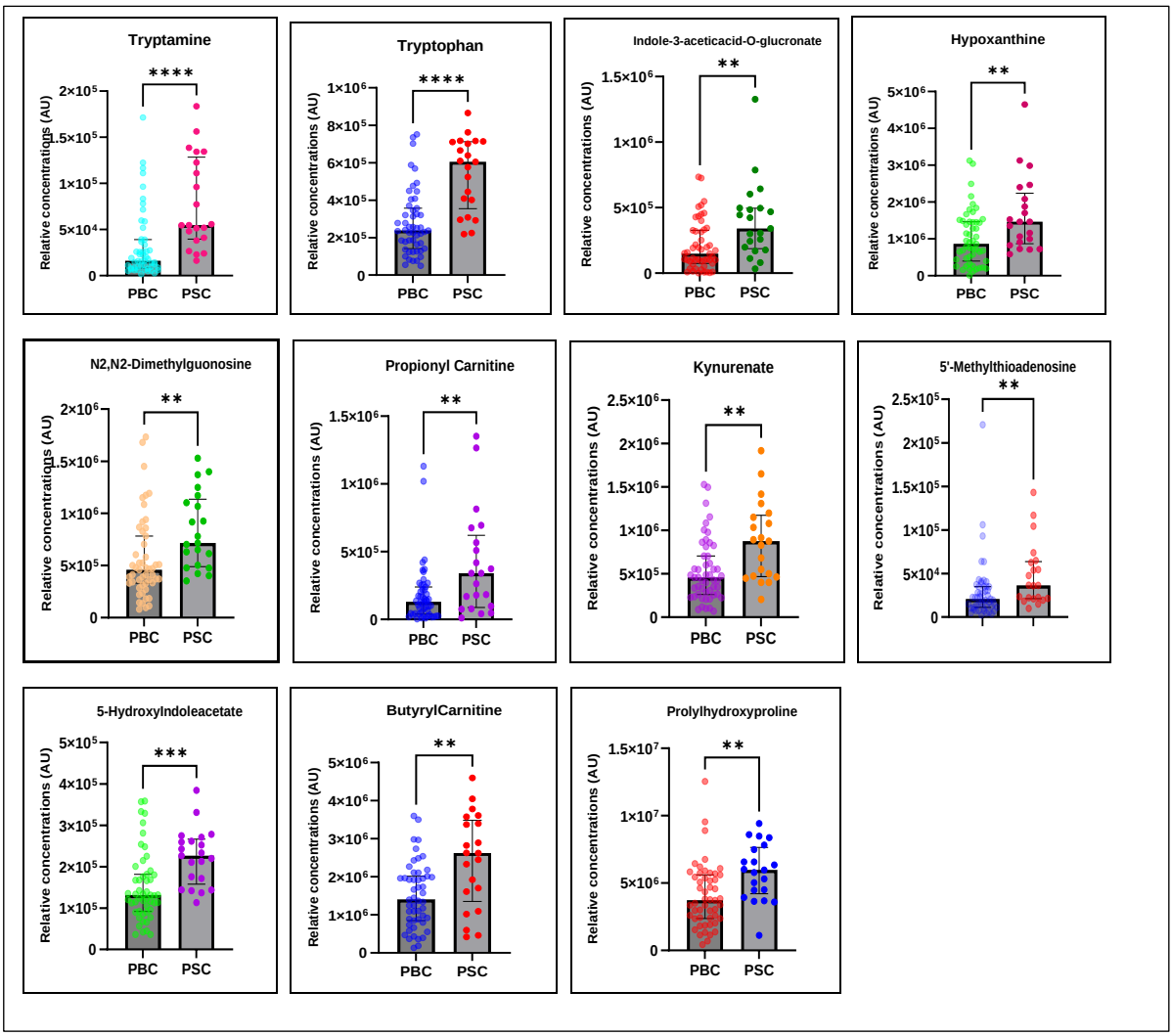


Figure 78: Targeted metabolomics: Panel to compare individual metabolites measured in urine in patients with PBC [n=51] and PSC [n=21].

The X-axis shows the relative serum concentrations expressed in Arbitrary Units (AU). p-value: ns= no significant, <0.05*, <0.01**, <0.001***, <0.0001****.

Faecal analysis PBC vs PSC:

- **Faecal Bile Acids (negative ion mode)**

A 6-component PCA model (R^2X=0.784, Q^2=0.589) attained visual discrimination between patients with PBC and PSC, in faecal bile acids on mass spectrometry. This was confirmed subsequently on a 2-component OPLS DA model, with the following statistics: (1+1+0), R^2X=0.378, R^2Y=0.448, Q^2=0.281, CV-ANOVA p=7.33527e-06 (Table 58). Permutation testing demonstrated that the model was valid in that cut-offs for R^2 and Q^2 were lower in the randomly permutated model (999 permutations) (Figure 79). Metabolites with a VIP score of ≥ 1 was used to predict their influence on class separation between the two study groups (Table 59). Resonances **increased in patients with PBC** were Lithocholic acid, Lithocholic acid-3-sulfate, 5-alpha-cholanic-acid-3-one, 3-ketocholanic-acid, 5-beta-cholanic-acid-3-beta 12-alpha-diol, 3-alpha-Hydroxy-12 Ketolithocholic Acid, Glycodeoxycholic Acid, 5-beta-Cholanic Acid 12-alpha-ol-3-one, Lithocholenic Acid, Glycolithocholic Acid (Table 60).

				CV-ANOVA p-value
PCA- X Coordinates	A	R^2X	Q^2	
	6	0.784	0.589	7.33527e-06

	A	R^2X	R^2Y	Q^2
OPLS-DA Coordinates	1+1+0	0.378	0.448	0.281

Table 58: PCA and OPLS-DA coordinates of Faecal bile acids negative ion mode PBC (n=51) vs PSC (n=21).

A= no of principal components, R^2 predicts goodness of fit and Q^2 goodness of prediction of the model. Significant p-values (<0.05)

