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The discovery and validation of metabolites as candidate biomarkers for the diagnosis of hepatocellular carcinoma

Mei Ran Abellona U

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Department of Metabolism, Digestion and Reproduction
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Abstract

Mei Ran Abellona U, *The discovery and validation of metabolites as candidate biomarkers for the diagnosis of hepatocellular carcinoma.*
(Under the direction of Professors Elaine Holmes, Simon Taylor-Robinson and Jeremy Nicholson)

Hepatocellular carcinoma (HCC) constitutes a major disease burden worldwide. Much of its high mortality-to-incidence ratio can be attributed to late diagnosis, resulting in poor survival. Motivated by the need to develop a novel non-invasive diagnostic test to improve the chances of early diagnosis, this thesis makes use of metabonomic technologies to discover and validate metabolites as potential novel diagnostic markers for HCC. First, a systematic review of the literature on the topic was conducted to collate all published evidence of metabolites that were reported to be discriminatory for HCC. A bespoke risk of bias assessment tool was developed for metabonomic studies and a weighted score system was implemented to rank metabolites based on the strength of evidence. This resulted in a ranked list of metabolites with the greatest potential to be followed up for validation for each of the sample types (tissue, blood and urine). Then, validation of urinary metabolites with the greatest potential concluded from the systematic review was performed using data acquired from a UK cohort. None of the previously reported difference between HCC and cirrhosis groups could be reproduced, indicating the current lack of candidate markers specific for HCC detectable in urine. Finally, an exploratory analysis of serum ^{1}H-NMR data from a UK and a Nigerian cohort was performed. Common and different alterations in metabolite levels between the two cohorts were compared. Glutamine-to-glutamate ratio was identified as a potential marker with the best discriminatory power between HCC and cirrhosis patients and this was validated using an independent cohort from the Gambia. This work adds to the ongoing effort to elucidate metabolites with the best potential to be further validated with the goal of developing a novel diagnostic test for HCC.

To all who has faith in me

Table of contents

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Statement of originality

I declare that the work presented in this thesis and the research to which it refers are the product of my own work. Any work by other people are fully acknowledged and referenced.

Contributions of others are described below:

Dr Eric Yi-Liang Shen developed the literature search strategy, and Dr Eric Yi-Liang Shen, Dr Alzhraa Alkhatib and Dr Caroline Cartlidge contributed in data extraction and the development of the risk of bias assessment for the systematic review (Chapter 2).

Study design, participant recruitment and sample collection had been completed by research teams at various sites where the studies were conducted under the leadership and coordination of Professor Simon Taylor-Robinson (Chapters 4 and 5).

List of figures

List of tables

List of Abbreviations

1D	1-dimensional
^{1}H-NMR	Proton nuclear magnetic resonance
AAA	Aromatic amino acids
AFP	Alpha-fetoprotein
ARLD	Alcohol-related liver disease
ATP	Adenosine triphosphate
AUROC	Area under receiver operator characteristic curve
B_0	External magnetic field
BCAA	Branched chain amino acids
BCLC	Barcelona Clinic Liver Cancer
BMI	Body mass index
CE-MS	Capillary-electrophoresis mass spectrometry
CEUS	Contrast-enhanced ultrasound
CHB	Chronic hepatitis B
CHC	Chronic hepatitis C
COSY	Correlation spectroscopy
CPMG	Carr-Purcell-Meiboom-Gill
CT	Computed tomography
EASL	European Association for the Study of the Liver
FDA	Food and Drug Administration
FDR	False discovery rate
FID	Free induction decay
FWER	Family-wise error rate
GC	Gas chromatography
Gln/Glu	Glutamine: glutamate
GLOBOCAN	Global Cancer Observatory
h	Planck's constant
$\hbar$	Reduced Planck's constant
H_0	Null hypothesis
H_1	Alternative hypothesis
HBsAg	Hepatitis B surface antigen
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HILIC	Hydrophilic interaction
HIV	Human immunodeficiency virus
HMDB	Human metabolomics database
HPLC	High performance liquid chromatography
HSQC	Simple quantum coherence spectroscopy
JRES	*J*-resolved spectroscopy

JUTH	Jos University Teaching Hospital
k	Boltzmann's constant
I	Spin quantum number
LC	Liquid chromatography
LC-MS	Liquid chromatography-mass spectrometry
LD	Liver disease
log_2FC	log_2 fold change
LPC	Lysophosphatidylcholines
m	Magnetic quantum number
M	Net nuclear magnetic moment
m/z	mass-to-charge
MRI	Magnetic resonance imaging
NAFLD	Non-alcoholic fatty livery diease
NASH	Non-alcoholic steatohepatitis
NMR	Nuclear magnetic resonance
nOe	Nuclear Overhauser effect
NOESY	Nuclear Overhauser effect spectroscopy
OPLS	Orthogonal partial least squares
P	Angular momentum
PAR	Population attributable risk
PC	Principal component
PCA	Principal component analysis
pFDR	FDR-adjusted p-value
*p*FDR	$p_{\text{False discovery rate}}$
Phe-trp	Phenylalanyl-tryptophan
p_i	Loadings vector
ppm	Parts per million
PRISMA DTA	Preferred reporting items for systematic review and meta-analysis of diagnostic test accuracy studies
PROLIFICA	Prevention of LIver FIbrosis and Cancer in Africa Study
QC	Quality control
RF	Radiofrequency
RoB	Risk of bias
ROC	Receiver operating characteristic
RP	Reversed phase
RSD	Relative standard deviation
RT	Retention time
SN	Sensitivity
SP	Specificity
STOCSY	Statistical total correlation spectroscopy
STORM	Subset optimisation by reference matching
T	Temperature in Kelvin
TCA	Tricarboxylic acid

t_i	Scores vector
TMAO	Trimethylamine *N*-oxide
TOCSY	Total correlation spectroscopy
TOF	Time-of-flight
TSP	3-(trimethylsilyl- [2,2,3,3,-$^2H_4$]-propionic acid
UHPLC	Ultra-high-pressure liquid chromatography
UPLC	Ultra-performance liquid chromatography
v	Frequency
v_L	Lamour frequency
x_i	Predictor variable
α	Significance level
β_0	Intercept
β_i	Regression coefficient
γ	Gyromagnetic ratio
δ	Chemical shift
ΔE	Energy difference
μ	Magnetic moment

Chapter 1 – Background

1.1 Hepatocellular carcinoma: a global health perspective

1.1.1. Clinical manifestations of HCC

Hepatocellular carcinoma (HCC) is malignancy arising from hepatocytes in the liver. It is the most common form of primary liver cancer, accounting for 90% of cases (1). Most HCC cases occur as a late-stage manifestation of advanced chronic liver disease as a result of known underlying aetiology. These can be broadly categorised as viral, lifestyle-modifiable factors, carcinogen-exposure, and less frequently, autoimmune or hereditary conditions. Many of the underlying causes of chronic liver diseases increase the risk of developing HCC indirectly, through chronic damage and inflammatory response, leading to fibrosis and subsequently cirrhosis of the liver, while others exert a direct carcinogenic effect. The most common risk factors for HCC, namely chronic hepatitis B or C infection, alcohol-related liver disease, and non-alcoholic fatty liver disease, each induces inflammatory response that leads to hepatitis which progresses to fibrosis and cirrhosis overtime. As such, the majority (70-90%) of HCC cases occurs on a background of cirrhosis (2) and HCC is the leading cause of death in patients with cirrhosis (3).

Chronic hepatitis B (CHB) and C (CHC) infections are viral causes of HCC. 73.4% of all HCC cases can be attributable to these two causes (4). CHB alone accounts for more than half of HCC cases worldwide (5). Hepatitis B virus (HBV) is a blood-borne virus most often acquired through mother-to-child transmission or in early years of life. In highly endemic regions in East Asia and sub-Saharan Africa, acquisition early in life are usually asymptomatic. However, between 30-90% of infected individuals become

chronic carriers of the virus, depending on the age at the time of infection (6). HCC incidence in CHB carriers with or without cirrhosis is 3 and 1 per 100 person-years, respectively (6).

CHC infection is the second leading cause of HCC and is the leading cause of HCC in Western countries (7). Unlike HBV, hepatitis C virus (HCV) is most commonly transmitted via contaminated blood products and injected drug use. 75-80% of HCV infected individuals become CHC carriers, with a 10-20% chance of developing cirrhosis in 20 years and an annual risk of 1-4% in developing HCC once cirrhosis is established (8). CHB and CHC promote HCC through chronic inflammation, leading to fibrosis and cirrhosis, but also possess viral factors (such as the HBx protein in HBV or the core protein in HCV) that contribute to oncogenic transformation directly (9). CHB induced HCC without cirrhosis is more common in African American and Asian populations (accounting for up to 40% of the cases as opposed to 9% in Caucasian populations (10) and CHB has been shown to transform hepatocytes into malignant cells without associated inflammation or fibrosis and although the mechanisms are not fully understood, they are thought to involve epigenetic changes and the ability of HBV to integrate into the host genome causing genomic instability (11).

Prolonged excessive consumption of alcohol results in alcohol-related liver disease (ARLD) and is the second leading cause of HCC in Western nations (7). ARLD comprises the spectrum of conditions from alcoholic steatosis, chronic fibrosis and cirrhosis, with an annual incidence of 2.1-5.6% once cirrhosis is established (12).

Non-alcoholic fatty liver disease (NAFLD) is the umbrella term that encompasses steatosis, which is the accumulation of fat in the liver, and non-alcoholic steatohepatitis (NASH), which is the presence of inflammation and liver injury in addition to the accumulation of fat. It is considered the hepatic manifestation of metabolic syndrome as a result of obesity (13). Over time, NAFL progresses to NASH and the chronic inflammation and liver injury leads to fibrosis and subsequently cirrhosis. NASH patients with cirrhosis have a cumulative risk of developing HCC as high as 12.8% over 7 years (14). Half of the NASH-induced HCC arise in patients without cirrhosis (15), suggesting that NAFLD/NASH can promote carcinogenesis in the absence of cirrhosis.

On many levels, the metabolic signature of HCC is a progression of the metabolic dysfunction found in cirrhosis. For example, elevated serum lactate, triglycerides and choline concentrations corresponding to lactate dehydrogenase and alkaline phosphatase are elevated in both conditions but the increase in concentration is more marked in HCC patients compared to cirrhotic controls (16). Similarly, the distribution of metabolites across liver tissue, as measured by mass spectrometric imaging, was similar in cirrhosis and HCC with some slight metabolic differences. For example, some phospholipids and fatty acids were found in higher abundance in the cirrhotic condition, whereas choline, glycerophospholipid, beta-alanine, arginine and proline metabolism and arginine biosynthesis, showed a gradation of increase in relative concentrations from healthy control to cirrhosis to HCC (17). This thesis examines the metabolic patterns associated with HCC on a background of cirrhosis but also considers the HCC profile without a cirrhotic background, acknowledging that there is an increasing

number of cases arising from conditions such as NAFLD that do not always develop cirrhosis prior to HCC.

Aflatoxin B1 is a carcinogenic mycotoxin produced by a few fungal species in the genus of Aspergillus that grow in food stored in humid conditions and is a common contaminant of foods such as grains, corn and legumes (18). It is a potent hepatocarcinogen by forming covalent bonds with DNA after being metabolised in the liver. It is thought to play a causative role in 4.6-28.2% of all HCC cases, mainly in sub-Saharan Africa, Southeast Asia and China (19). Another environmental exposure risk is iron overload due to consumption of home-brewed alcohol with high iron content, which is common in rural areas in sub-Saharan African (20). It was found to elevate HCC risk by 10-fold even after controlling for viral hepatitis and aflatoxin exposure (21).

Less frequent causes of HCC include hereditary and autoimmune-related liver diseases. Hereditary haemachromatosis is characterised by the accumulation of iron in the liver and is associated with a 20-fold increased risk of HCC (22). Autoimmune hepatitis and primary biliary cholangitis are autoimmune causes of HCC, which have lower (23) or similar (24) risk of developing HCC once cirrhosis is established, compared to other causes, respectively. Budd-Chiari syndrome, a rare liver condition due to the obstruction of hepatic veins, is also a risk factor for HCC (25).

Aside from the above-mentioned causes of chronic liver injury that heighten the risk of developing HCC, advanced age, male sex and tobacco use are also independent risk factors. As with all cancers, which is a disease of the cumulation of genetic mutations and chromosomal aberrations leading to malignant transformation, the risk of

developing HCC increases with age. The male-to-female ratio of HCC range between 2-4:1 in different populations. The phenomenon is explained by androgenic hormone being HCC-promoting (26) and that male sex may be associated with greater exposure to environmental carcinogen (2). Tobacco use (27) and diabetes (28) have also been shown to be independent risk factors of HCC.

The different risk factors of HCC have synergistic effect. Co-infection of human immunodeficiency virus (HIV) with HBV (29) or with HCV (30) result in extra risk of HCC and co-infection of HBV and HCV (31) is also associated with higher risk than individuals with mono-infection alone. Tobacco use, alcohol intake and obesity have also been shown to have synergistic effects on the risk of developing HCC (27).

In summary, HCC is a late-stage manifestation of many different chronic liver conditions that promotes HCC development either indirectly or directly. The various pathological processes leading to HCC are summarised in Figure 1.1.

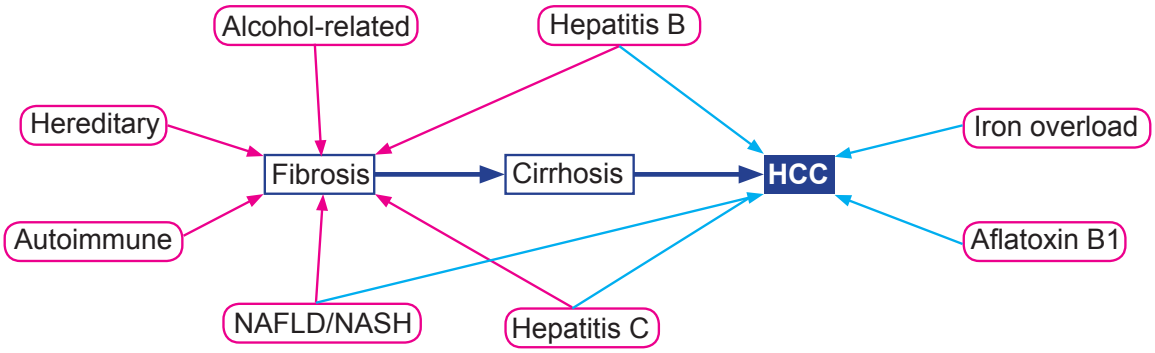

Figure 1.1. Underlying causes of HCC. While most risk factors promote HCC development through chronic inflammation leading to fibrosis and subsequently cirrhosis (pink arrows), others exert a direct effect (light blue arrows). NAFLD: Non-alcoholic fatty liver disease; NASH: non-alcoholic steatohepatitis.

1.1.2. Global burden of HCC: distribution and trends

The latest GLOBOCAN (an online database providing global cancer statistics as part of the International Agency for Research on Cancer's Global Cancer Observatory) estimates that primary liver cancer is the sixth (fifth in men) most common cancer worldwide and the fourth (second in men) most common cause of cancer-related death (32), with an estimated annual incidence of 841,000 and mortality of 782,000. It is among the top three leading causes of disability-adjusted life-years lost in men and is the second leading cancer (after lung cancer) cause of years of life lost (33). With HCC being the major subtype of primary liver cancer, accounting for up to 90% of cases (1), it constitutes substantial healthcare burden globally.

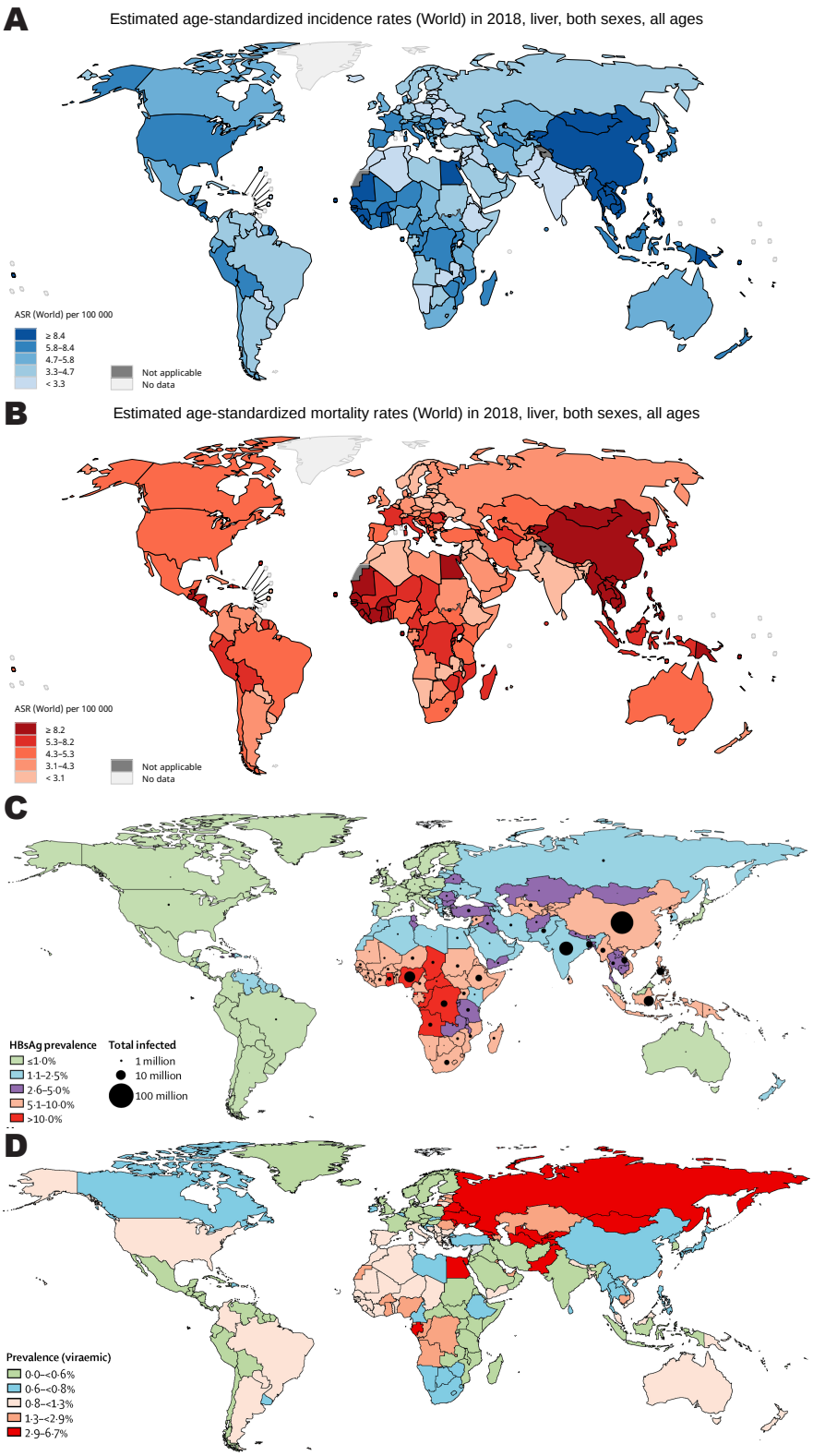

Figure 1.2. Global distribution of HCC. (A) Incidence and (B) mortality of HCC, source: GLOBOCAN 2018 (28). Regions with high HCC burden reflect the prevalence of chronic hepatitis B and C infections (C & D, respectively), adapted from Refs (34, 35).

The geographical distribution of HCC incidence is heterogenous and largely reflects the distribution of the prevalence of underlying liver conditions (Figure 1.2). CHB, responsible for over half of the HCC cases worldwide, is highly endemic in East Asia and sub-Saharan African and is responsible for these regions to have the highest incidence of HCC at over 20 per 100,000. Egypt and Mongolia are the countries with the highest prevalence of CHC in the world at over 12% (36, 37), which constitutes the high incidence of HCC in these two countries. These countries with high CHB or CHC prevalence altogether account for over 80% of the HCC cases worldwide (38) and China alone accounts for 50% of all HCC cases (39).

The pattern of HCC incidence is changing due to effective interventions for preventable causes of HCC and the rise of other risk factors. With the development and implementation of an effective HBV vaccine as part of national immunisation programs since the 1980s in East Asia, the prevalence of HBV infection in the birth cohorts has since dropped dramatically and has been shown to be associated with the decline in HCC cases due to CHB in these countries (40). However, despite the availability of an effective vaccine, due to incomplete coverage of HBV immunisation in sub-Saharan Africa, the prevalence of HBV infection remains high (41, 42).

The introduction of direct-acting antiviral therapy which offers high cure rate has been shown to lower the risk of *de novo* development of HCC (43), which is expected to reduce the contribution of CHC to HCC in the future (44). However, a few studies have found an association between direct-acting antiviral therapy and HCC suggesting that in some instances the therapy may itself contribute to the aetiology of HCC (45).

The burden of diabetes, obesity and metabolic syndrome are rising worldwide (46). The liver manifestation of these conditions, NASH/NAFLD was reported to be the most rapidly growing cause of HCC among patients with resectable HCC (47) and those listed for transplant (48) and was predicted to be the leading cause of HCC in the United States by the year 2030 (49). The rise of metabolic syndrome-related HCC is one of the major factors of the increase in incidence in previously low-risk countries (44).

In summary, HCC has the highest prevalence in East Asia, sub-Saharan Africa, Egypt and Mongolia due to the high endemicity of CHB or CHC infections. With the introduction of effective newborn HBV vaccine, there has been a decline in incidence in East Asia. However, the effect has not yet been observed in sub-Saharan Africa owing to incomplete immunisation coverage. A similar decline in CHC-related HCC is expected in the coming decades due to the introduction of direct-acting antiviral therapy for CHC, which has achieved high cure rates. On the other hand, incidence in previously categorised low-risk countries is rising due to the rise in obesity. Overall, the global burden of HCC persists but with a changing geographical pattern.

1.1.3. Surveillance and diagnosis of HCC

HCC may be detected by various methods each with its own advantages and disadvantages (Table 1.1).

Table 1.1. Comparison of various methods for detecting HCC. Based on the European Association for the Study of the Liver (EASL) Clinical Practice Guidelines (1).

	Advantages	Disadvantages	Availability in resource-limited settings	Invasiveness
Biopsy	Gold standard, allow for subtyping and differential diagnosis	Pain, risk of bleeding, risk of tumour seeding	Unlikely	Invasive
Dynamic magnetic resonance imaging	High specificity	Costly, risk of allergic reaction to contrast agent	Unlikely	Non-invasive
Contrast-enhanced computed tomography	High specificity	Costly, radiation exposure	Unlikely	Non-invasive
Ultrasound	Low-cost, risk-free	Operator-dependent, subject to machine quality, not applicable for obese patients	Likely, although machine quality may be poor and operator training may be insufficient	Non-invasive
Serum alpha-fetoprotein	Low-cost	Low sensitivity	Likely	Non-invasive

Liver biopsy is the current gold standard for unequivocal diagnosis of HCC. It allows for the delineation of HCC from intrahepatic cholangiocarcinoma, the second most common form of primary liver cancer. Histopathological examination and staining also allow for the subtyping of tumour. However, it is invasive and is associated with pain, risk of bleeding, seeding of tumour cells along the needle track (50) and has a false-negative rate as high as 30% (51).

Diagnosis of HCC via non-invasive criteria, mainly imaging techniques, is generally only applicable to patients with pre-existing cirrhosis (1). Contrast-enhanced computed tomography (CT) or dynamic magnetic resonance imaging (MRI) are two imaging modalities. HCC lesions display high contrast uptake during the arterial phase, followed by characteristic ‘wash out’ in the venous phase. For nodules between 1-2

cm, CT and MRI each has specificity of close to about 90% and sensitivity of about 70% (52). However, the high cost of MRI, radiation risk associated with CT scans and the risk of allergic reaction to contrast agents prevent their routine use as a surveillance method (1) and they are not available in resource-limited settings.

Ultrasound is a favourable method for detecting HCC as it is non-invasive, free of radiation exposure and risk-free. Meta-analyses estimated that ultrasound has a pooled sensitivity of 84-94% for detecting HCC in cirrhotic patients but the sensitivity is much lower (47-63%) for early HCC (53, 54). However, the accuracy of US performance depends on the level of operator training, the quality of the ultrasound machine and may not be applicable for obese patients (55).

Alpha-fetoprotein (AFP) is the only established serum biomarker that has been shown to be of diagnostic value for HCC. It is a protein found in high levels in the fetus and also in most HCC patients but is otherwise absent in adults. A cutoff at 20ng/mL has specificity of 90% and sensitivity of about 60% (56). However, since there is no evidence that AFP provides additional value to the detection of HCC than using ultrasound alone, its use is no longer recommended in European and American guidelines (1, 57).

In addition to AFP, few other serum biomarkers such as des-gamma-carboxy prothrombin and lens culinaris agglutin-reactive AFP, have been assessed as potential serum biomarkers. However, their accuracy has not been found to be superior to AFP and hence they are not in routine clinical use (58, 59).

The use of these different methods in the surveillance and diagnosis of HCC depends on the clinical settings. European and American guidelines recommend six-monthly surveillance using ultrasound for high-risk patients (1, 57). Diagnosis can be established by one positive imaging with confirmation by alternative imaging, or biopsy in cases where imaging results are inconclusive (1, 57).

However, in resource-limited settings, early diagnosis remains a challenge (42). A blood test for AFP and / or an ultrasound scan alongside clinical observations (enlarged upper right quadrant) may be the only available options (60). Given that most HCC cases occur in resource-limited settings, the inadequate performance of AFP and the limitations of ultrasound highlight the need for new low-cost tests for detecting HCC.

1.1.4. The need for novel non-invasive diagnostic tool for HCC

The stage of HCC at diagnosis determines the clinical management and treatment outcome. Barcelona Clinic Liver Cancer (BCLC) staging (Figure 1.3) is one of the most common staging systems for HCC as it links best treatment options with prognosis based on clinical evidence (61, 62) . Staging is determined by the size and multiplicity of the tumour, as well as the preservation of liver function, determined by Child-Pugh score (63). Curative treatment options (such as radiofrequency ablation, transplant or resection) are only viable if diagnosis is made early when tumours are smaller than 3 cm with preserved liver function (1). Overall survival decreases as diagnosis is made at later stages. In resource-limited settings, resection and liver transplant options may not be available. Nevertheless, affordable curative treatment options, such as percutaneous ethanol injection may be available, but only if diagnosis is made early (60).

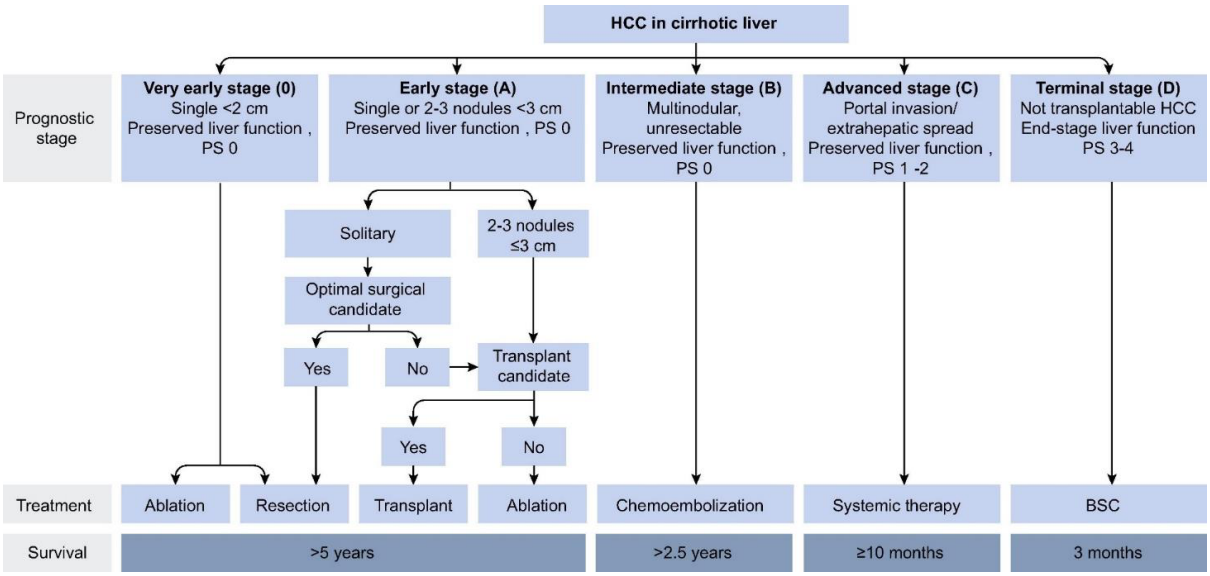

Figure 0.3 The Barcelona Clinic Liver Cancer staging system with overall survival. The BCLC system operates on a five-stage model based on the number and size of tumours, liver function, and performance status (based on child-Pugh score) of patients. For each stage, there is a recommended treatment strategy. Reproduced and adapted from Refs 1 and 64, with permission.

As most HCC cases occur in resource-limited settings where there is difficulty to diagnose HCC early, it is a major reason for the high mortality-to-incidence ratio worldwide. Given that HCC mostly affects adults of productive age, especially in regions where the burden is most serious, the availability of a novel non-invasive tool for diagnosis, and possibly surveillance or screening depending on the healthcare setting, confer immense value in alleviating the burden of HCC. The availability of a novel low-cost non-invasive diagnostic test for HCC would not only be of immense value in resource-limited settings by allowing diagnoses to be made earlier to improve survival, it would also be beneficial in countries where there are surveillance programs in place by improving early detection rate.

1.2. The role of metabolism during tumorigenesis of HCC

1.2.1. Metabolic reprogramming necessary to sustain cell proliferation

The re-arrangement of energy metabolism necessary to sustain chronic cell proliferation has been recognised as an emerging hallmark of cancer (65). The three key purposes of the metabolic reprogramming are to meet cancer cells' increased energy, biosynthesis and redox needs during malignant transformation (66).

One of the best described metabolic alteration attributable to cancer is that cancer cells in culture prefer glycolysis for generating ATP, metabolising glucose to lactate, rather than using the oxygen-dependent oxidative phosphorylation even when oxygen is abundant. This aerobic glycolysis was the first described by Otto Warburg (67) and was eponymously termed the 'Warburg effect' (68). It has been suggested that it leads to greater survival during unstable oxygen supply and that the intermediates from

glycolysis may act as substrates to sustain biosynthesis necessary for cell proliferation (65, 66).

Apart from the reliance on glucose, dependence on glutamine is another well-characterised feature of cancer cells. Despite the shift towards aerobic glycolysis, the tricarboxylic acid (TCA) cycle is often intact (66) and glutamine can serve as an alternative fuel source to produce ATP via the TCA cycle (69). This process, called glutaminolysis, also act as an important source for the biosynthesis of amino acids, lipids and nucleotides and for sustaining glutathione pool for the maintenance of redox balance (68).

1.2.2. Metabolic regulation as targets of tumour suppressor genes and oncogenes

Given the necessity for cancer cells to reprogram their metabolism, it is not surprising that many downstream targets of oncogenes and tumour suppressors have a role in metabolic regulation.

In genomics and exome sequencing studies of HCC, the tumour suppressor gene *TP53* and the β-catenin gene *CTNNB1* were found to be the most frequently mutated genes in HCC tumours, each occurring in more than a third of the cases (70, 71). The tumour suppressor p53 is known to regulate glucose uptake and central energy metabolism (72). The loss of tumour suppressor p53 leads to the downregulation of the glycolysis inhibitor TIGAR, which consequently promotes glycolysis (73). A deficiency in, or dysregulation of p53 can also lead to the downregulation of TCA cycle via SCO2 (synthesis of cytochrome C oxidase 2). Thus, p53 has been recognised as

a major force behind the glycolytic phenotype of cancer cells (65). On the other hand, Wnt/β-catenin signaling can be linked to glycolysis, the TCA cycle and glutaminolysis both directly and indirectly (74).

As such, metabolic reprogramming is a crucial process during tumourigenesis and is the direct result of genomic alterations in cancer cells. To support such metabolic activities, tumour cells need to interact with biomolecules available in circulation, through uptake or secretion. Additionally, rapid cell proliferation in tumour is accompanied by necrosis, resulting in cellular contents being released (65). Taken together, this leads the formation of the hypothesis that tumourigenesis leaves a detectable signature in biofluids.

1.3. Metabonomics

1.3.1. Metabonomics: the 'omics' of molecular phenotype

The term 'metabonomics', first coined by Nicholson and colleagues (75), was defined as 'the quantitative measurement of the dynamic multiparametric metabolic response of living systems to pathophysiological stimuli or genetic modification'. It differs from the term metabolomics, which is the complete characterisation of the collection of metabolites in biological sample (76), in that metabonomics focuses on the elucidation of changes as a result of biological or disease processes. The following discussion uses the term metabonomics. Nonetheless, metabolomics largely applies due to the overlapping nature of the two terms.

Advancement in various technologies in recent decades have forged the formation of various 'omics' fields. This includes genomics, which studies the collection of genes;

transcriptomics, the collection of expressed genes; and proteomics, the collection of proteins. Such comprehensive study of biological systems gave rise to a new paradigm. Contra to previous 'reductionist' approaches focusing on particular components and its relation to others in a 'bottom-up' manner, the 'systems' approach allows for holistic and integrated understanding of the biological phenomenon in a 'top-down' manner (77). Since components in a biological system do not work in isolation, the systems approach has the benefit of better understanding the formation of emergent properties and response to perturbation through studying the interaction and behaviours of all components in a system (78).

According to the central dogma of molecular biology (79), genetic information encoded in DNA is transcribed into messenger RNA and is in turn translated into polypeptide chains and hence proteins to execute biological functions. The outcome of the process is the development and maintenance of the phenotype of the living cell and consequently, the whole organism. In this context, metabonomics can be considered the ultimate 'omic' of the predecessing 'omics' as it represents the end point of the central dogma characterising phenotypes at the molecular level – the molecular phenome. Hence, metabonomics has been proposed to be a complement and augmentation of the other 'omics' fields (80).

The definition of the subject of interest of the field of metabonomics is not clear cut. However, it typically includes any small compounds (smaller than 2,000 Da) that may be found in biological systems. They may be metabolites derived from endogenous metabolism of the organism, or of dietary or of xenobiotic origin (81). While lipidomic, the study of the diverse forms of lipids is generally considered a sub-field of

metabonomics (82), polypeptides are considered to be under proteomics, and thus not within the scope of metabonomics.

Metabonomic analysis can be classified into two strategic approaches, targeted or untargeted. Targeted analysis involves the quantification of a pre-defined set of metabolites while untargeted analysis involves the global unbiased profiling of compounds present in a sample (83). Targeted analysis allows for the absolute quantification due to the use of calibration curves of individual compounds. The untargeted approach has the advantage of allowing discovery of novel metabolites associated with the biological or clinical condition in question but face the issues of more complex data handling, the need for metabolite identification and its semi-quantitative nature (83).

1.3.2. Enabling technologies for metabonomics

The advent of the field of metabonomics has been facilitated by the advancement of various technologies in analytical chemistry. Nuclear magnetic resonance (NMR) spectroscopy and liquid or gas chromatography (LC / GC) coupled to mass spectrometry (MS) are the most common methods employed in the field. Both allow for accurate and precise measurements of a large collection of small molecules using a small volume of liquid (50-600μL, which may be urine, serum or other biofluids, homogenised tissue or cell extract) or solid (typically <1mg of tissue) samples (84). Each of these methods has its advantages and disadvantages. Also, due to the diversity of chemical compounds present in a biological sample that no single assay can encompass, the use of different methods to analyse the same sample has the benefit of maximising coverage.