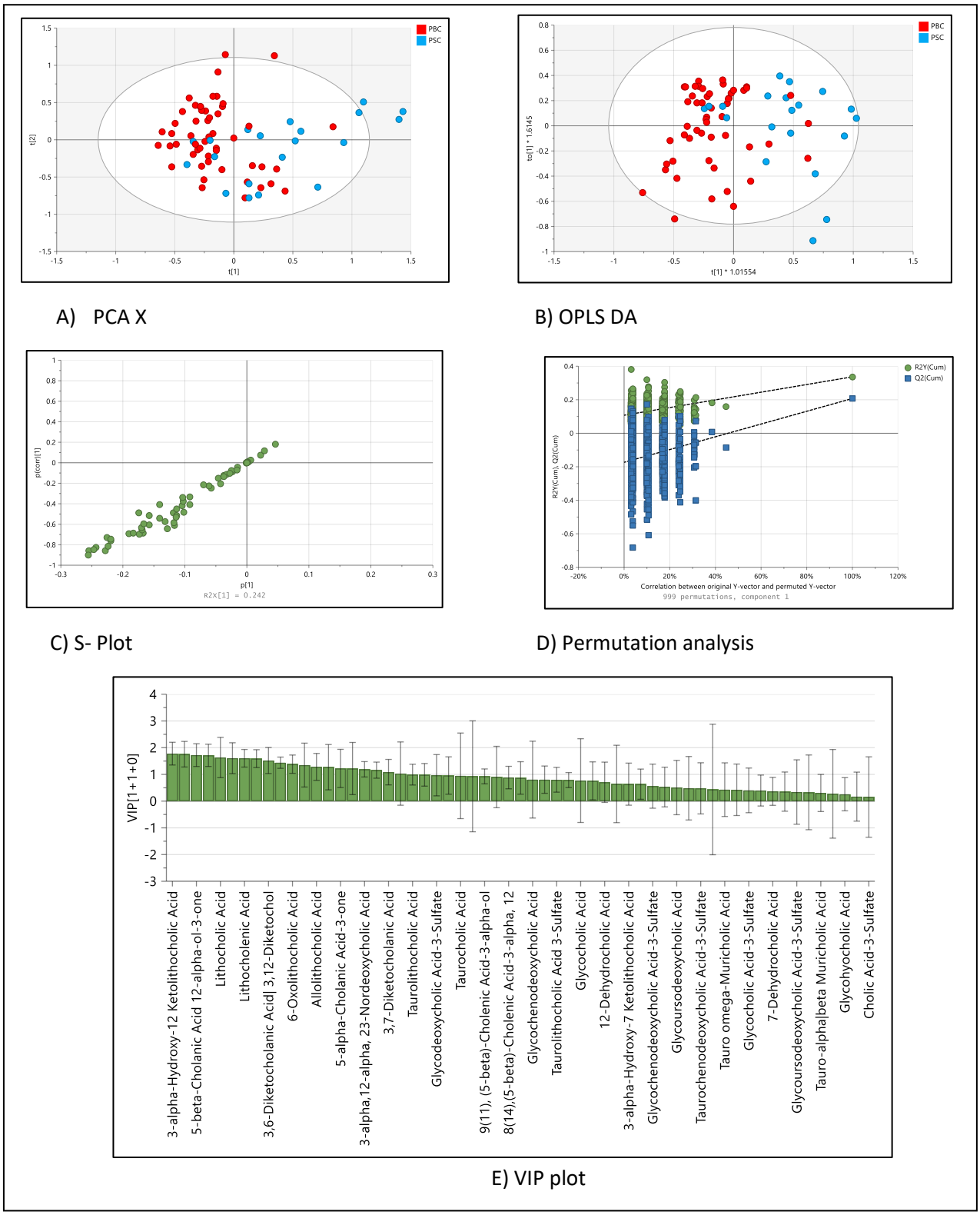


Figure 79: Fecal BA profile models PBC (n=51) vs PSC (n=21)

These multivariate models demonstrate discrimination of patients with PBC and PSC. (A) Principal components analysis (PCA) scores plot [6-component model R^2X=0.784, Q^2=0.589]. (B) Orthogonal projection least squares discriminant analysis (OPLS-DA) scores plot [(1+1+0), R^2X=0.378, R^2Y=0.448, Q^2=0.281]. (C) S-line loading plot (D) Permutation analysis (E) VIP-scores plot

Glycodeoxycholic Acid	1.72
3-alpha-Hydroxy-12 Ketolithocholic Acid	1.66
Glycolithocholic Acid	1.64
5-beta-Cholanic Acid 12-alpha-ol-3-one	1.60
5-beta-Cholanic Acid-3-beta, 12-alpha-diol	1.60
Lithocholic Acid	1.57
Lithocholenic Acid	1.53
3-Ketocholanic Acid	1.52
3,6-Diketocholanic Acid| 3,12-Diketocholanic Acid	1.44
3-alpha,12-alpha, 23-Nordeoxycholic Acid	1.43
Hyodeoxycholic Acid	1.38
6-Oxolithocholic Acid	1.33
Lithocholic Acid 3-Sulfate	1.29
Taurodeoxycholic Acid	1.26
Allolithocholic Acid	1.23
5-alpha-Cholanic Acid-3-one	1.21
Isoallolithocholic Acid	1.20
Glycolithocholic Acid-3-Sulfate	1.13
Glycodeoxycholic Acid-3-Sulfate	1.11
Taurodeoxycholic Acid-3-Sulfate	1.10
3,7-Diketocholanic Acid	1.10
Taurolithocholic Acid 3-Sulfate	1.05

Table 59: Faecal BA metabolites with VIP >1 (PBC (n=51) vs PSC (n=21)

	PBC (median + IQR)	PSC (median + IQR)	p-value Mann-Whitney
Glycodeoxycholic Acid	29806 (8400 –50035)	3335 (566.1 – 7072)	<0.0001
3-alpha-Hydroxy-12 Ketolithocholic Acid	1026220 (475088 – 1857329)	77099 (10386 – 1104440)	0.0004
Glycolithocholic Acid	11926 (5865 – 23160)	1224 (452.5 – 7094)	<0.0001
5-beta-Cholanic Acid 12-alpha-ol-3-one	460665 (162180 – 807921)	26970 (6162 – 350134)	<0.0001
5-beta-Cholanic Acid-3-beta, 12-alpha-diol	1370886 (506886 - 2544219)	118479 (11047 – 1195334)	0.0002
Lithocholic Acid	31740469 (15679845 – 42696379)	5760324 (175358 - 15027126)	<0.0001
Lithocholenic Acid	33559 (20090 –58148)	4673 (816.6 – 10874)	<0.0001
3-Ketocholanic Acid	1698654 (663561 – 2811258)	221537 (21666 – 636614)	<0.0001
Hyodeoxycholic Acid	97912 (40858 – 247884)	21124 (2972 – 94340)	0.0002
6-Oxolithocholic Acid	27650 (11093 – 90502)	5491 (1973 – 28256)	0.0008
Lithocholic Acid 3-Sulfate	500381 (30712 - 5765829)	39402 (11540 – 82234)	0.0006
Taurodeoxycholic Acid	2444 (1223 – 8163)	1017 (614.7 – 1694)	<0.0001
5-alpha-Cholanic Acid-3-one	1194 (7130 – 39476)	1898 (655.7 - 11123)	0.0007
Glycodeoxycholic Acid-3-Sulfate	1004 (708.1 – 2159)	610.8 (211.7 - 911.5)	0.0017
Taurodeoxycholic Acid-3-Sulfate	567.7 (184.2 - 819.1)	101.2 (0.000 - 718.2)	0.04

Table 60: Mass spectrometry- faecal bile acid metabolites in PBC and PSC

Mass spectrometry observed faecal bile acid metabolites with associated intensity differences (Median + IQR) in patients with PBC [n=51] and PSC [n=21]. Statistical significance was determined by a non-parametric Mann-Whitney test, comparing ranks. Significant p-values (<0.05) are highlighted in green.