NMR spectroscopy takes advantage of the magnetic spin nature of atoms. When subjected to a strong external magnetic field and radio frequency pulses, atoms with uneven spin relax to ground energy state at different frequencies depending on its molecular structure, hence giving rise to signals that inform its structure and quantity. In metabonomic phenotyping, proton (^{1}H) NMR is used most often due to the ubiquity of protons in biological compounds. NMR spectroscopy has the advantage of being a highly reproducible non-destructive method requiring minimal preparation. However, compared to MS, it has lower sensitivity, which may be partially overcome by increasing the number of scans or by the use of a spectrometer with stronger magnetic fields.

Liquid chromatography (LC) or gas chromatography (GC) coupled to MS are the other main analytical technologies used. The first chromatographic step separates the complex mixture of compounds according to their physical and chemical properties. By being coupled to a mass spectrometer, the partially separated compounds can be detected with high sensitivity. The separation method determines the class of compounds separated and detected. For example, GC allows for the separation of compounds that can be derivatised to become volatile (e.g. amino acids), hydrophilic interaction (HILIC) LC allows for the separation of polar compounds and reversed phase (RP) LC allows for the separation of non-polar compounds (e.g. lipids). Modern ultra-high-pressure LC (UHPLC) systems, which utilises smaller solid phase particles (smaller than 2 micron) and higher pressure (up to 15,000psi), permits high resolution separation of compounds in a complex mixture (85). Together coupled-MS systems have the advantage of being highly sensitive but suffer from the disadvantages of

samples being destroyed during analysis, lower reproducibility and more complicated data handling due to batch effects (for LC) and more time-consuming sample preparation (for GC).

The principles of NMR and LCMS, which are the two analytical platforms used for sample analyses in this thesis, are described in greater detail in Section 3.1.

In addition to advances in analytical chemistry that made simultaneous, sensitive and accurate measurement of small metabolites possible, accompanying development in data analysis and modelling was also crucial to advances in the field. This includes unsupervised and supervised multivariate statistical modelling algorithms, such as principal component analysis (PCA) (86) and orthogonal partial least squares (OPLS) (87) which identify latent variables of data by maximising the variance or the covariance, respectively, providing overviews of data in a reduced dimension. Whilst other multivariate statistical methods such as neural networks or genetic algorithms can be applied to '-omics' data, the transparency of the influence of the input variables make the linear projection methods such as PCA and OPLS an appropriate choice for modelling spectroscopic data. Also, statistical tools were developed for aiding metabolite assignment, an example of which is statistical total correlation spectroscopy (STOCSY) (88) which identifies NMR peaks with high covariance and correlation. Computational methods used for analysis in this thesis are described in greater detail in Section 3.2.

1.4. Scope of thesis

Given the global burden of HCC and the unmet need for novel non-invasive diagnostic test illustrated in Section 1.1, and that HCC tumourigenesis involves metabolic reprogramming, which may leave a detectable signature in easily accessible biofluids described in Section 1.2, this project makes use of the advent of metabonomics (Section 1.3) to search for potential diagnostic biomarkers for HCC.

The term ‘biomarker’ is a portmanteau of ‘biological markers’. It may be broadly defined as any objectively measured characteristics that indicate a biological or pathological process, or response to therapeutic intervention (89). In the context of diagnosis for HCC, since most HCC cases develop on a background of cirrhosis, biomarkers should be able to delineate HCC cases from healthy control as well as from cirrhotic patients. Additionally, the ideal biomarker should be universal – that it is applicable regardless of genetic, environmental and aetiological factors.

Therefore, the overarching hypothesis of the thesis is that HCC tumourigenesis involves aberrant metabolism that leaves a unique metabolic signature that is detectable in easily accessible biofluids such as urine or blood. The responsible metabolites can be identified and can serve as diagnostic biomarkers for HCC that are superior to currently available methods in terms of sensitivity and specificity. To test this hypothesis, a comprehensive metabonomic investigation was performed using samples collected from sub-Saharan Africa and the United Kingdom.

The aim of the project is to identify and validate metabolites in either urine or blood that can be used as robust diagnostic biomarkers for detecting HCC. The ultimate goal is to develop a low-cost, rapid point-of-care diagnostic tool for HCC.

The specific objectives are:

- To comprehensively collate from the literature previous reports of metabolites that have been found to be discriminatory
- To perform metabolic phenotyping of urine and serum samples using ^{1}H-nuclear magnetic resonance (NMR) spectroscopy and ultra-performance liquid chromatography (UPLC)-mass spectrometry (MS)
- To evaluate the performance of previously reported biomarkers using the current data as an independent validation of the published potential markers
- To analyse metabolic profiles to identify discriminatory metabolites
- To assess whether the identified biomarkers are specific to HCC independent of genetic, environmental and aetiological factors by validating the results using samples from different populations

Chapter 2 presents a systematic review of published literature on the topic to date.

Chapter 3 describes the experimental and computational methods used in subsequent chapters.

Chapter 4 presents an attempt to validate urinary markers concluded from the systematic review (Chapter 2) using NMR and LCMS data generated from a UK cohort.

Chapter 5 presents an exploratory analysis comparing serum metabolic profiles from a UK and a Nigerian cohort in order to identify common alterations, with validation performed using a Gambian cohort.

Chapter 6 provides an overall discussion and general interpretation of the results.

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Chapter 2 – Discriminatory metabolites as candidate biomarkers for the diagnosis of hepatocellular carcinoma: a systematic review

Abstract

Background and aims: Metabolic reprogramming needed to sustain hepatocellular carcinoma (HCC) development forms the rationale for the search for novel diagnostic biomarkers for HCC in metabonomic studies. This review aims to systematically collate metabolites that have been reported to be dysregulated and identify any that may be probable candidate biomarkers for HCC.

Methods: Eligible studies were metabolic profiling studies that analysed tissue, blood or urine samples comparing the concentration of metabolites in biological matrices of participants with HCC with those in corresponding control groups: healthy, pre-cirrhotic liver disease, cirrhosis, or non-tumour tissue. Searches were conducted on Medline and EMBASE for reports published up to 5th February 2019. A bespoke risk of bias tool for metabonomic studies was developed and implemented. Discriminant metabolites in each sample type were ranked using a weighted score with the direction and extent of change, and the risk of bias of the reporting publication taken into account.

Results: A total of 84 eligible studies were included in the review, which consisted of 15, 54 and 9 studies investigating tissue, blood and urine respectively, and 6 studying more than one sample type. High ranking metabolites by their weighted score for each sample type included a diverse collection of metabolites such as energy metabolites, bile acids, acylcarnitines and lysophosphocholines. There was high coherence between studies among high-ranking discriminatory metabolites in tissue, but insufficient data and coherence for urine and blood.

Conclusions: While no metabolite can be concluded definitively as potential markers for HCC, these first-in-kind systematically compiled ranked lists of discriminatory metabolites provide a valuable resource for informing future studies. Studies with standardised design are necessary to validate existing findings and to move research forward beyond exploratory investigations. (Funding: None; PROSPERO Registration: CRD42018095412)

2.1. Rationale

Hepatocellular carcinoma (HCC) is a major contributor of disease burden globally with the majority of cases occurring in low-and-middle-income countries (1, 2). Methods to diagnose HCC include: percutaneous liver biopsy, which is invasive and has associated risks (3); imaging technologies such as contrast-enhanced computed tomography or magnetic resonance imaging, which are not readily available where the burden is the heaviest; ultrasound, which is operator-dependent (4), and serum alpha-fetoprotein (AFP), which has suboptimal accuracy (4). Despite poor performances, US and AFP are still heavily relied upon in resource-limited areas in Africa (2). Since HCC is largely asymptomatic at its early stages, diagnoses are often late, resulting in poorer survival. Therefore, a more accurate and low-cost diagnostic marker is an unmet medical need that has huge potential impact of alleviating the healthcare burden and overall survival of HCC (5).

Metabonomic techniques utilise advanced analytical chemical technology, such as nuclear magnetic resonance (NMR) spectroscopy and gas or liquid chromatography-mass spectrometry (GC/LC-MS), to detect and quantify compounds in complex chemical mixtures simultaneously. Its application to analyse clinical samples, such as

tissue extract or biofluids, holds promise to provide novel solutions to diagnostic and other clinical needs (6). The search for novel diagnostic biomarkers in HCCs using metabonomic methods has been motivated by the understanding that substantial metabolic reprogramming is necessary for malignant cells to drive and sustain tumorigenesis (7). In HCC, quantities of different metabolites are likely deranged due to changes in central energy metabolism (cancer cells' preference for anaerobic respiration, even when oxygen is available: the Warburg hypothesis) and lipid profile to support rapid cell membrane turnover (8, 9).

To date, numerous studies have been published reporting discriminatory metabolites between HCC and control groups with the aim to identify potential diagnostic biomarkers. However, the huge volume of publications has not yet been systematically reviewed to gather the evidence and identify contradictory reports, with published reviews either suggesting the diagnostic promise of metabonomic technology with examples (10), targeted metabolite panels (11) or focussing on specific analytical platforms or biofluids (12).

This chapter presents a systematic collation of metabolites that have been found to be statistically discriminatory between HCC and control groups in tissue, blood or urine with the purpose of identifying any probable diagnostic biomarker with the best potential to robustly identify HCC. To accomplish that, a systematic review was conducted in accordance with the PRISMA-DTA statement (13). A bespoke risk of bias assessment was developed for metabonomic studies and implemented to assess the included studies. A weighted score system was developed to rank metabolites based on the number of reports of significant difference, the fold-change reported, the

concordance in the direction of change among reports, and the risk of bias of the reporting publication(s). This resulted in a ranked list of metabolites that have been most frequently and consistently reported to be discriminatory for each sample type, representing metabolites that should be followed up in future validation studies.

2.2. Methods

2.2.1. Strategy of literature search

Eligible studies were human case-control studies that satisfied the following criteria: i) the study compared patients diagnosed with HCC to one or more of the following control groups: healthy controls, any pre-cirrhotic liver disease (e.g. chronic hepatitis, NAFLD, etc.), cirrhosis and non-tumour tissue (for tissue studies); ii) investigated liver tissue, blood (serum or plasma) and/or urine samples, and; iii) analysis of the samples and report of the metabolites found to be statistically significantly different between HCC and the control groups. Exclusion criteria were studies that investigated compounds of specific dietary components (such as from tea), xenobiotics (such as aflatoxin exposure), hormones (such as androgen levels) and reactive oxygen species. Studies were limited to original research articles published in English.

Literature searches were conducted on the databases MEDLINE and Embase via Ovid. The search strategies, which included both MeSH terms and keywords, were developed by Dr Eric Yi-Liang Shen with a librarian and were validated manually (Appendix A Table 1). Studies published up to 5th February 2019 were retrieved using the search strategies, with additional papers identified manually through searches on PubMed and Google Scholar. This systematic review was performed in accordance to the PRISMA-DTA statement (13) (Appendix A Table 2) and had been registered on PROSPERO: CRD42018095412 (14).

2.2.2. Study selection and data extraction

Initial screening using titles and abstracts, and subsequent full-text screening were performed by two investigators independently at each stage (myself with Dr Alzhraa Alkhatib and myself with Dr Eric Yi-Liang Shen, respectively). Included studies were then divided into four groups and were extracted by investigators (myself with Dr Alzhraa Alkhatib, Dr Eric Yi-Liang Shen and Dr Caroline Cartlidge) and were reviewed independently. Any discrepancy or disagreement was resolved through discussion. For each article, two sets of data were extracted: study characteristics and the discriminatory metabolites reported. Data extracted for study characteristics were: the number of participants in each study group recruited (main and validation cohort separately); the country where the study was conducted, the underlying aetiology of the liver disease of HCC patients; the condition of sample collection, whether the control groups were matched according to sex and age; the analytical method used, and the source of funding. For the reported discriminant metabolites, only those found to be statistically significant, defined by the study were extracted. Data items include compound name, control group compared, statistical test used for determining significance, direction of change (increased or decreased in HCC), fold change (where available), and *p*-value (or equivalent). To unify synonyms of the names of the compounds reported, the reported names were matched with those in the Human Metabolome Database (15) for non-lipids or LIPIDMAPS (16) for lipids. Those not listed in either of these databases or those that were not individual chemical entities (e.g. groups of compounds or ratios) were unified manually.

Table 2.1. A bespoke risk of bias assessment for metabonomic studies.

No.	Item	Category	Risk of bias
Domain 1 – Design of experiment			
1.1	Number of HCC cases	$n \geq 50$	Low
		$10 \leq n < 50$	Medium
		$n < 10$	High
1.2	Are participant characteristics reported by study group (HCC, cirrhosis, etc.) for each cohort (discovery & validation)	Yes	Low
		No	High
1.3	Are the diagnostic criteria for HCC and other liver conditions (where applicable) stated?	Stated – HCC confirmed with 2 modalities of imaging (Any 2 of MRI, CT, CEUS) or histologically proven	Low
		Stated – other methods	Medium
		Not stated	High
1.4	Are inclusion and exclusion criteria stated?	Stated	Low
		Not stated	Medium
1.5	Were potential confounders (e.g. sex, age) discussed and taken into account in data analysis? (Note 1)	Discussed and taken into account	Low
		Discussed	Medium
		Not discussed	Medium
1.6	Was there validation using an independent cohort?	Yes	Low
		No	Medium
Domain 2 – Chemical analysis			
2.1	Are the conditions of sample collection, storage and transportation stated?	Stated, no concern	Low
		Stated, concern present	Medium
		Not stated	High
2.2	Were samples randomised prior to analysis?	Yes	Low
		Not stated	Medium
2.3	Is pre-analysis sample processing stated?	Stated, no concern	Low
		Stated, minor concern present	Medium
		Stated, major concern present	High
		Not stated	High
2.4	Was a pooled quality control sample used and its reproducibility shown?	Yes – shown	Low
		Yes – not shown	Medium
		Not mentioned	High

		Not applicable	NA
2.5	Does the reporting of chemical analysis meet minimum reporting standards (Note 1)	No concern Minor concern present Major concern present	Low Medium High
Domain 3 – Data analysis			
3.1	Is the data analysis workflow clearly described?	Yes Unclear, minor Unclear, major	Low Medium High
3.2	Is there concern regarding the workflow?	No concern Minor concern present Major concern present	Low Medium High
3.3	Were the levels of significance corrected for multiple testing?	Yes No	Low High
Domain 4 – Reporting of discriminant metabolites			
4.1	Level of confidence in metabolite identification (Note 2)	1 – identified 2 – putatively annotated 3 – putatively characterised compound class 4 – unknown	Low Medium High High
4.2	Is the variability (e.g. interquartile range) of metabolite level reported?	Yes No	Low Medium
4.3	Is the precise *p*-value (or equivalent) reported?	Precise value stated Only range reported Direction of change only	Low Medium High

Note:

1. Methods for taking confounders into account include controlling in adjusted models or by stratified analysis
2. Basic information of experimental setup and condition required for the reporting of each analytical technology, see Sumner, *et al.*, 2007 (20)
3. As defined in Sumner, *et al.*, 2007 (20)

CEUS: Contrast-enhanced ultrasound; CT: computed tomography; HCC: hepatocellular carcinoma; MRI: magnetic resonance imaging.

2.2.3. The development of risk of bias assessment

A bespoke tool to assess the risk of bias of the studies reviewed was developed based on established tools (17, 18) and minimal reporting standards for metabonomic studies (19, 20) (Table 2.1). Each publication was assessed according to four domains: 1) study design, 2) chemical analysis, 3) data analysis and 4) the reporting of discriminant metabolites, each containing three to six items. For each item, criteria for high, medium and/or low risk were defined and were assigned -1, 0 and +1 point respectively. The total score from the risk of bias (RoB) assessment was the sum of all four domains. The RoB assessment was developed, trialled and modified based on feedback provided by investigators who performed data extraction. Once finalised, it was implemented in the same manner as the data extraction process.

2.2.4. Synthesis of results

In order to synthesise the extracted list of discriminatory metabolites across publications reviewed, a weighted score system was developed to incorporate four factors: vote count (an upvote, given the value of +1 if a metabolite was reported to be significantly higher in HCC than in the control group compared, and -1 for a downvote), the extent of change represented by $\log_2$ fold change ($\log_2$FC), RoB of the publication reporting the finding and an overall discordance penalty. For each metabolite in each comparison (HCC vs healthy, HCC vs non-cirrhotic liver disease, HCC vs cirrhosis or HCC tumour vs matched non-tumour liver tissue) in each sample type,

$$Weighted\ score = p\sum_{i=1}^{n} C \cdot R$$

where,

n is the number of publications reporting significant differences between HCC and controls,

p is a penalty for discordant report, calculated by |*sum(upvote,downvote)*| / *n*. For example, a metabolite that was reported to be significantly higher in HCC compared to healthy control in four studies but lower in one study, is allocated a penalty *p* = (4-1) / 5 = 0.6);

R is the total score from the RoB assessment, and

C is extent of change, defined as $\log_2$FC, if fold change was reported; otherwise an estimate using the median of $\log_2$FC values reported in each particular comparison and direction of change was used.

Thus, a positive weighted score indicated a metabolite to be predominantly reported to be higher in HCC compared to control and vice versa; the higher the absolute value, the more studies reported the change, and the greater the extent of change, with the reported direction of change being more consistent and/or the lower the risk of bias in the reporting studies. Reported metabolites in each sample type were then ranked by the absolute value of the weighted score. For urine and blood, metabolites were ranked by the sum of the weighted score across the three comparisons (HCC vs healthy, HCC vs non-cirrhotic liver disease, HCC vs cirrhosis). Data analysis and visualisation were carried out in R (version 3.6) (21).