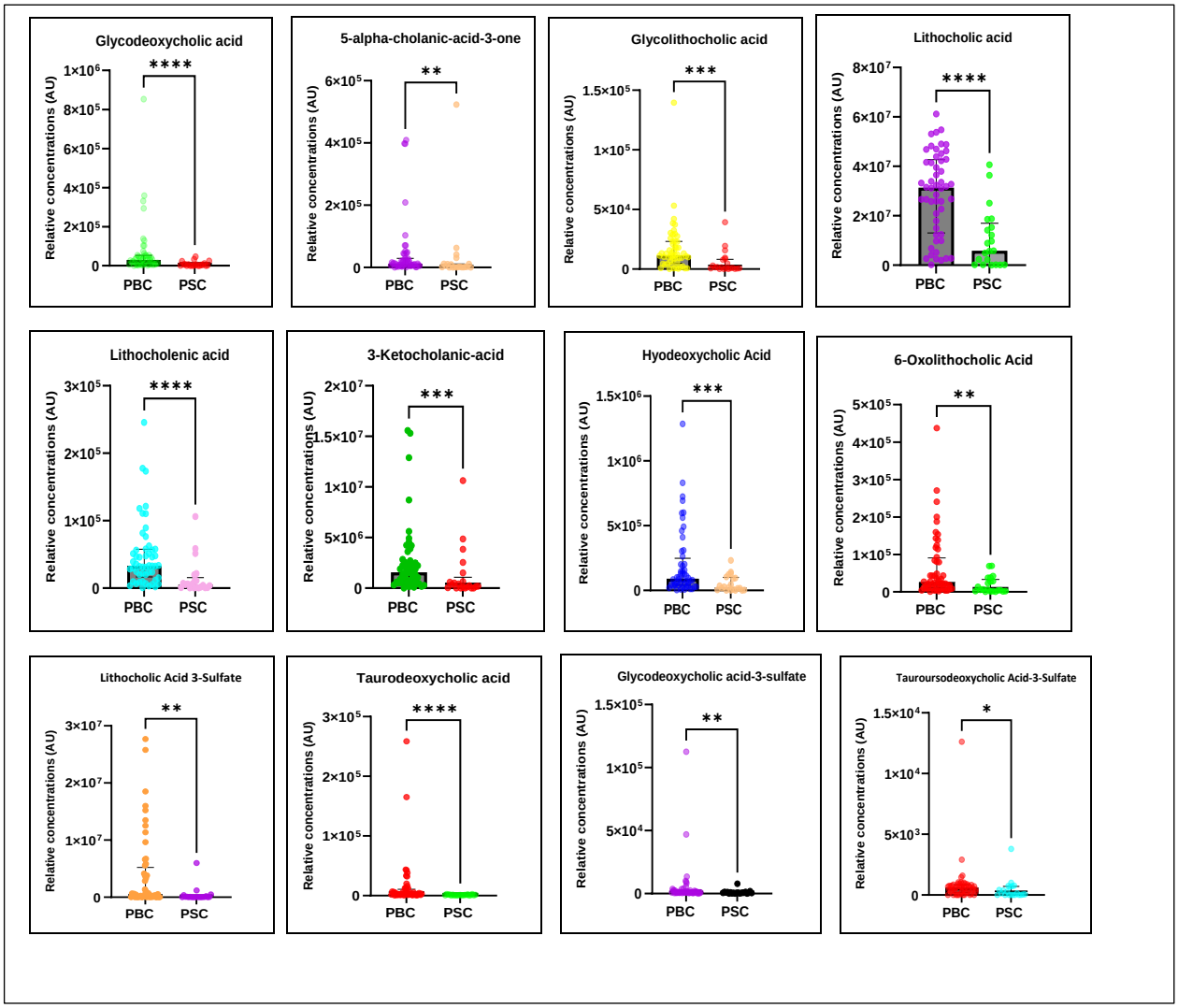


Figure 80: Panel to compare individual bile acid metabolites measured in faeces in patients with PBC [n=51] and PSC [n=21].

The X-axis shows the relative serum concentrations expressed in Arbitrary Units (AU). p-value: ns= no significant, <0.05*, <0.01**, <0.001***, <0.0001****.

The total bile acids were 7-fold and 10-fold higher in PBC and PSC respectively compared to HV.

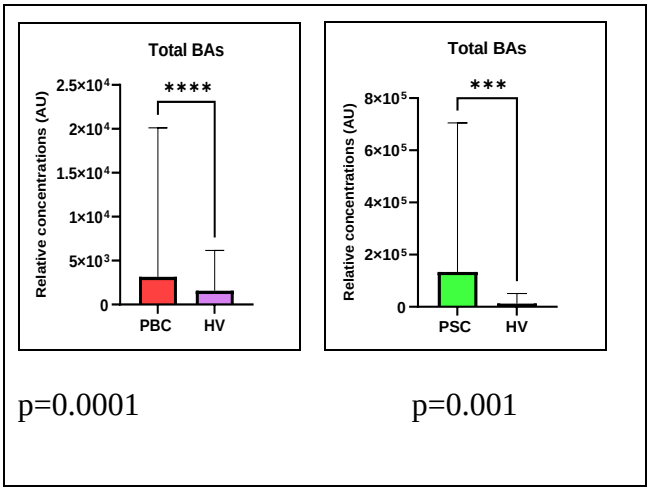
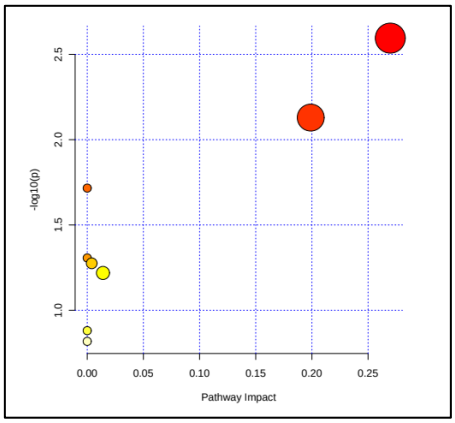


Figure 81: Levels of total BAs compared in PBC and PSC group with HV.

p-value: ns= no significant, <0.05*, <0.01, <0.001***, <0.0001****.**

Pathway analysis:

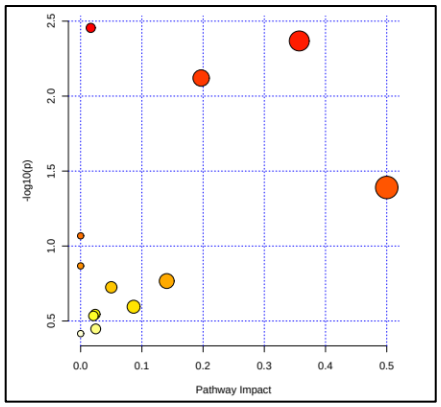
For serum lipids, highest impact pathways are sphingolipids and glycerophospholipids metabolism (Figure 82).



	Total	Expected	Hits	Raw p	-log10(p)	Holm adjust	FDR	Impact
Sphingolipid metabolism	21	0.08	2	2.54E-03	2.60E+00	2.13E-01	2.13E-01	0.27
Glycerophospholipid metabolism	36	0.14	2	7.42E-03	2.13E+00	6.16E-01	3.12E-01	0.20
Linoleic acid metabolism	5	0.02	1	1.92E-02	1.72E+00	1.00E+00	5.38E-01	0.00
alpha-Linolenic acid metabolism	13	0.05	1	4.94E-02	1.31E+00	1.00E+00	8.46E-01	0.00
Glycosylphosphatidylinositol (GPI)-anchor biosynthesis	14	0.05	1	5.31E-02	1.28E+00	1.00E+00	8.46E-01	0.00
Glycerolipid metabolism	16	0.06	1	6.05E-02	1.22E+00	1.00E+00	8.46E-01	0.01
Arachidonic acid metabolism	36	0.14	1	1.32E-01	8.80E-01	1.00E+00	1.00E+00	0.00
Steroid biosynthesis	42	0.16	1	1.52E-01	8.18E-01	1.00E+00	1.00E+00	0.00

Figure 82: Pathway analysis Serum Lipids (PBC vs PSC)

For Urine metabolites, the highest impact pathways are Tryptophan, Phenylalanine and Tyrosine metabolism (Figure 83).



	Total	Expected	Hits	Raw p	-log10(p)	Holm adjust	FDR	Impact
Purine metabolism	65	0.67	4	3.51E-03	2.46E+00	2.95E-01	1.80E-01	0.02
Phenylalanine metabolism	10	0.10	2	4.29E-03	2.37E+00	3.56E-01	1.80E-01	0.36
Tryptophan metabolism	41	0.42	3	7.58E-03	2.12E+00	6.21E-01	2.12E-01	0.20
Phenylalanine, tyrosine and tryptophan biosynthesis	4	0.04	1	4.07E-02	1.39E+00	1.00E+00	8.55E-01	0.50
Aminoacyl-tRNA biosynthesis	48	0.50	2	8.55E-02	1.07E+00	1.00E+00	1.00E+00	0.00
Arginine biosynthesis	14	0.14	1	1.36E-01	8.67E-01	1.00E+00	1.00E+00	0.00
Pentose and glucuronate interconversions	18	0.19	1	1.71E-01	7.66E-01	1.00E+00	1.00E+00	0.14
Citrate cycle (TCA cycle)	20	0.21	1	1.88E-01	7.25E-01	1.00E+00	1.00E+00	0.05
Alanine, aspartate and glutamate metabolism	28	0.29	1	2.54E-01	5.95E-01	1.00E+00	1.00E+00	0.09
Glyoxylate and dicarboxylate metabolism	32	0.33	1	2.85E-01	5.45E-01	1.00E+00	1.00E+00	0.02
Cysteine and methionine metabolism	33	0.34	1	2.92E-01	5.34E-01	1.00E+00	1.00E+00	0.02
Tyrosine metabolism	42	0.43	1	3.57E-01	4.47E-01	1.00E+00	1.00E+00	0.02
Primary bile acid biosynthesis	46	0.47	1	3.84E-01	4.16E-01	1.00E+00	1.00E+00	0.00