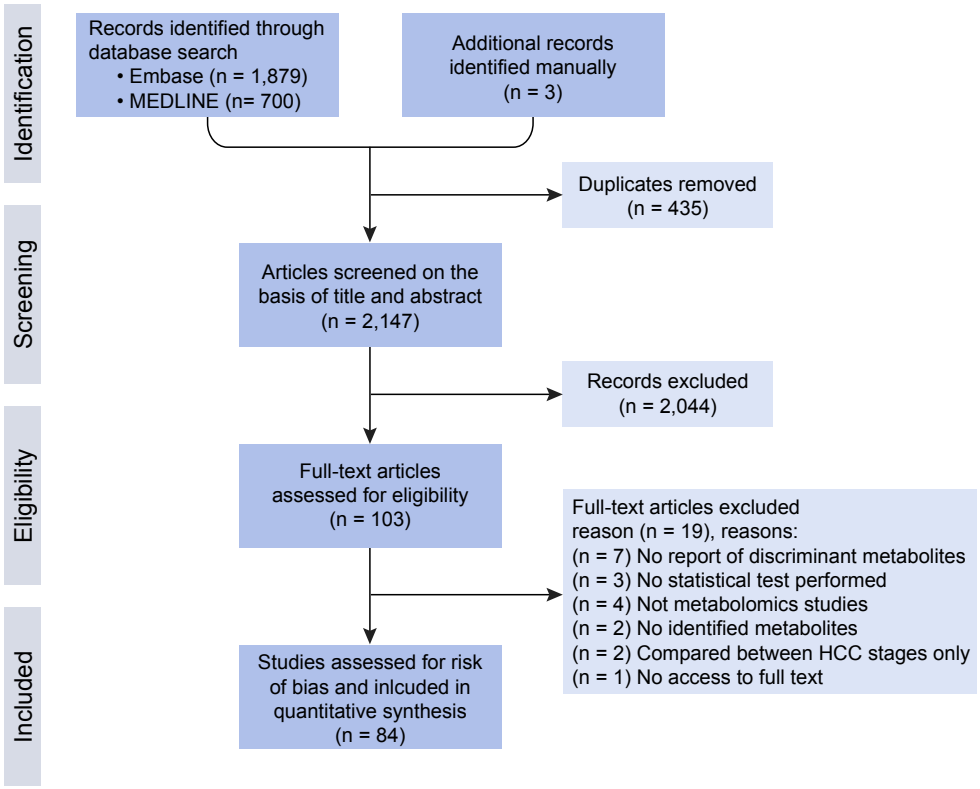

Figure 2.1. PRISMA flow diagram of study selection. Based on the PRISMA diagnostic test accuracy (PRISMA-DTA) tool (13).

2.3. Results

2.3.1. Studies reviewed

A total of 2,144 non-duplicated citations were identified from Medline and EMBASE, with an additional three identified manually (Figure 2.1). After excluding 2,044 citations on the basis of titles and abstracts, full-text of 103 articles were retrieved and reviewed for eligibility. Nineteen of the studies were excluded with reasons provided (Figure 2.1). Finally, a total of 84 studies (22-105) were included in the systematic review, of which 15, 54 and 9 analysed tissue, blood and urine samples respectively, and 6 studies presented findings of two sample types (5 blood and tissue, and one blood and urine, Figure 2.2A). The number of HCC cases in the studies ranged between 5 and 361. Studies using blood samples had the highest median HCC cohort size (*n* = 34),

compared to *n* = 28 for urine and *n* = 29 for tissue (Figure 2.2B). Over half of the studies (*n* = 47) were conducted in China, with USA having the second highest number of studies (*n* = 10, Figure 2.2C). For over half of the studies, the HCC cases either had chronic hepatitis B or C as the main underlying aetiology (Figure 2.2D). For studies using urine or blood samples, most had healthy volunteers or cirrhosis as control groups. Nineteen of the twenty studies that analysed tissue samples had matched non-tumor tissue from HCC patients as the control sample (Figure 2.2E). LC-MS was the most common analytical method used (Figure 2.2F). Details of individual studies are shown in Appendix A Table 3.

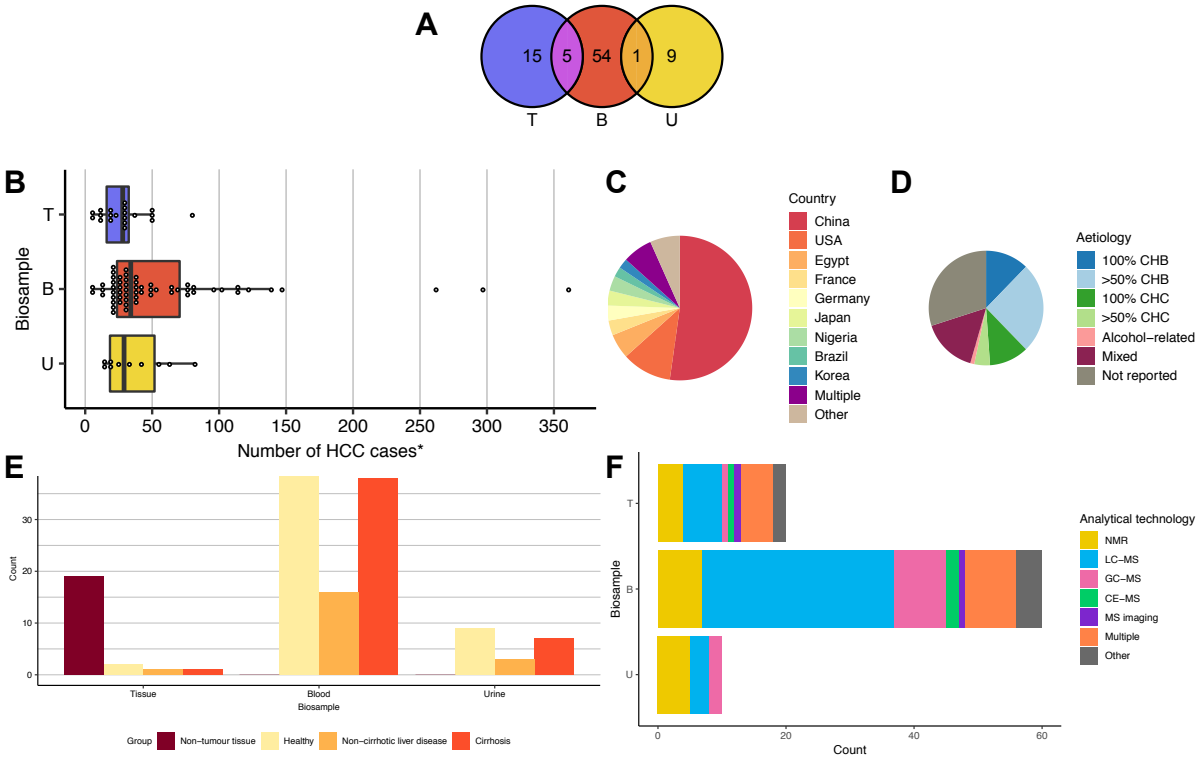

Figure 2.2. Summary characteristics of the included studies. (A) Biosamples analysed. (B) Number of HCC cases (excluding validation cohort). (C) Country where study was conducted in. (D) Underlying aetiology of HCC cases investigated. For each biosample type, (E) Control groups compared. (F) Analytical platform used. Note for (B, E, F): studies that analysed more than one biosample type are presented twice, once in each biosample. Abbreviations: hepatitis B (CHB); hepatitis C (CHC); capillary-electrophoresis mass spectrometry (CE-MS); gas chromatography mass spectrometry (GC-MS); liquid chromatography mass spectrometry (LC-MS); nuclear magnetic resonance spectroscopy (NMR).

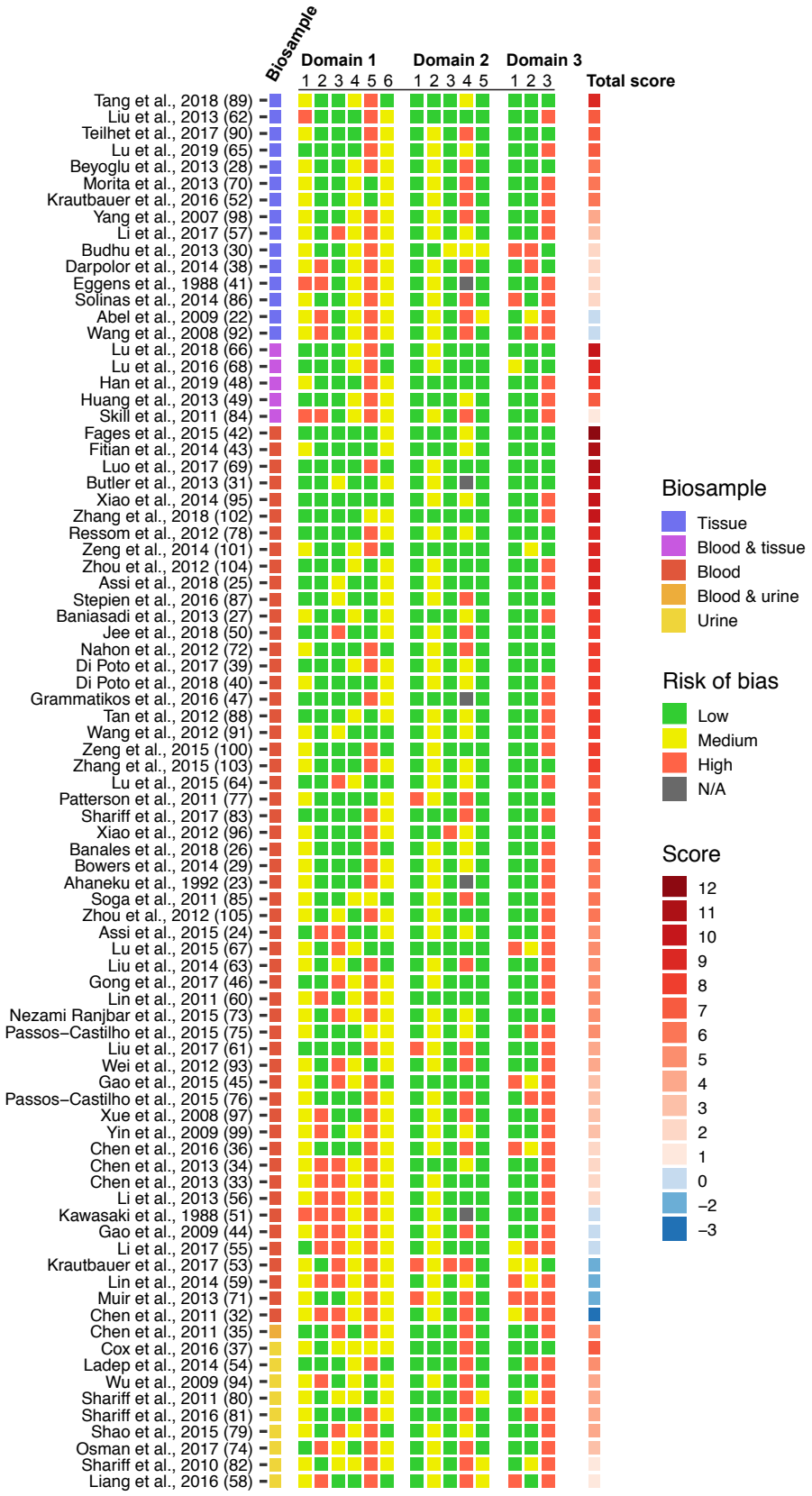

Figure 2.3. Risk of bias assessment of included studies. Individual studies were sorted according to biosample studied and total score (the higher the score, the lower the risk of bias). Reference number displayed in (brackets).

2.3.2. Risk of bias assessment

In order to assess the RoB of the studies reviewed, while there is no readily-available tool for metabonomic studies, a domain-based bespoke tool incorporating items from existing RoB tools (17, 18) along with requirements from published reporting standards in the field of metabonomics (19, 20) was developed (Table 2.1). Domains 1, 2 and 3 assessed the overall methodological and reporting concerns of the publication and gave a maximum total point of 14 (Figure 2.3). The reviewed studies scored an average of 5.1 points. The average points for studies that analysed blood samples and tissue samples were 5.6 and 4.8, respectively. Studies with urine samples had the lowest average point of 3.8; lower than the overall average (one-sample t-test $p = 0.050$). There was a weak correlation (Spearman's $\rho = 0.325$, $p = 0.003$) between the year of publication and RoB points, suggesting RoB of published studies was on a trend of lowering risk of bias over time.

In terms of study design, 20/60 studies that analysed blood samples had a study size of the HCC group of 50 or greater. Only 1/20 and 3/10 studies for studies that analyzed tissue and urine, respectively, had 50 or more HCC cases. Nearly a quarter of the studies (20/84, 24%) did not report basic demographic or clinical characteristics of participants separately for each study group. There were also 20/84 (24%) that did not state diagnostic method used for HCC cases. Twenty-five studies (30%) discussed how potential confounders (age, sex or underlying aetiology) may have affected findings: 21 had taken them into account in their statistical analysis, using adjusted models or by demonstrating that potential confounders had no effect on the findings. A total of 21 (25%) of studies had independent validation cohorts, of which, 19 were

validation of findings from analysis of blood samples, and one each for tissue and urine.

Concerns in Domain 2 regarding chemical analysis included Items 2.2 and 2.4. Only 22 / 84 (26%) papers explicitly stated that samples were randomised. A total of 44/79 studies (56%) stated the use of a pooled quality control (QC) sample, of which 18 of them showed reproducibility of QCs across the analysis (five of the studies used targeted LC-MS analysis only, for which QCs were not required). Of particular concern in Domain 3 on data analysis was the small proportion (23/84, 27%) of publications implementing multiple testing correction (106, 107) for determining statistical significance.

Domain 4 (Table 2.1) on the reporting of discriminatory metabolites was metabolite-specific, as the score differed for each metabolite reported in each publication. For example, a Level 1 identification (20) (the highest in confidence) may have only been performed for a subset of reported metabolites. Less than half of the studies (40/84, 48%) had any discriminant metabolites with identification made with Level 1 in confidence, suggesting the majority of studies relied on metabolite identification based only on matching to reference databases.

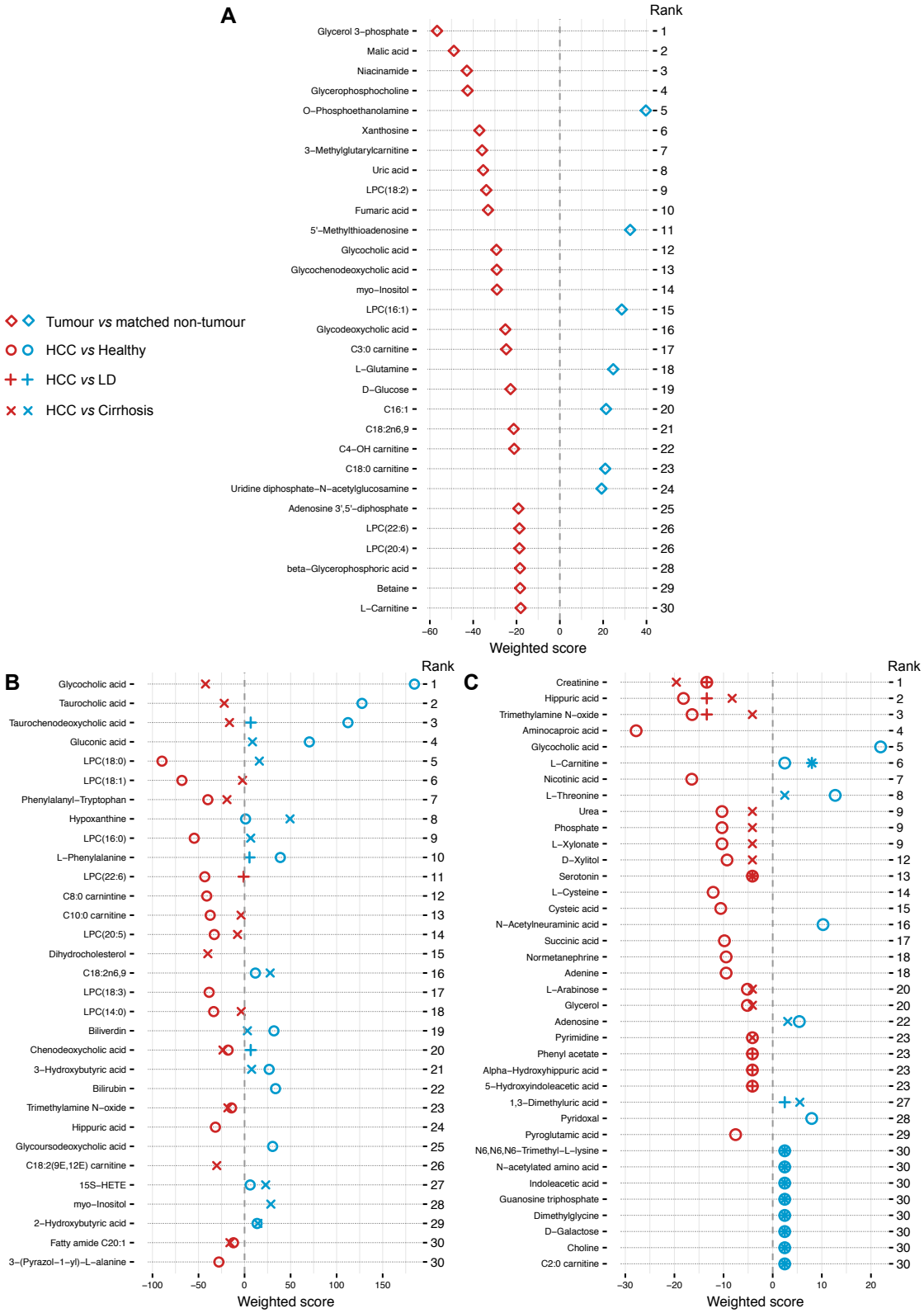

Figure 2.4. Top 30 metabolites ranked by |weighted score| in each sample type. (A) Tissue. (B) Blood. (C) Urine. A positive value suggests that the consensus among the reports is that the metabolite is higher in HCC than in the control group, vice versa. The weighted score combines $\log_2$(Fold change) values, the risk of bias of the reporting publication and a penalty for contradictory reports of direction of change. LD: non-cirrhotic liver disease.