Figure 83: Pathway analysis Urine metabolites PBC vs PSC

5.2 Discussion:

PBC and PSC are the two most common cholestatic liver diseases. Though the underlying laboratory biochemical alterations are similar, the two disease processes vary significantly in terms of their severity and prognosis, PSC being more aggressive in its disease course(Kim et al., 2000b, Crosignani et al., 2008, Silveira and Lindor, 2008a). Despite ongoing efforts, only treatment options are available for PBC UDCA, OCA, and fibrates and none for PSC. Moreover, there are no specific biomarkers for the diagnosis and prognostication of PSC. Hence, there is an unmet need to identify metabolic signatures in these two disease processes to improve the diagnostic and therapeutic efficacy.

The study found significantly high levels of total bile acids in both PBC and PSC. These findings are consistent with the underlying disease process and have been substantiated in previous studies (Tang et al., 2015a, Lian et al., 2015a, Yang and Duan, 2016, Hegade et al., 2019b, Tietz-Bogert et al., 2018). The study from Trottier et al in PBC and PSC patients showed

an increase in total bile acids in PBC and PSC patients(Trottier et al., 2012). They further showed a 13-fold increase in primary bile acids in cholestatic patients. There was a 3-fold increase in taurine conjugate in PBC patients giving a glycine to taurine conjugate ratio of 1, however, this ratio was maintained (3:1) in PSC patients. Notably, none of the patients in this study were on UDCA. In this study, these changes were not observed because patients in both PBC and PSC study groups were on therapeutic doses of UDCA. UDCA effectively improves the bile acid profile to normalisation in PBC (Dilger et al., 2012, Crosignani et al., 1991, Poupon et al., 1993). Both glycine and tauro conjugates are cytotoxic, however tauro conjugates by activating Phosphatidylinositol 3-Kinase-dependent cell survival pathway are believed to be less cytotoxic(Marion et al., 2012, Woolbright et al., 2015, Rust et al., 2000a, Hirano et al., 2006). This may contribute to a potentially more aggressive disease progression in PSC patients. No significant alterations in secondary bile acids were seen in the PSC group compared to HV, similar to previous studies(Trottier et al., 2012, Bell et al., 2015a). An increase in secondary BAs in the PBC group was seen in this study which is different from previously reported studies. As secondary bile acids are metabolised in the intestine, the findings may indicate alterations in the gut bacteria in our group in addition to the effect of UDCA.

Significantly higher levels of HCA in the cholestatic groups were seen in this study. This is in line with the findings of previous studies(Bell et al., 2015a). HCA is present in very low concentrations (1-3%) in human serum and trace quantities in human urine and faeces(Spinelli et al., 2016). But they are increased in cholestasis. HDCA undergoes glucuronidation using UDP-glucuronosyltransferase in human liver(Sacquet et al., 1983, Marschall et al., 1987). HCA has been suggested as a marker of metabolic disorder, with levels being low in diabetes and obesity(Zheng et al., 2021b). HCA has been shown to impact glucose metabolism by its inhibitory action on FXR(Zheng et al., 2021a).

Lipid profile showed significantly higher levels of cholesterol, TGs, SMs and PCs in both cholestatic groups, with changes more pronounced in PBC. These findings are consistent with previous reports(Kowdley, 1998, Mortiaux and Dawson, 1961). Higher levels of carnitines were observed in PBC compared to PSC. Their key function is to transport fatty acids to mitochondria for energy production. It has been well established that mitochondrial dysfunction related to the impairment of beta-oxidation of fatty acids plays a central role in cholestatic liver disease(Lang et al., 2001, Arduini et al., 2012, Lang et al., 2002). In general Bile acids inhibit carnitine Acetyltransferase activity in hepatic peroxisomes(Sekas and Paul, 1992, Sekas and Paul, 1989, Wächter et al., 1999). Studies have reported increased carnitine levels in cirrhosis

of varying aetiology (Amodio et al., 1990). Lack of carnitine is associated with hepatic steatosis and enterocyte apoptosis(Sonne et al., 2012, Bowyer et al., 1988).

The raised serum level of carnitine in our study is consistent with a previous study by Bell et al (Bell et al., 2015a). It is important to note patients in both the studies were on UDCA which modulates bile acid metabolism. Yang et al in the study of their cholestatic mice models demonstrated increased carnitine levels in alphanaphthyl isocyanate (ANIT, intrahepatic PBC model) and 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC, intrahepatic PSC model) (Yang et al., 2018a). These differential levels of carnitine in the PBC and PSC groups may be explored as a potential biomarker in distinguishing these two disease processes in larger studies.

Various differential metabolites have been identified in the urine of PBC and PSC patients. Reduced levels of carnitine metabolites and guanosine in the urine of PBC patients. Similar reduced levels of these metabolites were demonstrated by Tang et al in the urine of PBC patients when compared to that of healthy controls(Tang et al., 2015a). Guanosine (a purine nucleoside) metabolites have been linked with oxidative stress-related DNA damage. Mitochondrial dysfunction, inhibition of beta-fatty oxidation and oxidative stress underlie the pathogenesis of PBC causing liver damage(Sasaki et al., 2008, Zhao et al., 2017). Increased urinary levels of hydroxyphenyllactate have been seen in Neonatal intrahepatic cholestasis caused by citrin deficiency (NICCD) and may be a potential biomarker to investigate the pathogenesis of the adult cholestatic liver disease(Zhao et al., 2011). We also noted raised urinary levels of adipate and suberate in both PBC and PSC patients, significantly higher in PSC compared to PBC. Both metabolites are products of the omega-oxidation pathway, which kicks in situations of beta-oxidation dysfunction and/or carnitine deficiency. These metabolites warrant further investigation in larger cohorts with varying stages of the disease process and may be useful in designing prognostic models.

Chapter 6: CONCLUSIONS

6.1 Conclusions:

This chapter summarizes the main conclusions drawn from the research study presented in this thesis. PBC is a progressive cholestatic liver disease, and the therapeutic options available for managing PBC-related cholestasis and its symptoms are limited. The research project aimed to investigate the distinct metabolic profiles of patients with PBC, AMANL, and symptom-specific PBC in their serum, urine, and fecal samples.

Firstly, the study examined the baseline metabolic profiles of PBC and AMANL patients in comparison to those of the healthy general population.

Chapter 2.2 compared the metabolic profile of PBC patients with Healthy Volunteers. A distinguishing pattern was seen in the serum Bile acids, serum lipids and faecal bile acids in the PBC cohort when compared to healthy volunteers. Total and secondary bile acids were significantly higher in the serum of the PBC cohort. The majority of the study patients were on UDCA therapy. We also found significantly raised levels of cholesterol, TGs, SMs and PCs in PBC patients. The findings are in line with the previous reports.

Chapter 2.3 compared the metabolic profile of AMANL patients with Healthy Volunteers. No significant difference was seen in the metabolic profile of serum, urine, and faeces of these two groups. Therefore, the AMANL group behaved metabolically like a healthy population with the only difference being positive for AMA antibody. However, given the small number of AMANL patients, the study may be underpowered to tease out the difference between the two groups.