2.3.3. Discriminatory metabolites between HCC and control groups

A total of 2,302 entries of differential metabolites were extracted from the 84 studies reviewed. The aim was to produce a list of metabolites that were found to be up- or down-regulated in a consistent manner for each sample type by incorporating three factors. First, the number of times each metabolite was reported to be significantly changed in one direction, as opposed to the other by taking the sum of vote counts (+1 for up-regulation in HCC compared to the control group, -1 for down-regulation). Alternatively, to incorporate the extent of change, log base-2 of fold change (log_2FC) values were used in place of vote count, where fold change values were reported. For entries without fold change values reported, they were approximated by an estimate of the median of reported fold change value for each direction of change in each comparison. Second, each report's contribution for a metabolite was scaled by the risk of bias (RoB) score. The RoB results from Domains 1-3 for the reporting publication were added to the Domain 4 metabolite-specific results, giving a total 4-domain RoB score of a maximum of 17 points (+1 for Low, 0 for Medium and -1 for High RoB for each of the 17 RoB assessment items). Metabolite entries with a final RoB score of zero or below (n = 48) were removed from the final analysis. Third, a discordance penalty was applied to penalise metabolites that had contradictory reports: the number of reports of change in one direction versus reports of change in the other direction. The top 30 metabolites with the highest absolute values in weighted score as well as metrics from intermediate steps leading to the final weighted score are listed in Appendix A Tables 4-6.

There were 699 entries of reported discriminant metabolites of 476 unique compounds in tissue, 684 of which were based on the comparison of HCC tumour (T) *vs* matched

non-tumour (NT) liver tissues and 15 others were from comparisons between tumour and non-matched healthy liver tissues. Because of the paucity of data from non-matching comparisons, subsequent analysis focused only on the T *vs* matched NT comparison. Of the 684 entries, 275 entries reported up-regulated metabolites, whereas 409 were down regulated. A total of 288 entries reported fold change. The median log_2FC for up-regulation was 0.807 (range: 0.202 to 3.856) and -0.811 (range: -0.105 to -2.251) for down-regulation. These values were used as estimates in the weighted score calculation for entries without reported fold change values.

For blood, there were 1376 entries of 590 unique compounds. There were 410, 125 and 841 entries for HCC *vs* cirrhosis, HCC *vs* liver disease (LD) and HCC *vs* healthy control, respectively. The median log_2FC of up-regulated and down-regulated entries were 0.516 (based on 121 entries, range: 0.029 to 3.101) and -0.515 (based on 115 entries, range: -0.022 to -3.322) for HCC *vs* cirrhosis and 0.872 (based on 127 entries, range: 0.070 to 6.521) and -0.737 (based on 212 entries, range: -0.059 to -0.737) for HCC *vs* healthy. For HCC *vs* LD, because there were only nine entries with fold-change, the median of all log_2FC values reported in blood were used as estimates in weighted score calculations instead (0.669 for increased metabolites in tumor, range: 0.029 to 6.521; -0.655 for decreased metabolites, range: -0.021 to -5.644).

There were 222 entries of 126 unique compounds reported in urine. This includes 73 entries of HCC *vs* cirrhosis, 40 entries of HCC *vs* LD and 109 entries of HCC *vs* healthy control. Due to the lack of studies reporting fold change values (a total of 31 entries from a single publication only), median of log_2FC from these 31 (0.614 for up-regulated metabolites, range: 0.333 to 5.492; -1.03 for down-regulated metabolites, range: -

0.269 to -3.474) were used for weighted score calculation for all urine entries, regardless of comparison.

For each entry of discriminant metabolites reported, the log_2FC (or an estimate of which) was scaled by the RoB score of the reporting publication. Subsequently, all reports of a metabolite in a comparison were summed. Finally, to further penalise metabolites that reported significant change in opposite directions, the sum was weighted by a penalty, corresponding to the fraction of difference between the number of contradictory reports, to produce the final weighted score. Using this approach, a ranked list produced from comparison of HCC T *vs* matched NT show that highest-ranking metabolites included the decrease of glycerol 3-phosphate, malic acid and niacinamide in tumor tissues (Figure 2.4A). Other high-ranking metabolites included bile acids (glycocholic acid, glycochenodeoxycholic acid and glycodeoxycholic acid), all of which were decreased in tumor tissues, and free fatty acids (including C16:1, C18:2n6,9), lysophosphatidylcholines (including LPC(18:2), LPC(16:1), etc.) and acylcarnitines (C3:0 carnitine, C4-OH carnitine, etc.), which had different directions of change depending on chain length and the number of double bonds.

For metabolites in urine or blood, ranking was made by the summation of the weighted score of all three comparisons (HCC *vs* healthy, HCC *vs* LD, HCC *vs* cirrhosis). This was based on the assumption that an ideal metabolite should discriminate HCC from all three of the control groups in the same direction. Hence, metabolites that showed strong discrimination with all three groups in the same direction were favored using this method.

The top three highest ranking metabolites in blood were primary bile acids: glycocholic acid, taurocholic acid and taurochenodeoxycholic acid (Figure 2.4B). However, the pattern of having a high positive weighted score in HCC *vs* healthy, but a negative score in HCC *vs* cirrhosis suggested that levels of these bile acids in HCC were between those in healthy and cirrhosis patients. Other high-ranking metabolites (gluconic acid, hypoxanthine) had a high weighted score for one comparison, but a low score for another, indicating a different degree of change depending on the control group compared. Only a few metabolites, such as trimethylamine *N*-oxide (TMAO) and 2-hydroxybutyric acid had similar scores for both the HCC *vs* healthy comparison and the HCC *vs* cirrhosis comparison, indicating the evidence of change in HCC compared to healthy and cirrhosis were similar.

In urine, the weighted scores had lower values, owing to fewer number of studies investigating urine (Figure 2.4C). High ranking metabolites included creatinine, hippuric acid and TMAO, all of which were lower in HCC. Unlike metabolites in blood, all urine metabolites with reports of more than one comparison showed uniform direction of change across different comparisons, albeit the extent of which may differ.

2.4. Discussion

The aim of this systematic review was to comprehensively compile a database of all reported discriminatory metabolites for HCC compared to control groups in tissue, blood and urine, and to identify any metabolites that may be potential biomarkers that should be investigated in future studies. A total of 84 publications were identified to be eligible for inclusion in the review after a two-stage screening process (title and abstract screening, followed by full-text screening). Data were extracted from each

eligible publication and risk of bias (RoB) was assessed using a bespoke tool for metabonomic studies, which was developed based on existing RoB tools and minimal reporting standards specific for metabonomic studies. Finally, a weighted score system was implemented to rank metabolites according to their frequency of reported to be significantly deregulated in HCC, the extent of change, consistency in the direction of change reported, and RoB of reporting publications. Using this approach, a ranked list of metabolites, discriminatory for HCC, was produced for each sample type. While there was not a single metabolite, or a combination of metabolites that could be concluded definitively as potential diagnostic markers, this body of work produced a resource for future research on this topic.

The systematic extraction of data and the assessment of RoB in this review highlighted heterogeneity in published study design and incomplete reporting of essential aspects relevant to metabonomic studies. As the metabolome is prone to influence from dietary intake and time of day (108), non-fasted, random sampling of biofluids may complicate data mining processes. For the majority of studies, the omission of reporting of sample randomisation and performance of quality control samples prevented assessment of analytical reproducibility. Given that analytical methods used in metabonomic studies allow measurement of many compounds at once, the number of variables tested was usually far greater than the number of observations. This “variables >> samples” ($n >> K$) problem is prone to false positives and necessitates the use of multiple testing corrections when testing for the significance of candidate biomarkers (109). However, it was only adopted in 27% of studies. Finally, reliance on online or in-house databases for metabolite identification, without demonstrating confirmation using chemical standards or orthogonal supporting evidence, risks erroneous assignments (109).

Taken together, these factors highlight the necessity for future studies to adhere to standardised study design, or at minimum, reporting that meets minimal standards to reduce risks of bias.

As a result of heterogeneity in the way studies were conducted, it was necessary to exercise caution to synthesise the findings. The weighted score system developed for this review was based on the assumption that the more times a metabolite was found to be significantly changed and the more consistent the direction of change was reported in different cohorts, the more likely it is that the metabolite could be a potential marker of clinical utility. The weighted score system was an improvement over previous synthesis of metabonomics studies (110), in which only vote count was used, i.e. only number of reports with direction of change but not taking into account the RoB of the reports and the extent of change observed. Log_2FC or an estimate of which using the median of reported fold change values was used in place of simple vote count to incorporate extent of change. In order to take into account RoB of studies and to minimise bias against larger studies, fewer in number but with greater statistical power (111), the RoB score was incorporated to scale the log_2FC. Finally, a penalty was applied for metabolites with reports of significant changes in opposite directions.

The resulting ranked lists of metabolites associated with HCC for each sample type (tissue, blood (serum or plasma) and urine) display metabolites that were reported to be changed consistently in multiple studies (Figure 2.4 & Appendix A Tables 4-6). In tissue, all top 30 metabolites had 100% concordance in the reported direction of change (discordance penalty value of 1, *i.e.* no penalty applied). Such high degree of agreement is promising in that it suggests similar patterns of change in tumour tissues

across different cohorts, which warrants further biological interpretation and investigations for mechanistic understanding of the disease. For both blood and urine, most metabolites do not have reports for all three comparisons (HCC *vs* cirrhosis, HCC *vs* liver disease and HCC *vs* healthy control) and some metabolites showed different directions (e.g. several bile acids in blood) and extent of change in different comparisons (e.g. hypoxanthine in blood, and creatinine in urine). Therefore, due to insufficient evidence and lack of coherence across studies, no definitive potential non-invasive markers can be concluded at this stage.

Despite the inconclusive finding, the ranked lists of discriminatory metabolites provide important insight for informing future research. Findings from the studies reviewed reveal that metabolites involved in various metabolic processes are altered in HCC. This suggests that there is no shortage of discriminatory metabolites, but the key question in this field of research is to choose the one or a panel of metabolites that can best serve as diagnostic markers for HCC. This selection process should be informed by having three biological considerations taken into account.

Firstly, candidate markers should reflect HCC tumorigenesis, rather than a secondary effect related to HCC development. Therefore, there should be evidence that any potential marker found in biofluids originated from the tumour. To this end, the strategy of testing for concurrent changes in tissue and in circulation, as adopted by four of the studies reviewed (48, 49, 68, 69), is one approach. However, since studies with such design are relatively scarce, comparing metabolites reported from tissue studies to those reported in biofluid studies serves as a good starting point. Differences in metabolite level in tumour tissue compared to differences in blood may be affected by

various processes including uptake, secretion (or release due to necrosis), synthesis, degradation or other metabolic reactions at the cellular level. Different patterns of alteration in tissue and biofluid may reflect different underlying processes. A metabolite that is found to be higher in tumour tissue and higher in circulation may suggest heightened synthesis accompanied by release. Unfortunately, the two highest ranking metabolites found to be higher in tumour tissue compared to matched non-tumour tissue, O–Phosphoethanolamine and 5'–methyltioadenosine, have not been reported in blood studies. On the other hand, a metabolite found to be increased in tumour tissue and decreased in circulation may reflect an increase in uptake. L-glutamine is one example (Appendix A Table 5). This is supported by the well-established understanding that cancer cells (including HCC) rely on L-glutamine as an energy source with increased uptake through the upregulation of glutamine transporter ASCT2 (112, 113). Metabolites found to be lower in both tumour tissue and blood in HCC patients, such as malate which is a tricarboxylic acid cycle intermediate, may reflect downregulation of the relevant pathway. Alternatively, for compounds known to be synthesised and secreted by hepatocytes, lower levels in both blood of HCC patients and in tumour tissue may suggest failure for tumour cells to maintain their synthetic functions leading to lowered overall level in circulation. Fibroblast growth factor 19, which is upregulated in cholestatic and cirrhotic conditions, downregulates bile acid synthesis and promotes tumourigenesis in the liver (114). This supports the observation that the primary bile acid, glycocholic acid, was found to be lower both in tumour tissue as well as in blood of HCC patients. Finally, for metabolites found to be lower in tumour tissue but increased in circulation, such as myo-inositol and L-carnitine, the likelihood of their change in blood being a direct effect of HCC is low unless there is active heightened secretion, evidence for which is lacking. The above discussed

metabolites may be of greater interest to be followed up in future blood studies due to their concurrent reports of significant change in previous blood and tissue studies.

Secondly, candidate markers should be specific to HCC, rather than being a marker of general liver damage. Therefore, ideally, a metabolite should only display altered levels in HCC, but not in cirrhotic patients, i.e. the extent and direction of change being similar across the three comparisons (HCC vs Healthy, HCC vs LD, HCC vs Cirrhosis). However, many metabolites reported in the studies reviewed, e.g. phenylalanyl-tryptophan (phe-trp), show progressive decrease in the healthy, cirrhosis and HCC groups (69). The three top ranking metabolites in blood, the primary conjugated bile acids, glycocholic acid, taurocholic acid and taurochenodeoxycholic acid, have the most notable patterns of alteration across the three comparisons of top-ranking metabolites. Their increase compared to healthy control is likely due to cholestasis that is frequent in patients with liver disease, while their decrease compared to cirrhotic patients likely reflects the reduced capacity of livers with HCC to synthesise them, as discussed earlier. Due to their levels being intermediate between healthy individuals and cirrhosis patients, the applicability of these bile acids as biomarker, at least on their own, is limited despite being top ranking. Given that 80% of HCC develop from patients with cirrhosis (115) and cirrhosis alone account for substantial metabolic changes in the body (116), future research efforts should focus on the HCC *vs* cirrhosis comparison and only use the other two comparisons to confirm findings. This strategy may help avoid identifying metabolites that are markers of liver damage, rather than markers specific for HCC.

Thirdly, the marker should be universal, i.e. valid independent of genetic, environmental, dietary or aetiological factors. For this, validation studies should be conducted in cohorts in different geographical locations and with different underlying aetiologies (Figure 2.2 C & D). The study conducted by Luo *et al*. (69) is the largest serum study to date and concluded that phenylalanyl-tryptophan (phe-trp) and glycocholic acid as markers for delineating HCC from cirrhosis. Despite having a validation study to confirm the findings, phe-trp has only been reported in one other study (43). Future profiling efforts should also specifically target these previously shortlisted metabolites to confirm their validity in different cohorts.

In addition to biological considerations, technical and practical considerations should be taken into account for HCC marker selection and validation. In terms of technical considerations, if a panel, rather than a single metabolite, is necessary to perform as a diagnostic test with sufficient accuracy, efforts should aim to minimise the number on the panel and take into account ease of detecting all marker compounds simultaneously and accurately in a single assay. As for practicality, given that HCC has the highest incidence in areas with limited resources (2, 3), cost, resource availability and logistics should be considered. A urinary test may be more easily implemented than a blood test.

As illustrated above, future research efforts on this topic should be guided by existing evidence and informed by biological understanding. In addition to standardisation of study design and adherence to minimal reporting standards, future metabonomic studies should be designed in a hypothesis-driven manner with the above discussed considerations taken into account. The systematically compiled ranked lists of

metabolites presented here for each sample type (Figure 2.4 & Appendix A Tables 4-6) provide an informative resource for electing metabolites to be further investigated.

A limitation of this review is the novelty of the metrics used. A bespoke tool for assessing RoB was developed to overcome the lack of an existing one for metabonomic studies. Opinions may differ regarding the relative importance of the different items in their contribution to a publication's risk of bias. However, it was developed by modifying existing tools (17, 18) with items for minimal reporting standards of metabonomic studies (2, 3) incorporated in an iterative process with the final version being a consensus reached by all investigators. Similarly, the weighted score system for ranking discriminatory metabolites was first-of-its-kind. It was developed to circumvent the heterogeneity in study design, chemical analysis and data analysis across studies which present a major challenge for synthesising the findings. Ranking of the resulting final score, as well as ranking using primary data and the intermediate steps are provided for reference (Appendix A Tables 4-6).

Another limitation is that the studies reviewed were subject to publication and observer biases. All studies reported one or more statistically significant discriminatory metabolites between HCC and control group(s). Even those that were excluded in the full-text screening step were not due to the absence of discriminatory metabolites. Rather, they were, for example, publications focused on targeted assay development where statistical tests were not reported and therefore confidence in the results could not be ascertained. A limitation of metabonomics, unlike some other 'omics', is that the choice of analytical method or assay used affects the collection of compounds detected or measured due to the chemical diversity of the metabolome. As such, the

dominance of bile acids or acylcarnitines may be due to investigators' choice of using targeted methods for these compounds. By using a scoring system that favours the number of coherent reports, it may be biased towards these metabolites that were selectively investigated in more studies. However, since investigators likely chose to assay certain classes of metabolites with well-supported reasons, the resulting ranked list based on the weighted score should still be valid.

The application of metabonomics to address clinical questions has been heralded as a promising strategy for identifying novel biomarkers for diagnostic and prognostic purposes. However, to date, contributions from the field have not yet been translated to new biomarkers endorsed by clinical guidelines for use in the clinic. The main question of the review, seeking to identify novel diagnostic biomarkers for HCC is amongst the most popular clinical questions researched in metabonomics. Along with the need for validating existing evidence with biological, technical and practical considerations taken into account, the standardisation of study design and adherence to minimal reporting standards are crucial for the field to move beyond exploratory studies to phase two clinical trials.

2.5. Conclusions

This systematic review aimed to survey reported discriminatory metabolites to identify potential diagnostic biomarkers for HCC. While there is not any metabolite that can be definitively concluded to be potential biomarkers, this review has led to a systematic compilation of reported discriminatory metabolites which offers a valuable resource for guiding future research on this topic. Validation studies, standardised study designs

and publications meeting minimal reporting standards are crucial for advancing the field beyond exploratory studies.

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Chapter 3 – Methodology

This chapter provides the theoretical basis of the experimental and computational methods used in subsequent chapters.