Further, the study was conducted around the three principal hypotheses:

Hypothesis 1: **AMA-positive patients show a differential biomarker profile in the urine, serum, and faeces of those who develop liver disease compared to those who do not develop liver disease. (PBC vs AMANL)**

Chapter 3 compared the metabolic profile of AMANL patients with PBC. Total bile acids and secondary were significantly higher in the PBC group. Significant differences were seen in the metabolic profile of these two groups in the serum and faecal secondary bile acid levels. The resonances increased were metabolites of UDCA and secondary bile acids (specifically LCA).

Alterations in UDCA levels reflect the fact that most of our PBC patients were on therapeutic doses of UDCA.

Hypothesis 2: **Symptoms in PBC are associated with distinct metabolic signature(s).**

Chapter 4 compared the metabolic profile of PBC patients with specific symptoms of pruritus, fatigue, and cognitive impairment to those who were asymptomatic. No significant differences in the metabolic profile of those with fatigue and cognitive impairment were seen when compared to asymptomatic patients. However, in PBC patients with cholestatic pruritus Hyocholic acid metabolites were significantly raised in serum and faeces. Due to the exploratory nature of the study, no cause-and-effect relationship can be drawn. However, this finding of differential rise in HCA in PBC pruritus warrants further investigation in a larger cohort of PBC pruritus patients.

Hypothesis 3: **PBC has a distinct biofluid metabolic profile when compared to cholestatic control (PBC vs PSC)**

Chapter 5 compared the metabolic profile of PBC with PSC patients. Significant differences were seen in the metabolic profile of these two groups in the serum lipids, faecal bile acid and urine metabolite levels. Total, primary, and secondary bile acids were significantly higher in PSC patients reflecting a greater degree of cholestasis. The relatively higher proportion of glycine conjugates in PSC patients may explain the more aggressive nature of this disease. UDCA is known to alter bile acid composition and glycine to tauro-conjugate ratio. Most of the PSC patients in our study were on UDCA. Despite this no alterations were seen in glycine: tauro conjugates, which indicate an alternative pathogenesis mechanism may be at play. The resonances increased in serum lipid profile in PBC were TGs, SMs, Carnitines, ceramide, glycerophospholipids and cholesterol levels with the highest impact on sphingolipids and glycerophospholipids metabolism pathways. Higher levels of serum carnitine and lower levels of urine carnitine were found in PBC patients. The findings are in line with previous studies.

In summary, the thesis describes:

1. PBC and AMANL patients showed distinguishing serum and faecal (secondary) bile acid profiles, largely owing to the UDCA therapy in the PBC group (in this study).

2. PBC pruritus patients show differentially raised levels of serum and faecal HCA, and further studies are warranted to explore this. Whether raised HCA levels result in pruritus or there is complex genetic-environmental play in this subset of patients causing raised HCA levels, needs to be studied further.
3. PBC and PSC patients have differential serum lipids, faecal bile acid and urine metabolite. UDCA does not show a significant effect on bile acid profile in PSC, indicating alternative pathogenetic mechanisms. Our findings are in line with previously reported studies.

6.2 Future directions:

The research studies conducted as part of my thesis were focused primarily on addressing the role of metabolomics in the study of the expression of overt PBC in immunologically and genetically "at risk" individuals and PBC-associated symptoms. I believe the work presented in this thesis has provided novel insight into this area. However, several aspects could not be covered or completed in our studies. I believe these could be incorporated in future larger cohort studies as below.

1. Explore metabolomic signatures in treatment-naive PBC patients when compared to the AMANL group.

In our studies, the majority of PBC patients were on UDCA treatment. Therefore, we did not have the opportunity to explore the treatment-naïve PBC patients. Exploring metabolomic signatures in treatment naïve PBC may provide valuable insights into disease mechanisms, progression, and potential therapeutic targets. Metabolomic studies may unravel key metabolites and pathways linked to lipids, amino acids, bile acids, and inflammatory markers. These may, in turn, help identify unique metabolomic profiles in treatment-naive patients. Also, such mechanistic information in the development of PBC may aid in identifying therapeutic targets for early therapy and alter the course of the disease.

2. Explore metabolomic signatures, following AMANL patients in time for those who develop PBC compared to the ones who do not develop PBC.

As explored in this thesis, AMA-positive patients with normal LFTs compared to PBC have a distinct metabolic profile. However, this was only studied in a cross-sectional way. The majority of patients in the PBC group were on established UDCA treatment, potentially affecting the results. Conducting longitudinal metabolomic studies over time may help clarify how metabolomic signatures evolve with disease progression. In addition, combining metabolomics with other omics approaches may provide a more comprehensive understanding of if and how AMANL patients develop PBC.

3. Explore HCA as a potential biomarker for cholestatic pruritus. Is this a cause of pruritus' or a result of more complex genetic/environmental factor interaction?

In our study, HCA levels were raised in both PBC and PSC. However sub-group analysis showed levels were differentially raised in the PBC pruritus group.

The pathogenesis of cholestatic pruritus remains incompletely understood. The role of bile acids (total or specific type of bile acids) as pruritogen(s) has been proposed in previous studies but no specific pruritogen has been yet confirmed. Future studies could assess this further and explore if levels of Hyocholic acid correlate with the severity of cholestasis and associated pruritus. Furthermore, if HCA has any influence on TGR5 activation (signalling pathways related to itch) should be investigated.

Current evidence suggests OCA has a dose-dependent effect on pruritus and bezafibrate therapy alleviates pruritus. Future studies may assess the effect of OCA and bezafibrate on serum levels of HCA and if changes in HCA levels pre- and post-therapy correlate with itch severity. Future studies may also be designed to understand the correlation between dynamic changes in HCA levels (pre-and-post treatment) and any structural/ functional changes in the target receptors. Nasobiliary drainage improves pruritus in cholestasis. Studies are required to explore HCA levels in bile.

Another pertinent question that needs exploring is- are the alterations in HCA specific to chronic cholestasis or they are also associated with acute cholestatic conditions like Intrahepatic cholestasis of pregnancy or DILI? Metabolomic studies have shown differential alterations in bile acid levels in different grades of DILI severity. A study of hereditary intrahepatic cholestasis reported HCA in a diagnostic panel to distinguish between ABCB11- mutated cohort from HCs (AUC=0.95). Therefore, studies of HCA in different models of symptomatic acute and chronic cholestasis may provide better insight into the mechanism.

4. Explore the relationship of alterations in gut microbiome in relation to the metabolic alterations specifically relating to symptoms in the PBC and PSC group.

In our study, we did not explore the role of the gut microbiome and its interaction with metabolic profiling. However, it is known that in cholestatic diseases, there is some evidence of an association between cholestasis and gut microbes. The interplay between gut microbiome and metabolome may lead to significant metabolic/clinical and symptomatic consequences. PBC and PSC, although both cholestatic diseases have diverse phenotypes and disease courses. This may be related to distinct dysbiosis and complex interactions between gut bacteria and bile acids circulating in the enterohepatic circulation. Fatigue, cognitive dysfunction and pruritus which are common symptoms of PBC and PSC may be related to changes in the gut microbiome

that affect systemic inflammation and metabolic pathways. Therefore, future microbiome profiling studies should examine the changes in the gut microbiome in relation to disease progression and symptom development, in both cross-sectional and longitudinal ways.

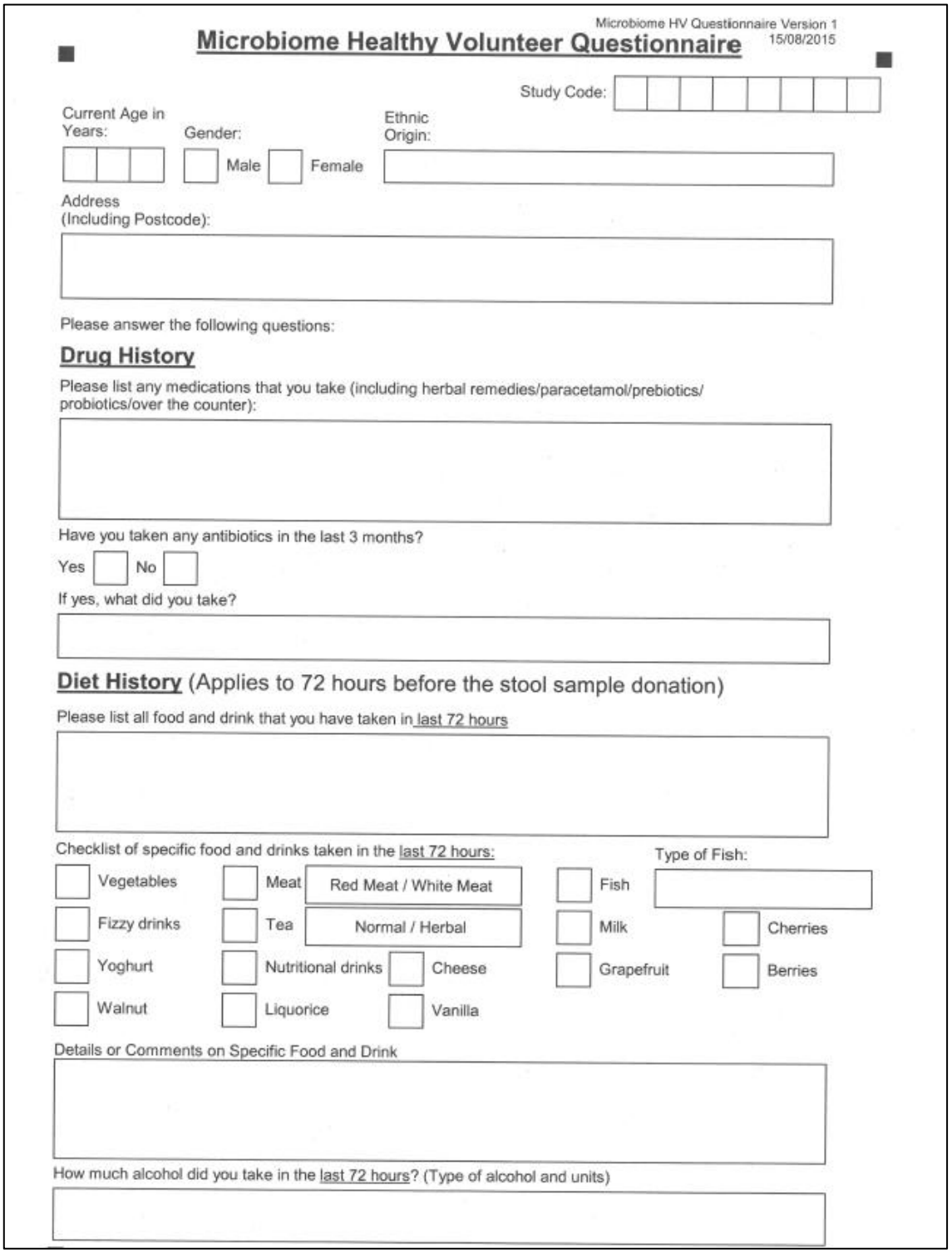
5. Prospective evaluation in all three study groups to explore change in the microbiome over time and with relevant clinical interventions.

In our studies, we did not have pre and post drug-therapy samples. Currently there are no clinical interventions/medical therapies to target and alter the metabolome or modulate gut microbiota for PBC and PSC patients. This is largely due to the lack of mechanistic insights in how specific microbial taxa and their metabolites influence pathology and systemic symptoms. Large prospective cohort studies will be needed to explore the shift in gut microbiome in relation to disease progression and how interventions (e.g. dietary, probiotics, drugs) influence the change and affect clinical outcomes in PBC and PSC patients.

Finally, I believe that it is important to utilize the scientific knowledge obtained from metabolomics in real-world clinical practice. Currently, metabolomic analysis requires bulk processing of samples and is often time-consuming and laborious. Future efforts need to focus on designing assays, which are cheap, and able to provide rapid results with high sensitivity and specificity for rapid applications in clinical areas.

7. LIST OF APPENDICES:

Appendix 1: Microbiome Healthy Volunteer Clinical Questionnaire



Microbiome HV Questionnaire Version 1
15/08/2015

Microbiome Healthy Volunteer Questionnaire

Study Code: ☐☐☐☐☐☐☐☐

Current Age in Years: ☐☐☐

Gender: ☐ Male ☐ Female

Ethnic Origin:

Address (Including Postcode):

Please answer the following questions:

Drug History

Please list any medications that you take (including herbal remedies/paracetamol/prebiotics/ probiotics/over the counter):

Have you taken any antibiotics in the last 3 months?

Yes ☐ No ☐

If yes, what did you take?

Diet History (Applies to 72 hours before the stool sample donation)

Please list all food and drink that you have taken in last 72 hours

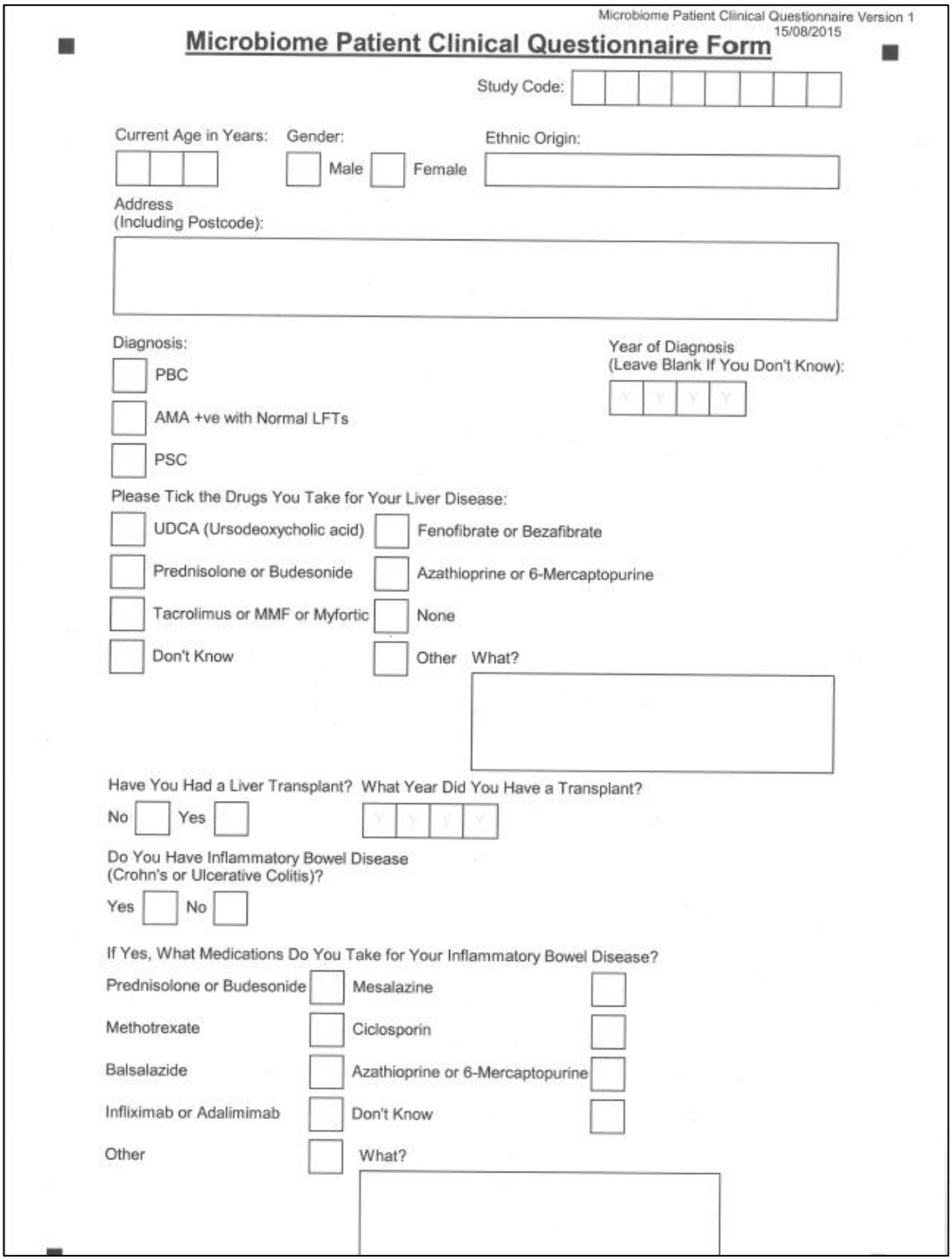
Checklist of specific food and drinks taken in the last 72 hours:

☐ Vegetables	☐ Meat	Red Meat / White Meat	☐ Fish	Type of Fish:	
☐ Fizzy drinks	☐ Tea	Normal / Herbal	☐ Milk	☐ Cherries	
☐ Yoghurt	☐ Nutritional drinks	☐ Cheese	☐ Grapefruit	☐ Berries	
☐ Walnut	☐ Liquorice	☐ Vanilla			

Details or Comments on Specific Food and Drink

How much alcohol did you take in the last 72 hours? (Type of alcohol and units)

Appendix 2: Microbiome Patient Clinical Questionnaire



Microbiome Patient Clinical Questionnaire Version 1
15/08/2015

Microbiome Patient Clinical Questionnaire Form

Study Code: ☐☐☐☐☐☐☐☐

Current Age in Years: ☐☐☐ Gender: ☐ Male ☐ Female Ethnic Origin: ☐

Address
(Including Postcode): ☐

Diagnosis:
- ☐ PBC
- ☐ AMA +ve with Normal LFTs
- ☐ PSC

Year of Diagnosis
(Leave Blank If You Don't Know): ☐☐☐☐

Please Tick the Drugs You Take for Your Liver Disease:
- ☐ UDCA (Ursodeoxycholic acid)
- ☐ Prednisolone or Budesonide
- ☐ Tacrolimus or MMF or Myfortic
- ☐ Don't Know
- ☐ Fenofibrate or Bezafibrate
- ☐ Azathioprine or 6-Mercaptopurine
- ☐ None
- ☐ Other What? ☐

Have You Had a Liver Transplant? No ☐ Yes ☐ What Year Did You Have a Transplant? ☐☐☐☐

Do You Have Inflammatory Bowel Disease
(Crohn's or Ulcerative Colitis)?
Yes ☐ No ☐

If Yes, What Medications Do You Take for Your Inflammatory Bowel Disease?
- Prednisolone or Budesonide ☐
- Methotrexate ☐
- Balsalazide ☐
- Infliximab or Adalimimab ☐
- Other ☐ What? ☐
- Mesalazine ☐
- Ciclosporin ☐
- Azathioprine or 6-Mercaptopurine ☐
- Don't Know ☐

Study Code: | | | | | | | | |

Have You Had a Colectomy
(Part or All of Your Bowel Removed)?

Yes ☐ No ☐

Do You Suffer from Fatigue?

Yes ☐ No ☐

Do You Suffer from Itch?

Yes ☐ No ☐

Do You Take Medications for Itch?

Yes ☐ No ☐

Please answer the following questions:

Drug History

Please list any medications that you take (including herbal remedies/paracetamol/prebiotics/ probiotics/over the counter):

Have you taken any antibiotics in the last 3 months?

Yes ☐ No ☐

If yes, what did you take?

Diet History (Applies to 72 hours before the stool sample donation)

Please list all food and drink that you have taken in last 72 hours

Checklist of specific food and drinks taken in the last 72 hours:

☐ Vegetables
☐ Meat Red Meat / White Meat
☐ Fish
Type of Fish:
☐ Fizzy drinks
☐ Tea Normal / Herbal
☐ Milk
☐ Cherries
☐ Yoghurt
☐ Nutritional drinks
☐ Cheese
☐ Grapefruit
☐ Berries
☐ Walnut
☐ Liquorice
☐ Vanilla

Details or Comments on Specific Food and Drink

How much alcohol did you take in the last 72 hours? (Type of alcohol and units)

Appendix 3: SOP for blood sample collection, processing, and storage

SOP 2: Blood sample collection, processing and storage

SOP number: 2
SOP full title: Blood sample collection, processing and storage
SOP effective: 28/01/2016

SOP HISTORY
First version

1. BACKGROUND/INTRODUCTION

2. PURPOSE
This SOP details the procedure for blood sample collection, processing and storage.

3. SCOPE
Applicable to all personnel working on the study. Staff should be GCP trained and be on the delegation log.

4. PROCEDURE: Please follow the following steps:

1. Using standard blood sample collecting procedure collect following samples:
   a) Two plain serum/no additive tubes- 4 mls each
   b) One lithium heparin tube-4mls
2. Inverted each bottle eight times for thorough mixing of the blood and additive
3. Place the sample bottles on ice (light excluded) or in a +4°C refrigerator for a minimum of 30 minutes before processing.
4. Process the samples after >30 minutes but <2½ hours after collection.
5. Record the collection and processing time on the CRF.
6. Spin the samples at +4°C at 1000g for 10 minutes.
7. Record the time of centrifuging on the CRF.
8. Store 250µL aliquots of the supernatant in 1.0ml 2D bar-coded sample storage tubes with screw caps (Fluid X); aiming for a minimum of 4 aliquots from each vacutainer tube.
9. Discard the vacutainers containing the sediment.
10. Samples should be labelled as follows:
    a) Patients study number; followed by
    b) The alphabetic code for the sample (S = red cap serum; H = lithium heparin plasma); followed by
    c) The aliquot number.
11. Store the labelled samples at -80°C as soon as possible.
    (NOTE: Short term storage in a standard -20°C freezer is acceptable, pending transfer to a -80°C freezer).
12. Samples should be sent to Imperial.

5. REVIEW AND MONITORING OF THIS DOCUMENT: Review if blood sample collection procedure is altered.

6. REFERENCES: Local material transfer agreement.

Appendix 4: SOP for Urine sample collection, processing, and storage

SOP 3: Urine sample collection, processing and storage

SOP number: 3
SOP full title: Urine sample collection, processing and storage
SOP effective: 28/01/2016

SOP HISTORY
First version

1. BACKGROUND/INTRODUCTION

2. PURPOSE
This SOP details the procedure for Urine sample collection, processing and storage.

3. SCOPE
Applicable to all personnel working on the study. Staff should be GCP trained and be on the delegation log.

4. PROCEDURE

Please follow the following steps:

1. Collect second void urine sample at-least 15mls using plain, additive free universal container on the day of research visit.
2. Place the sample bottles on ice (light excluded) or in a +4°C refrigerator for a minimum of 30 minutes before processing.
3. Process the samples after >30 minutes but <2½ hours after collection.
4. Record the collection and processing time on the CRF.
5. Spin the samples at +4°C at 1000g for 10 minutes.
6. Store six 2mL aliquots of urine supernatant in Eppendorf tubes.
7. Discard any remaining urine, cellular debris etc. remaining in the urine tube.
8. Label the samples as follows:
   a) Patient's PBID number, followed by
   b) The alphabetic code for the sample (U = urine), followed by
   c) The aliquot number.
9. Store the labelled samples at -80°C as soon as possible.
   (NOTE: Short term storage in a standard -20°C freezer is acceptable, pending transfer to a -80°C freezer).
10. Samples should be sent to Imperial.