3.1. Experimental methods

3.1.1. Nuclear magnetic resonance

3.1.1.1. The phenomenon of magnetic resonance

NMR makes use of the quantum mechanical properties of certain atoms. Nuclei of atoms of certain isotopes with an odd total number of protons and neutrons have a non-zero spin quantum number (I). I is related to the magnetic quantum number (m) in the following manner: a nucleus with the spin quantum number I has magnetic quantum numbers $m = I, I - 1, I - 2, \ldots -I$. Thus, a nucleus with a spin quantum number I has $2I + 1$ possible energy states. Each proton has $I = ½$ and therefore has 2 spin states (one aligned with the magnetic field and one opposing), with a magnetic quantum number $m = \pm ½$.

The angular momentum (P) of the spinning nature of nuclei with non-zero spin gives rise to a non-zero magnetic moment (μ). The two are related through the gyromagnetic ratio (γ), a constant which is specific for each nucleus (for example, for proton, $\gamma = 26.75 \times 10^7\ T^{-1}\ s^{-1}$), where $\mu = \gamma P$. The angular moment at a particular axis (e.g. z-axis), can be calculated as $P_z = \hbar m$, where $\hbar$ is Planck's constant divided by 2π and m being the magnetic quantum number. Therefore, the magnetic moment of a nucleus at an arbitrary z-axis is related to the magnetic quantum number in the following manner:

$$\mu_z = \gamma P_z = \gamma \hbar m$$

When placed within an external magnetic field (B_0) along the z-axis, nuclei with magnetic moment behave as magnetic dipoles and align themselves either parallel or anti-parallel to the direction of the field. Those in low energy spin state (m = +½) are in the same direction as the direction of the magnetic field and those in high energy spin state (m = -½) are in the opposite direction. As the external magnetic field B_0 led the nuclei to have potential energy of $\pm \mu_z B_0$, depending on the spin states, the energy difference (ΔE) between the two spin states is as follows:

$$\Delta E = 2\mu_z B_0$$

The lower energy state is energetically favourable. The distribution of nuclei with the two spin states ($m = \pm ½$) is described by the Boltzmann distribution:

$$\frac{n_{high}}{n_{low}} = e^{-\frac{\Delta E}{kT}},$$

where n_{high} and n_{low} is the number of nucleus in the high and low energy states, respectively, k is the Boltzmann's constant and T is the temperature in Kelvin.

Given the population difference, the magnetic moments of the whole population of nuclei could be summarised by the net nuclear magnetic moment (M) (Figure 3.1).

The angular momentum of the nuclei in the magnetic field B_0 results in a precessional motion of the nuclei at a frequency known as the Lamour frequency (v_L):

$$v_L = \gamma B_0 / 2\pi$$

According to the Bohr frequency condition, the energy difference is related to frequency (v) through Planck's constant (h): ΔE = hv. Therefore, by stimulating the

equilibrium at a frequency sufficiently close to Lamour frequency, it is possible to perturb the populations of nuclei between the high and low energy spin states and alter the net nuclear magnetic moment.

In NMR spectroscopy, a sample is first placed within a constant magnetic field (B_0) at a strength depending on the magnet. Stimulation is achieved through a radiofrequency (RF) pulse at various lengths in time, which alters the net nuclear magnetic moment to certain angles from the equilibrium (typically, 90º or 180º). Once the RF pulse ceases, the nuclei relax back to the thermal equilibrium state and emits the absorbed radiation at various resonance frequencies which is detected by a detector coil. This time-domain free induction decay is converted to a frequency-domain spectrum through Fourier transform (Figure 3.1).

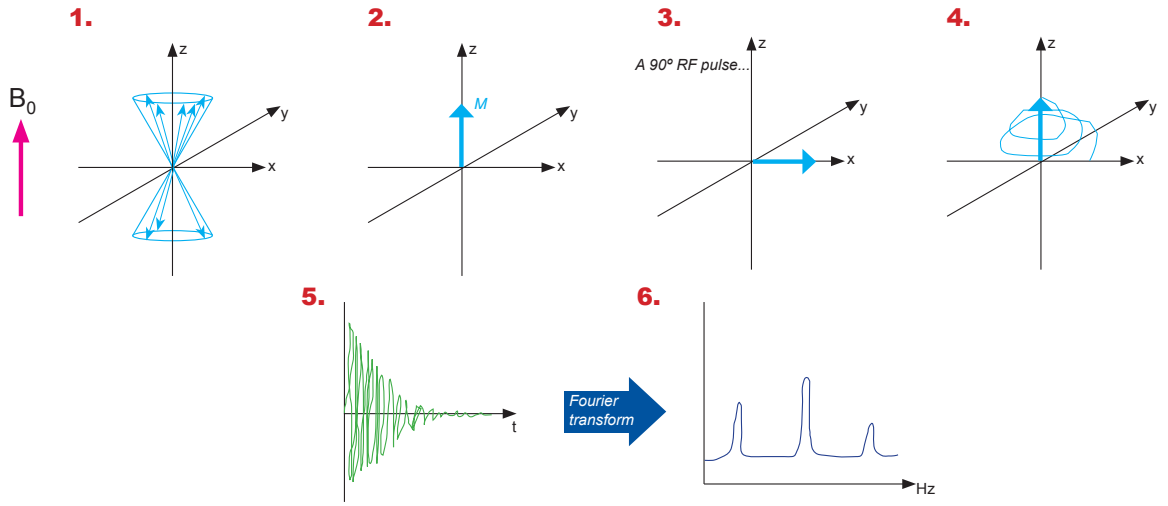


Figure 3.1. Schematic diagram of an NMR experiment. 1. When subjected to an external magnetic field B_0, nuclei with non-zero spin precess parallel (those in low energy state) or anti-parallel (those in high energy state) to the direction of the magnetic field according to the Boltzmann distribution. 2. Due to the difference in population, this can be summarised by a vector of net nuclear magnetic moment (M). 3. A radiofrequency pulse that matches the precession frequency can alter the net magnetic moment. 4. Once the pulse ceases, the nuclei relax and return to the thermal equilibrium state. 5. The radiation emitted during relaxation is detected by the detector coil of the spectrometer as a free induction decay (FID). 6. Fourier transformation converts the time-domain FID to a frequency-domain spectrum.

3.1.1.2. Properties of signals in a ^{1}H-NMR spectrum

The resonance frequency, i.e. frequency at which a nucleus relaxes back to thermal equilibrium, vary slightly depending on its local molecular environment. Such differences in resonance frequencies as well as the overall signal intensities are the key to structural and quantitative information provided by NMR, which is illustrated using lactate as an example here (Figure 3.2).

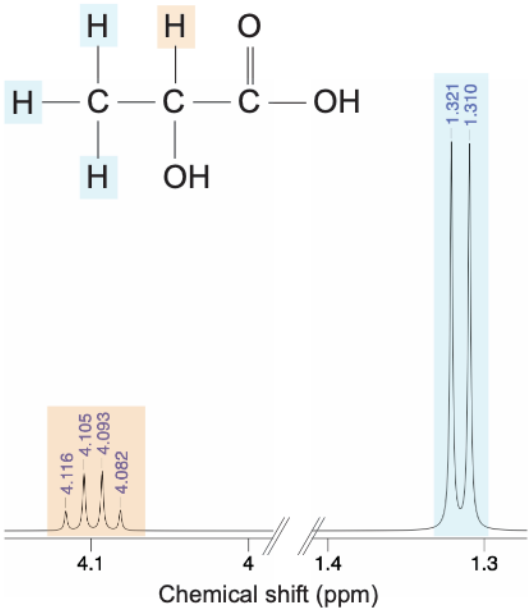


Figure 3.2. ^{1}H-NMR spectrum of lactate. The doublet at 1.3 ppm is the resonance peaks from the -CH_3 group (shaded in blue) and the quartet at 4.1 ppm is the resonance peaks from the -CH group (shaded in orange). The spectrum is adapted from the Human Metabolome Database (1).

Number of peak groups

In a molecule, protons with the same magnetic environment, as determined by the mirror and/or rotational symmetry of the molecule, contribute to the same signal. In lactate, the protons in the -CH_3 group are magnetically equivalent while the proton of

the -CH group is independent. Therefore, these two groups of protons are responsible for producing 2 groups of peaks in a ^{1}H-NMR spectrum.

Shielding and chemical shift

Since electrons surrounding a nucleus exert magnetic force that slightly alter the actual magnetic field experienced by the nucleus, the exact resonance frequency depends on the density of electrons surrounding the nucleus, a phenomenon called shielding. In ^{1}H-NMR, the electron density around protons in a benzene ring is much lower than those in a methyl (-CH_3) moiety as electrons are drawn to the *p* orbital of the ring. Therefore, a proton in a benzene ring is less 'shielded', experiences a stronger effective magnetic field and resonates at a higher frequency.

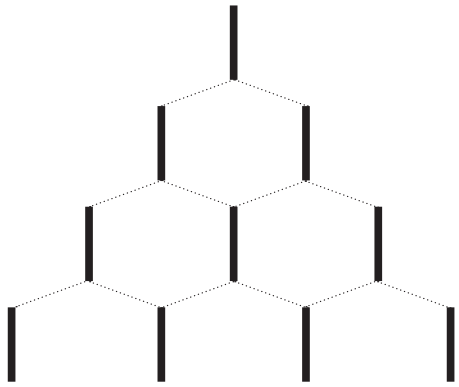
Because the Lamour frequency and hence the resonance frequencies are dependent upon the strength of the magnetic field B_0, signals detected in NMR are expressed as chemical shifts (δ) in parts per million (ppm) relative to signal from a reference compound to allow for comparison of spectra acquired from spectrometers with different magnet strengths. In ^{1}H-NMR applications in metabonomics, 3-(trimethylsilyl-[2,2,3,3,-$^{2}H_4$]-propionic acid) (TSP) is commonly used as the reference compound and chemical shifts of other compounds are expressed relative to the resonance frequency signal from TSP:

$$\delta = \frac{v - v_{TSP}}{v_{TSP}}$$

In the lactate example, protons in the -CH_3 group has a higher electron density than the proton in the -CH group, and thus have a lower chemical shift.

Spin-spin coupling

In addition to electrons, nuclei within close proximity with each other, but which are magnetically non-equivalent, also affect the effective magnetic field of each other. This causes slight differences in the chemical shift of each nucleus which manifests as peaks appearing as multiplets (groups of more than one peak). This is due to spin-spin coupling and the simplest type, first order spin-spin coupling, is described here. Protons within three bond distance from each other affect each other's effective magnetic field depending on the combination of possible spin state(s). For *n* number of protons, there can be *n* + 1 possible combination of spin states. For example, with two protons, the two protons may take any of the following combination of spin states: low/low, low/high and high/high. Since these spin states occur at a frequency of 1:2:1, the resulting peak of protons with 2 neighbouring protons is split into three at a 1:2:1 ratio. The multiplicity and the relative intensities of the split peak can be generalised by the n+1 rule and Pascal's triangle (Figure 3.3). The -CH signal in lactate is split into a quartet at 1:2:2:1 ratio by the three protons in the $-CH_3$ group and in return, the $-CH_3$ group signal is split into a doublet at a 1:1 ratio by the -CH proton.



Number of neighbouring protons	Multiplicity	Relative intensities
0	Singlet	1
1	Doublet	1:1
2	Triplet	1:2:1
3	Quartet	1:3:3:1

Figure 3.3. Relative intensities of multiplets due to spin-spin coupling follow Pascal's triangle.

Signal intensity

The intensity of a signal is proportional to the number of protons giving rise to the signal. Therefore, in the lactate spectrum, the peak integral of the -CH group quartet is a third of that of the $-CH_3$ doublet. More importantly in the context of metabolic profiling, peak intensity reflects the molar concentration of the compound in the sample, which allows for comparison of metabolite concentration between samples. An in-depth explanation of NMR theory can be found in textbooks such as Ref (2).

3.1.1.3. Metabolic profiling using ^{1}H-NMR

One of the main advantages of NMR as an analytical platform for the analysis of biofluids is that it requires relatively simple sample preparation. To analyse a biofluid sample in NMR, samples are prepared by centrifugation to remove any undissolved impurities, the addition of buffer and preparation into glass NMR tubes (3). Typically,

a phosphate buffer is used, which allows for pH buffering and contains the reference compound TSP, deuterated water, which is used for locking the magnetic field of the spectrometer, and sodium azide, which prevents microorganism growth in the sample.

A pooled quality control sample of equal volumes from each sample is prepared as a 'representative' sample to assess the repeatability of data acquisition during a run. Since NMR is a relatively reproducible analytical technique, the use of QC sample can be analysed much more sparingly, compared to an LC-MS experiment. At least one QC sample is used in every rack of 96 samples (3).

Urine samples are typically analysed using a standard one-dimensional pulse sequence that uses the first increment of the nuclear Overhauser effect (nOe) pulse sequence to achieve suppression of the water signal. Blood (which may be serum or plasma) samples are analysed using a Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence, which selectively enhances signals from small molecules to overcome the broad peaks due to the presence of macromolecules (lipids, protein and lipoproteins) (3). In addition to 1-dimensional NMR, 2-dimensional NMR pulse sequences may be used to aid structural assignment. These include *J*-resolved spectroscopy (JRES), where the projection of the peaks on to the chemical shift axis yields singlet peaks for each multiplet (i.e., homodecoupling all of the spin coupled multiplets), thereby reducing the spectral region occupied by each metabolite, COrrelation SpectroscopY (COSY) or TOtal Correlation SpectroscopY (TOCSY), which show cross-peaks between neighbouring magnetically equivalent groups of protons; and heteronuclear simple quantum coherence spectroscopy (HSQC), which shows cross-peaks between signals from different nuclei (e.g. ^{13}C and proton).

3.1.2. Liquid chromatography-mass spectrometry

3.1.2.1. Liquid chromatography

Liquid chromatography (LC) refers to the chromatographic technique that separates complex mixture based on analytes' interaction with a solid phase packed within a chromatographic column and a liquid mobile phase of solvents that flow through the column. Analytes that interact with the stationary phase more strongly than the mobile phase are retained for longer than those that interact less strongly. As such, analytes are separated based on their physical and chemical properties. The choice of the composition of the stationary phase and the mobile phase determines the type of analytes with certain physiochemical properties that is best separated.

A gradient elution refers to the change in the composition of mobile phase during a separation. Depending on the analyte's properties, this changed mobile phase composition may become more favourable to interact with than its interaction with the stationary phase and hence will elute. In this way, molecules elute at different times depending on the composition of the mobile phase. Typically, an LC method begins with high proportion of one mobile phase with gradual increase in the proportion of the other during a method of 5 to 20 minutes. Gradient elution is advantageous over isocratic elution, which is elution without varying mobile phase composition, as it allows separation of molecules with a greater range of physiochemical properties and is therefore the preferred method in metabonomic analyses.

Column-based LC relies on pumps to push pressurised mobile phase solvents together with the sample through a column that contains the stationary phase material.

High performance liquid chromatography (HPLC) refers to systems that can tolerate pressure up to 6000psi and particles within the column with a diameter of 5μm or greater. Ultra high-pressure LC (UHPLC), also known as ultra-performance LC (HPLC), is a more recent development, introduced in the 2000s, that refers to systems that can tolerate pressure up to 15,000psi with particle sizes below 2μm (typically 1.7-1.8μm) (4). This led to an improvement in separation resolution and sensitivity.

To optimise the separation of analytes with different physiochemical properties, combinations of columns with different stationary phase and different mobile phases may be used. The two major types of column commonly used for metabonomic studies are reversed-phase (RP) and hydrophilic interaction (HILIC).

RP-LC is so called because it has the opposite set up to 'normal'-phase that chromatographic technologies were initially developed using. Whereas the stationary phase is more polar than the mobile phase in normal-phase systems, it is the reverse in RP systems, with the stationary phase being less polar than the mobile phase. Columns for RP-LC are packed with silica with saturated aliphatic chains of certain length, typically with 8 or 18 carbons. Mobile phase gradient begins with high proportion of water-based solvent with gradual increase of an organic solvent (e.g. acetonitrile), leading to the separation of non-polar compounds.

HILIC-LC is a variant of normal-phase LC in that the stationary phase is more polar than the mobile phase. However, unlike normal-phase which relies on solvents that many polar compounds found in biological samples are not soluble in, HILIC is preferred as it relies of solvents (typically acetonitrile) that are already used for RP

methods. HILIC uses silica as the stationary phase with mobile phase gradient beginning with high proportion of organic solvent with gradual increase of a water-based solvent, leading to the separation of polar compounds.

3.1.2.2. Mass spectrometry

In a coupled system, a mass spectrometer functions as the detector of the output from UPLC. Mass spectrometry (MS) is a physical analytical technology that detects analytes based on its mass-to-charge (m/z) ratio. This is achieved by first ionising the molecules and then subjecting them to a mass analyser in order to be detected.

Ionisation

In order to be detected in MS, molecules first need to be ionised into gas-phase charged particles. Different ionisation methods may be used depending on the type of sample, e.g. matrix-assisted laser desorption / ionisation is suited for ionisation of solid samples. Electrospray ionisation is a common method used for liquid samples. It is a 'soft' ionisation method which causes little fragmentation and is therefore preferred in metabonomic studies. After elution from LC, the eluent which contains the analytes and mobile phase solvent is passed through a fine metal capillary. The tip of the capillary is connected to a counter electrode in close proximity with high voltage to create an electric field. This causes the eluent to disperse as fine aerosol of droplets with high charge accumulation after exiting the capillary. High flow rate of heated inert gas (typically nitrogen) flowing along the jet of aerosol facilitates the formation of the fine aerosol and solvent evaporation. As solvent evaporates, the repulsive force between charged particles causes the droplets to disintegrate into smaller droplets. This process is repeated until a point is reached when the repulsive force between the

charged particles exceed the cohesive force of the solvent and the residual charge in the droplet is transferred to the analyte molecule resulting in a charged ion. These charged ions then enter the MS analyser guided by the suction force of the vacuum of the mass analyser and the electric potential caused by the voltages applied within.

Mass analyser

The purpose of a mass analyser is to separate ions based on their m/z in order to be detected. There are two types of mass analysers commonly used: quadruple and time-of-flight.

A quadruple analyser uses two parallel pairs of cylindrical rods with opposite charge with static potentials (DC voltages) and alternating potentials (RF voltages) applied to create an oscillating electric field. When travelling through the electric field, ions with different m/z follow a different trajectory and oscillation frequency. By specifying the ratio of RF and DC voltages applied to the rods, only ions of certain m/z can pass through the quadrupole to be detected while others collide with the rods and are neutralised. In this way, ions may be specified to be detected or targeted for collision to allow for fragmentation,

In a time-of-flight (TOF) analyser, all ions are accelerated by the same amount of kinetic energy through the application of an electric pulse in a field-free region. As kinetic energy = ½ mv^2 where m is mass and v is velocity, this results in ions acquiring different velocities based on their m/z and therefore taking different amounts of time to reach the detector. Small ions of the same charge reach the detector faster than larger ions. A TOF analyser has the advantage of high sensitivity and mass accuracy

and the ability of measuring ions of a wide range of masses within very short period of time (nanoseconds).

A hybrid of quadrupole-TOF system is the first instrumentation of choice in metabonomic profiling as it features high mass accuracy and provides the option of selecting specific ions for fragmentation.

3.1.2.3. Metabolic profiling using LC-MS

Similar to NMR, sample preparation for LC-MS experiments involves centrifugation to remove any undissolved impurities. Solvents (usually the mobile phase at the start of LC method) are added to dilute the samples and prepare them for LC. Another centrifugation is performed after the addition of solvents to ensure that only liquid is transferred. This is especially important for samples containing macromolecules (e.g. plasma/serum) as they are precipitated due to the organic solvents added. This prevents the column from being blocked, leading to the buildup of pressure, poor chromatographic resolution and the whole system failing in the worst case.

As LC-MS data acquisition is more prone to shifts and drifts during a run, quality control samples are injected more frequently typically 1 every 7 to 10 samples (5). In order to assess linearity to dilution, dilution series are also run before and after sample analysis. In this way, the reproducibility of features may be assessed by the relative standard deviation (RSD = (standard deviation / mean) x 100%) of the intensity measured across QC samples and its correlation with the dilution factor in the dilution series.

3.2. Computational and statistical methods

3.2.1. Data processing of NMR and LC-MS spectra

Current automation programs of NMR spectrometers acquire and automatically process data, which includes baseline-correction, phasing, calibration to reference peak (3). This leaves relatively little additional processing necessary. Peaks of interest could be specified and integrated to estimate the concentration of the molecule in the sample. On the other hand, the nature of LC-MS gives rise to data that are three-dimensional and require peak-picking software to identify and quantify peaks. The R package *xcms* is an automated peak-picking algorithm that is commonly used (6).

Prior to further statistical analyses, metabonomic data require normalisation, which is a procedure to account for the difference in the overall signal intensities of variables from each sample in order to allow for comparisons between samples. The source of variation in intensity may biological (e.g. different dilutions of urine) or analytical (e.g. detector sensitivity during a run or pipetting error). Methods for normalisation include total area normalisation, which assumes that the total signal intensities should be identical across samples, or probabilistic quotient method, which assumes the median of signals should be identical instead (7). Normalisation is considered necessary for urine data as the concentration of urine depends on the hydration status of an individual. It is also considered beneficial for data from blood to account for analytical causes of variation (8).

3.2.2. Statistical analysis

3.2.2.1. Hypothesis testing

The goal of data analysis of a set of sampled observations is to make inference to generalise the conclusion to the general population from which the sampled observations were drawn.

A frequentist approach to statistical inference is a common approach where statistical relationships between variables are investigated through hypothesis testing. Null hypothesis (H_0) is the case if there is no association between the variables tested. Alternative hypothesis (H_1) is the case if there is a statistical association between the variables. *P* value is the probability of obtaining an outcome at least as extreme as the one observed if the null hypothesis was true. Null hypothesis is rejected, and the alternative hypothesis is accepted if the p-value is below a certain significance level (α), and the association is declared to be statistically significant. A value of 0.05 is often used as the acceptable α, which implies that there is a 1 in 20 chance of observing the outcome even if the null hypothesis was true.

As variables of interest may be binary (e.g. disease states), categorical (e.g. disease subtypes) or continuous (e.g. height), a number of different tests are developed to test for associations between different types of variables (Table 3.1). These can be classified as parametric or non-parametric. Parametric tests typically assume normal distribution and equal variance in the data while non-parametric tests do not make those assumptions and are hence more robust to outliers and skewed data. However, Welch's two sample t- test can be used to compare differences between two samples with unequal variances. Non-parametric methods bypass those assumptions by using

ranks of data instead of actual values. In metabonomic data, since each variable has different distribution and outliers are often present, the non-parametric Wilcoxon's rank sum test was the test of choice for hypothesis testing for statistical significance between groups in this thesis.

Table 3.1. A selection of statistical tests for comparing different types of variables.

Dependent variable	Independent variable	Parametric	Non-parametric
Continuous	**Binary**	t-test	Wicoxon's rank sum test
Continuous	**Categorical**	Analysis of variance	Kruskal-Wallis test
Continuous	**Continuous**	Pearson's correlation	Spearman's correlation

3.2.2.2. Multiple testing correction

There can be two types of errors in hypothesis testing. Type I error is the rejection of null hypothesis when it is true, i.e. a false positive, and type II error is the acceptance of the alternative hypothesis when it is false, i.e. a false negative.

By accepting an α of 0.05 for each test, if a large number of tests was conducted, the proportion of rejection of null hypothesis, i.e. a false positive, due to chance alone increases.

This is given by family-wise error rate (FWER), which is the probability of at least one test result being a false positive:

$$FWER = 1 - (1 - \alpha)^n$$

With 20 tests, the FWER is 64% and it becomes 99% with 100 tests. This illustrates the problem of multiple testing. Different procedures to control for FWER have been proposed. The most stringent of which is the Bonferroni method, which modifies α by dividing it by the number of tests conducted. It is considered too conservative and assumes each test to be independent, which is not satisfied in most cases in modern biological datasets given that variables are likely to be correlated.

Rather than focusing on controlling for FWER, an alternative approach of controlling for false discovery rate (FDR) instead was proposed. FDR is the expected proportion of false positive among those declared significant (9). There are different formulations to correct p-value to control for FDR. The Benjamini and Hochberg method (9) computes the FDR-adjusted p-value (pFDR) by first sorting all *n* p-values in descending order, which gives k, the rank of the p-value, and p_k, the k^{th} p-value, then:

$$pFDR_k = min\left(\frac{n \cdot p_k}{n - k + 1}, pFDR_{k-1}\right)$$

Another method was proposed by Storey and Tibshirani which introduced the concept of q-value, which is the probability of the variable being a false positive. Since the distribution of p-values would be uniform if all tests were truly null, the distribution of p-values could be used to estimate the proportion of true null hypothesis (π_0) which can then be used to scale pFDR to give q-value (10):

$$q_k = \pi_0 \cdot pFDR_k$$

Although there is subtle difference between the definitions of different measures for multiple testing correction (pFDR or q-value), in practice, the method of choice is often

the investigator's decision. Since a larger number of tests is required to estimate π_0, in this thesis, peak integrals from NMR (relatively fewer in number) was corrected using the pFDR method while data from LC-MS experiments were corrected to q-value, due to the availability of more variables to estimate π_0 in LC-MS.

3.2.2.3. Regression

Regression modelling is an alternative to statistical hypothesis testing to determine whether there is an association between variables of interest. The simplest form is a simple linear regression in which a response variable (y) is modelled by a predictor variable (x) by assuming a linear relationship:

$$y = \beta_0 + \beta_1 x + e$$

β_0 is the intercept, β_1 is regression coefficient, i.e. the slope, and e is the residuals not modeled by the linear equation. The coefficient β_1 is estimated by ordinary least squares which minimises the sum of squares of the residuals:

$$\beta_1 = \frac{\sum(x - \bar{x})(y - \bar{y})}{\sum(x - \bar{x})^2}$$

The simple linear regression could be extended to multiple linear regression, which allows for the inclusion of more than one predictor variables:

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \cdots + e$$

Wald test is a test to determine whether the regression coefficient of a given predictor variable is significantly different from 0, which allows for the evaluation of the contribution of each variable to the model. Since the regression coefficient (β_i) for each

predictor variable (x_i) is the change in y for each unit change in the corresponding x_i while holding all other predictor variables constant, it also offers as a method to assess and adjust for confounding.

Often times, the response variable of interest is not continuous and does not follow normal distribution, in which case, generalised linear regression can be used. Generalised linear regression allows for the response variable to be related the linear model via a link function. Logistic regression is one type of generalised linear model for a response variable that is binary (i.e. takes the value of 0 and 1), which is the method employed in thesis to predict the presence of HCC (1 for HCC cases, 0 for non-HCC cases).

In logistic regression, the link function is the natural log of the odds ratio (the probability of presence (Y = 1) over the probability of absence (Y = 0)), which is the response being modelled:

$$logit(p) = ln\left(\frac{p}{1-p}\right) = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \cdots + e$$

For logistic regression, parameter estimation is achieved by maximum likelihood estimation. The regression coefficients can be exponentiated to give the odds ratio, which can be interpreted as the change in the probability of Y = 1 for each unit increase in predictor variable.

3.2.2.4. Data transformation prior to multivariate statistical analysis

Since it is the relative values, rather than absolute values, that are of interest in order to identify patterns using multivariate statistical methods, data are transformed prior to analyses such as principal component analysis (PCA), which involves mean-centering and scaling. Data are commonly transformed in order to fulfil the necessary statistical assumptions such as normality of distribution. Mean-centering is the subtraction of each variable by their means to gather all variables around the origin to remove the effect of their absolute values. Scaling modifies the variance to reduce the influence of the range of variances between variables. Univariate scaling (dividing variables by their standard deviation) is a more extreme approach, which unifies the contribution of each variable to the model, regardless of their variance. This implies that a variable from a noise region contributes equally to the model as a variable from other 'real' signal. Pareto scaling (dividing variables by the square-root of their standard deviation) is another commonly used scaling method which reduce the influence of the difference in variance while still allowing for variables with greater variance to have a greater contribution.

3.2.2.5. Principal component analysis

PCA is a dimension-reduction multivariate statistical method commonly used to provide an unbiased overview of a metabonomic dataset (11). This allows for the assessment of the repeatability of data acquisition during a run using quality control samples, visualisation of the general patterns in the data and detection of outliers.

PCA summarises high-dimensional data (data with high number of variables, which may be correlated) into a set of orthogonal latent variables (variables that are uncorrelated to each other), known as principal components (PC). The first PC

represents the axis in the direction of the largest variance of the data and the second PC represents the axis in direction of the next largest variance that is orthogonal (i.e. uncorrelated) to the first, and so on for subsequent PCs (Figure 3.4 A & B).

Visually, PCA could be understood using a simplified case of dimension reduction of three variables into two. To represent a dataset of three variables in a two-dimensional plot, the most intuitive method is to find the 2-D plane at an orientation that the data is most spread out, i.e. with the largest variances. In this way, the original 3-D plot is best represented in the 2-D plot, with the distance of the points in the 3-D space from the plane as residuals not represented in the now reduced 2-D representation of the data.

PCA can be performed by singular value decomposition or eigenvector decomposition, through which the original data matrix X is decomposed to three matrices T, P and E:

$$X = T P' + E$$

The T matrix is known as the scores matrix which corresponds to each observation. The P matrix is known as the loadings and corresponds to each variable. Each vector in T (t_1, for PC1, t_2 for PC2, etc.) has the length of the number of observations, while each vector in P (p_1, for PC1, p_2 for PC2, etc.) has the length of the number of variables. Thus, linear combination of scores and loadings vectors of each PC (e.g. $t_1 p_1'$ for PC1) provides an approximation of X with the residuals (matrix E) as the variance not explained by the model (Figure 3.4C). In this way, plotting the scores from different PCs provides a visualisation of the patterns of the observations (samples) in the

reduced space while loadings provide information about the contribution of each variable to each PC (Figure 3.4 A & B).

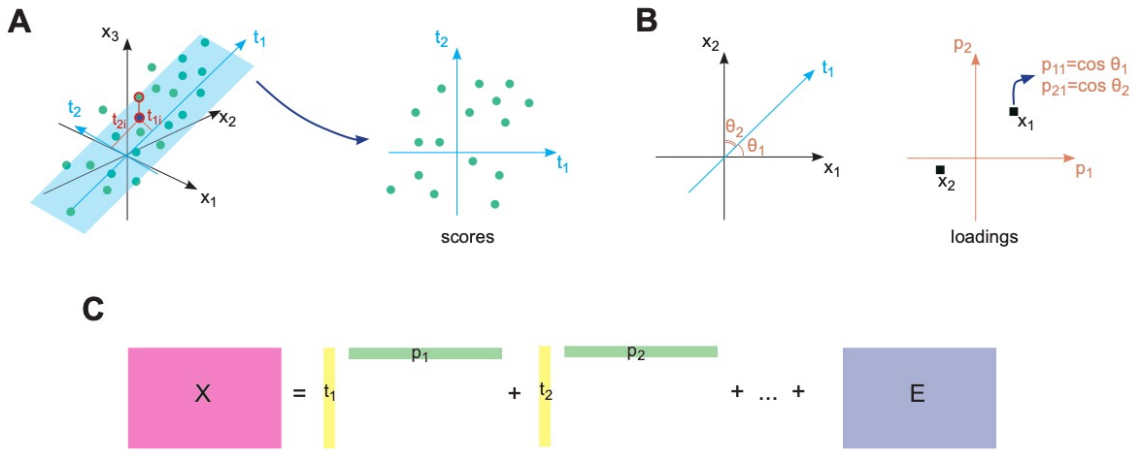


Figure 3.4. Visual explanation of principal component analysis. (A) Data in a higher dimensional space are projected on to a lower dimensional space through the identification of latent variables, known as principal components (PC). Shown here, the highlighted data point (green circle with red outline) in the 3-dimensional space is projected on to the 2-dimensional space (blue circle with red outline). The scores (t) are the coordinates of each sample in the new PC axes and (B) the loadings (p) are the contribution of the original variables to each PC. (C) The original matrix X is approximated by the matrix product of the scores (t_i) and loadings (p_i) vectors, with the E matrix containing the residuals from the approximation.

3.2.3. Measures of diagnostic accuracy

A diagnostic test is a binary classifier which classifies a case as positive (disease present) or negative (disease not present) based on a given input. There are a number

of measures for the performance of a binary classifier. One method that has been widely used in clinical diagnostic testing, is the receiver operating characteristic (ROC) curve is a plot used to visualise the relationship between sensitivity (SN) and specificity (SP). Ideally, it is desirable for a biomarker or diagnostic test to predict who has a disease or condition in 100% of cases. If a diagnostic is not sensitive, it will fail to detect individuals who have a disease and if it is not specific then it may indicate people have the disease when they really have another condition, thus it is important to maximize sensitivity and specificity in a diagnostic test. For ROC curves SN is the proportion of participants with the disease that test positive (true positives), i.e. patients who do have the disease; SP is the proportion of test negative cases among true negatives, i.e. patients who do not have the disease. The performance of the test can be obtained, by plotting SN versus (1 – SP). Thus the area under receiver operator curve (AUROC) has a value ranging between 0 and 1. A value of 1 represents a perfect test with no erroneous classification whereas 0.5 represents an uninformative test as this is the value expected from a classifier that gives random guesses.

The simplest way to determine the best cutoff (or level) for a diagnostic parameter is to use the ROC curve to identify the cutoff value with the highest sum of sensitivity and specificity. Different methods can be used for parametric and non-parametric cases and for comparing two or more binary classifiers with continuous variables. Options include the method by DeLong et al, which estimates the variance-covariance matrix of the differences in the AUROC between the classifiers (12) and Hanley and McNeil's method which compares two or more binary classifiers but for paired variables taking the correlation between the variables into account

(13). Since a single value or ratio was mostly used as the classifier in this thesis, the simplest method of using the sum of the highest sensitivity and specificity was applied.

3.2.4. Statistical methods for metabolite identification

Structural assignment of NMR data may be aided by statistical spectroscopy algorithms that take advantage of the collinearity of spectral signals. Statistical total correlation spectroscopy (STOCSY) was the first developed and is a widely used tool (14). By assuming that signals from the same molecule covary, it calculates the covariance and correlation of the driver variable specified with all spectral variables across a set of samples. This is visualised by a pseudo-spectrum plot which displays covariance as the intensity, coloured by the correlation coefficient. Signals from the same molecule as the signal of the driver variable are expected to show strong correlation. This allows for the rapid identification of all peak groups from the same molecule which facilitates structural elucidation. Multiple variations of STOCSY have been developed. One of which, subset optimisation by reference matching (STORM), allows a region of the spectrum, rather than a specific point, to be specified and selects a subset of samples enriched with the signal before performing an algorithm similar to STOCSY (15).

The typical workflow for metabolite assignment in NMR was as follows: a peak of interest was first used as the driver peak for STOCSY or STORM to identify any other peaks that showed high statistical correlation. This process was repeated using the identified associated peak to confirm the association and elucidate any other correlated peaks. This provided information to match against online (1) and in-house databases. Multiplicity and relation between peaks were clarified through the

inspection of data from 2D experiments. For the J-Resolved spectra, the projection of the peaks on to the chemical shift axis provides information on the number of protons contributing to each multiplet with information on the location / structure of neighbouring protons, whereas the 2D correlation sequences (COSY and TOCSY) show which protons are connected to each other along the backbone of a molecule. All non-general methods are provided within the chapter they apply to.