5. REVIEW AND MONITORING OF THIS DOCUMENT
Review if urine sample collection procedure is altered.

6. REFERENCES

Appendix 5: SOP for Stool sample collection, processing, and storage

SOP 4: Stool sample collection, processing and storage

SOP number: 4
SOP full title: Stool sample collection, processing and storage
SOP effective: 28/01/2016

SOP HISTORY
First version

1. BACKGROUND/INTRODUCTION

2. PURPOSE
This SOP details the procedure for Stool sample collection, processing and storage.

3. SCOPE
Applicable to all personnel working on the study. Staff should be GCP trained and be on the delegation log.

4. PROCEDURE

Please follow following steps:

1. Sample will be collected by the patient in 48 hours prior to the research visit.
2. Sample will be stored in the freezer at patients home
3. Frozen sample will be brought to the research visit by the patient.
4. Written instructions to collect , store and transport stool sample will be provided to the patient (attached information sheet)
5. Send frozen samples to Imperial College.

Stool Sample Collection instructions for the participants:
The participant should collect the stool sample as follows:

1. Unwrap the kit. Place the U-Tek® icepack in freezer. Write name, date and time on sample collection pots;
2. Open Faeces-Catcher® carefully in the direction of the arrows;
3. Pass as much urine as possible into toilet to empty the bladder;
4. Paste Faeces-Catcher® on back of toilet seat. Keep it as dry as possible and not in contact with water;
5. Put on disposable gloves. Position bottom over Faeces-Catcher® paper. Open bowels onto Faeces-Catcher® paper;
6. After opening bowels, take the sample collection pot without glycerol; open it and, using the 'scoop' on the container lid, scoop 1 full scoop of faeces into the container. Put the scoop into the tube and screw the lid securely on;
7. Take the second sample collection pot that contains glycerol, open it and, using the scoop on the container lid, scoop 1 full scoop of faeces into the container. Screw the lid securely closed and mix gently for about 10 seconds;
8. If any stool has got on the outside of the tubes, please wipe clean;

9. Place each faeces tube into a leak-proof transport container. Seal the leak-proof transport containers with their respective screw-caps;
10. Place each transport container and its contents into a brown paper bag. Then place each brown paper bag and its contents into a zip-lock plastic bag. Seal the bag;
11. Place the zip-lock plastic bags and their contents into the freezer;
12. Then dispose of the Faeces-Catcher® by loosening at both ends from toilet bowl and squeezing ends together;

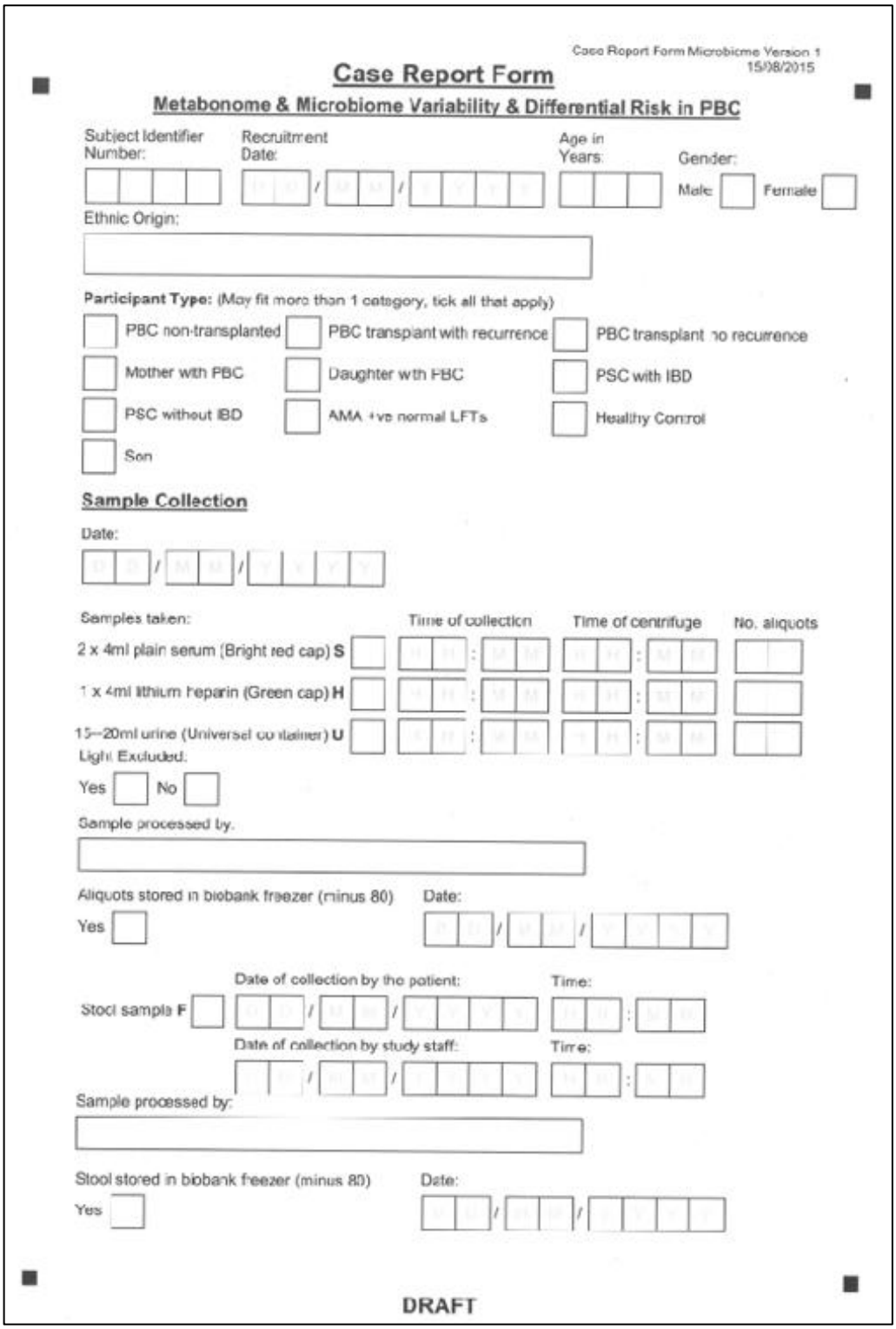
    Drop the Faeces-Catcher® into the toilet bowl and wait a moment for paper to soften with the water. It can then be easily flushed away. Dispose of disposable gloves in normal bin inside plastic bag if any contamination. Finally wash hands with soap and water.

5. REVIEW AND MONITORING OF THIS DOCUMENT

Review if Stool sample collection procedure is altered.

6. REFERENCES

Appendix 6: Case Report Form



Case Report Form Microbiome Version 1
15/08/2015

Case Report Form

Metabonome & Microbiome Variability & Differential Risk in PBC

Subject Identifier Number: | Recruitment Date: D D / M M / Y Y Y Y | Age in Years: | Gender: Male ☐ Female ☐

Ethnic Origin:

Participant Type: (May fit more than 1 category, tick all that apply)

- ☐ PBC non-transplanted
- ☐ PBC transplant with recurrence
- ☐ PBC transplant no recurrence
- ☐ Mother with PBC
- ☐ Daughter with PBC
- ☐ PSC with IBD
- ☐ PSC without IBD
- ☐ AMA +ve normal LFTs
- ☐ Healthy Control
- ☐ Son

Sample Collection

Date: D D / M M / Y Y Y Y

Samples taken:		Time of collection	Time of centrifuge	No. aliquots
2 x 4ml plain serum (Bright red cap) **S**	☐	H H : M M	H H : M M	
1 x 4ml lithium heparin (Green cap) **H**	☐	H H : M M	H H : M M	
15–20ml urine (Universal container) **U** Light Excluded.	☐	H H : M M	H H : M M	

Yes ☐ No ☐

Sample processed by.

Aliquots stored in biobank freezer (minus 80) Yes ☐ Date: D D / M M / Y Y Y Y

Stool sample **F** ☐ Date of collection by the patient: D D / M M / Y Y Y Y Time: H H : M M

Date of collection by study staff: D D / M M / Y Y Y Y Time: H H : M M

Sample processed by:

Stool stored in biobank freezer (minus 80) Yes ☐ Date: D D / M M / Y Y Y Y

DRAFT

8. REFERENCES:

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