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Chapter 4 – Multi-platform metabolic profiling of urine for the validation of reported discriminatory metabolites for HCC

Summary

Validation of previously reported discriminatory metabolites for a disease could be argued to be of equal importance as discovering novel ones. Using results extracted from the systematic review (Chapter 2), top ranking discriminatory metabolites for HCC based on urine profiles was targeted to be annotated in datasets acquired by ^{1}H nuclear magnetic resonance, reversed phase and hydrophilic interaction liquid chromatography-mass spectrometry in a UK cohort. Significant alteration of 10 metabolites reported to be altered in HCC compared to healthy controls were reproducibly validated. However, apart from these ten metabolites which were in agreement, seven others were found to be altered in the opposite direction as reports from the literature and none of the previously reported alterations in the HCC and cirrhotic comparison could be reproduced. The results highlight the absence of a potential diagnostic candidate marker in urine. Whilst there was some evidence of a metabolic signature of HCC and cirrhosis, HCC could not be differentiated from the underlying cirrhosis. Future studies should focus on the HCC and cirrhosis comparison with the aim of identifying metabolites specific to HCC and similar validation effort should be conducted in analysis of blood samples.

4.1. Rationale

Candidate diagnostic biomarkers have the greatest potential clinical utility if they are applicable universally. Therefore, it could be argued that it is of equal, if not greater, importance for any new cohorts to validate existing findings than to discover novel, previously unreported, discriminatory metabolites. Any discriminatory metabolites or panels of metabolites that are hypothesised to be potential biomarkers need to be validated in multiple independent cohorts with different genetic, environmental and aetiological backgrounds.

Using a cohort from the UK, this chapter presents an attempt to validate discriminatory urine metabolites based on information extracted from the systematic review in Chapter 2, drawn from published literature describing candidate biomarkers of HCC, in a pseudo-targeted manner. Metabolites with ranking among top 30 in any of the metrics used (based on number of reports in literature, fold change, risk of bias of publication) were targeted to be annotated in data generated from untargeted ^{1}H-nuclear magnetic resonance and RP and HILIC LC-MS spectral datasets profiled from urine samples. Results from statistical analyses of differences in relative concentration of these metabolites between HCC and either healthy or cirrhotic groups were compared to the findings from the systematic review.

4.2. Methods

4.2.1. Participant recruitment and sample collection

Participants were recruited from five hospitals in England (London, Manchester, Newcastle, Nottingham, Southampton). HCC diagnoses were made by two independent modalities of imaging or by pathological studies, confirmed by a multi-

disciplinary team. A total of 198 participants, consisting of 102 HCC patients, 42 patients with cirrhosis and 54 healthy volunteers with no known liver conditions were recruited. A spot urine sample was collected for each participant, along with the record of demographic and clinical data, current medication and dietary history for the past 72 hours through a standardised questionnaire. The Child-Pugh score and class for all patients and Barcelona Clinic Liver Cancer (BCLC) stage for HCC patients were determined based clinical data. Urine samples were kept on ice, stored at -80ºC within an hour of collection and transported on dry ice pending analysis. An informed written consent was obtained from each participant. The study was approved by Research Ethics Committee at Imperial College London (REC no.: 09/H0712/82).

4.2.2. Sample preparation, data acquisition and processing

A quality control sample (QC) was prepared by pooling an equal volume (50µL) from each sample. The samples were sorted into a randomised order using a computer based algorithm and centrifuged at 13,000 *g* at 4ºC for 5 minutes. NMR and LCMS analysis were performed in accordance with published protocols (1-3).

For NMR, a volume of 540µL of the supernatant from centrifugation and 60µL of deuterated phosphate buffer (1.5M KH_2PO_4, 2mM NaN_3, 1% 3-trimethylsilyl-2,2,3,3-$^2H_4$-propionic acid (TSP) in 99.9% D2O, pH 7.4) were mixed and transferred to 5mm SampleJet tubes (Bruker Biospin, Germany). NMR spectra were acquired using Bruker Avance III DRX 600MHz spectrometer with a BBI probe operating at 600.13MHz at 300K and an automatic sample handling SampleJet unit (Bruker Biospin, Germany). A QC sample was run every 47 samples to allow assessment of analytical reproducibility. For each sample, a standard 1-dimensional (1D) spectrum,

using the first increment of the nOe pulse sequence to achieve suppression of the water resonance, and a 2-dimensional *J*-resolved (JRES) spectrum, for establishing multiplicity of peaks, were acquired (3). Water suppression was achieved by continuous wave irradiation at 25Hz during relaxation delay and mixing time. For 1D experiments, a total of 32 transients were acquired in the spectral window of 20 ppm. An exponential line-broadening factors of 0.3Hz was applied prior to Fourier transformation. Spectra were recorded and processed by automatic baseline-correction, phasing, referencing to TSP at 0 ppm using TopSpin v3.2.3 (Bruker Biospin, Germany). NMR data were imported into R at a resolution of 0.001ppm using in-house script. Regions corresponding to TSP and noise at both ends (< 0.5 ppm and ppm > 9.5 ppm), water (4.7-4.877 ppm) and urea (5.486-6.050 ppm) were removed prior to subsequent analysis.

For LCMS experiments, analyses were performed on UPLC-MS systems with ACQUITY UPLC (Waters Corp., Milford, MA, USA) as the LC component and Xevo G2 Q-ToF MS (Waters Corp., Manchester, UK) as the MS component. The LC and MS components were coupled via an electrospray ionisation source and the MS detector was operating at positive ion detection mode. A QC sample was run every 11 samples and a series of diluted QC samples was also run at the beginning of each run.

For RP-LCMS, a 2.1 x 100 mm Acquity HSS T3 column (Waters Corporation, Milford, MA, USA) operating at 40ºC was used. Mobile phase solvents A and B were water and acetonitrile, respectively, with 0.1% formic acid. The flow rate was 0.5 mL/min and solvent gradient and MS setup was as described in Want, *et al.* (2). Samples were

prepared by diluting 100µL of sample with 100µL of water and the injection volume was 5µL.

For HILIC-LCMS, a 2.1 x 140 mm Acquity BEH HILIC column (Waters Corporation, Milford, MA, USA) operating at 40ºC was used. Solvent A was acetonitrile with 0.1% formic acid and solvent B was water with 20mM ammonium formate with 0.1% formic acid. The flow rate was 0.6 mL/min and solvent gradient and MS setup was as described in Lewis, *et al.* (1). Samples were prepared by diluting 100uL of samples with 300uL of acetonitrile and an injection volume of 5µL was used.

Raw LCMS data were converted to netCDF format for feature identification and integration using the R package *xcms* (4). Default parameters were used except for the following: in the chromatographic peak detection step using the centWave method, noise = 500, prefilter = c(7,5000), peakwidth = c(2,15) and integrate = 2; in the 'group chromatographic peak' step using the peakDensity method, minFrac = 0.1 and bandwidth = 1. Extracted features were filtered by relative standard deviation (RSD) of QC < 30% (in alignment with Food and Drug Administration (FDA) guidelines) and response to dilution with Spearman $\sigma > 0.8$ (1).

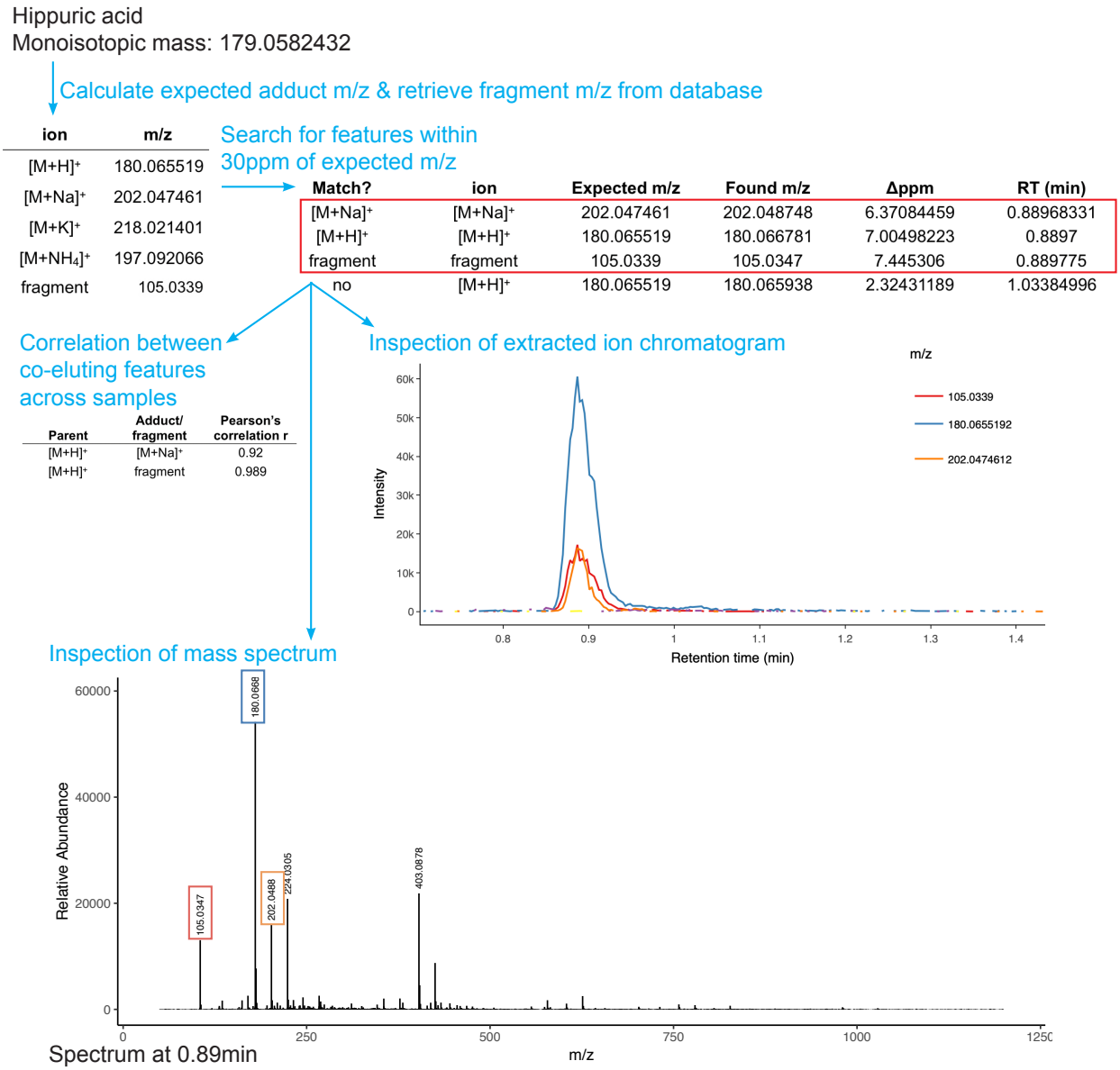

Figure 4.1. Method of metabolite annotation in LCMS using the annotation of hippuric acid in the HILIC dataset as an example. The monoisotopic mass as well as m/z of any fragment at collision energy of 0V was retrieved from online database (7). Then m/z of expected adducts were calculated. Features extracted using XCMS that are within 30ppm of expected m/z were shortlisted. These features with accurate mass-matching with or without co-eluting features (features with the same retention time) were identified as putative targets. Annotations were confirmed by inspection of the extracted ion chromatogram, mass spectrum and correlation between co-eluting features.

4.2.3. Metabolite annotation

Metabolite annotation in the NMR dataset was achieved by first retrieving ppm and multiplicity values from the Human Metabolomics Database (HMDB) (5) and in-house databases and confirmation by STOCSY (6) and inspection of JRES spectra to establish peak multiplicity.

Multiple lines of evidence were used for metabolite annotation in LCMS data (Figure 4.1). First, the expected mass-to-charge ratio (m/z) of the most commonly occurring adducts, $[M+H]^+$, $[M+Na]^+$, $[M+K]^+$ and $[M+NH_4]^+$ were calculated based on their monoisotopic mass retrieved from Metlin database (7). The m/z of fragments that occur at collision energy of 0V of were also retrieved (7). Features within 30ppm of the expected adduct and fragment m/z were shortlisted for inspection in the extracted ion chromatogram and the mass spectrum of the co-eluting peaks. Annotations were made by accurate mass matching of the parent ion, with additional confidence gained by the observation of other adduct or fragment peaks in raw data, the correlation of the parent ion with these adduct and/or fragment identified as features (Figure 4.1).

4.2.4. Statistical analysis

Each dataset was first normalised using the probabilistic quotient normalization method (8). Principal component analysis with mean-centering and univariate scaling was used to provide an overview of the data and to identify any outliers. Two-way comparisons between HCC compared to cirrhosis and HCC compared to the healthy control group were made using Wilcoxon's rank sum test. Multiple testing was accounted for using the Benjamini-Hochberg method for NMR peak integrals (9), and

by converting p-values to q-values for LC-MS features using the *qvalue* package in R (9). Although the precise definition of adjusted false discovery rate (FDR)-value (from Benjamini-Hochberg) and q-values differ, in practice, they are often used interchangeably (10). The method of choice was due to the availability of a greater number of features for testing in LCMS datasets, which allows for a better estimate of parameter required to compute q-values. A cutoff of < 0.05 was considered significant, which can be interpreted as accepting that there is a 5% probability of false positive in those metabolites declared to be significant.

Additionally, to delineate the effect of liver cirrhosis and the effect of malignant process as well as to remove potential confounding due to sex and age, two logistic regression models were built for each identified metabolite to model for the presence of HCC. In both models, age and sex were included as independent variables, with or without accounting for the severity of chronic liver disease:

Model 1: Presence of HCC ~ Intensity + age + sex

Model 2: Presence of HCC ~ Intensity + age + sex + Child-Pugh score

All data analysis procedures were performed in R (version 3.6) (11).

Table 4.1. Clinical characteristics of the cohort.

	HCC		Cirrhotic		Healthy control		
		n[a]		*n*[a]		*n*[a]	*p*-value[b]
n Total		102		42		54	
Demographic information							
Male sex, n (%)	82 (81%)	101	31 (74%)	42	23 (45%)	51	<0.0001[c]
Ethnicity, white, n (%)	92 (90%)	102	33 (79%)	42	40 (74%)	54	0.023[c]
Age, year*	69.3 (61.8,76.1)	101	55.2 (48.8,62.6)	41	36.7 (31.55,51.25)	51	<0.0001[d]
BMI, kg m^{-2}*	28.6 (25.3,32.4)	99	29.6 (25.8,35.4)	42	25.42 (22.3,28)	54	0.0003[d]
Co-morbidities							
Diabetes, n (%)	54 (53%)	102	12 (29%)	42	1 (2%)	54	
Hypertension, n (%)	42 (41%)	102	10 (24%)	42	4 (7%)	54	
Blood test results							
Alanine aminotransferase, IU L^{-1}	39 (26.5,59.5)	99	33 (26,56.75)	42			0.054
Alkaline phosphatase, IU L^{-1}	136 (90.75,174.25)	100	109 (80,125.75)	42			0.006
Albumin, g L^{-1}	35 (29.25,40)	102	34.5 (31,41.5)	42			0.915
Alpha-fetoprotein, ng L^{-1}	19 (4,124.5)	91	2.2 (2,4)	35			<0.0001
Bilirubin, µmol L^{-1}	17 (10,30.25)	102	18.5 (10,27.75)	42			0.921
Creatinine, µmol L^{-1}	71 (63,88.75)	98	71 (59,90)	41			0.784
International normalised ratio	1.1 (1.1,1.3)	71	1.2 (1.1,1.4)	29			0.099
Prothrombin time, s	14 (12,15.7)	86	15.2 (13.5,16.7)	37			0.024
Urea, mmol L^{-1}	5.25 (4.1,6.625)	100	4.5 (3.6,5.8)	41			0.063
Liver condition							
Cirrhotic, n (%)	85 (83%)	102	42 (100%)	42			
Child-Pugh Stage, n		102		42			
A	65		25				
B	33		15				
C	4		2				
HCC tumour condition							
BCLC stage, n		93					
0	7						
A	20						
B	14						
C	48						
D	4						
Aetiology, n		93					
CHB	5						
CHC	5						
ARLD	27						
NAFLD/NASH	33						
Autoimmue-related	7						
Mixed	8						
Other	9						
Multiplicity, n		101					
Single	57						
Multiple	42						
Diffuse process	2						
Diameter of largest tumour, n		89					
≤ 5	62						
>5	27						
Metastasis, n	3	102					

Data shown as median (interquartile range) with the exception of *, for which mean (±1 stand deviation) is shown. [a]number of data points available. [b]*p*-values of Mann-Whitney *U* test, unless stated otherwise. [c]χ^2 test. [d]1-way analysis of variance.

4.3. Results

4.3.1. Cohort characteristics

A total of 198 participants, comprising of 102 HCC patients, 42 cirrhotic patients and 54 healthy control individuals were studied. Demographic information and blood test results are summarised in Table 4.2. The proportion of male participants was not even across the study groups ($p < 0.0001$, χ^2 test) with the cirrhotic and HCC group comprising of an increasingly higher proportion of male participants compared to healthy control. The HCC and cirrhotic groups were also of more advanced age and had higher body mass indices (BMI, $p < 0.0001$ and $p = 0.0003$, respectively, 1-way analysis of variance) than healthy control.

The most common underlying cause of HCC in this cohort was non-alcoholic fatty liver disease (NAFLD) or non-alcoholic steatohepatitis (NASH), accounting for 33 (35%) of the cases, followed by alcohol-related liver disease (n = 27, 29%). Clinical biochemistry measurements between HCC and cirrhotic patients were largely matched with the exception of alkaline phosphate. Alpha-fetoprotein (AFP) differed significantly as expected. Using the commonly recommended cutoff of 20ng mL^{-1} (12), the AFP measured at the time of sampling has a sensitivity of 96% and specificity of 44%. With a higher cutoff of 200ng mL^{-1}, the specificity decreased to 22%, demonstrating its inadequacy as a biomarker.

4.3.2. Quality assessment of experimental data

The reproducibility of the experimental data was assessed by use of QC samples run at regular intervals between samples. Overlay of raw data and the overview of the processed data in PCA shows that QC samples co-mapped tightly for most datasets, with the exception of the HILIC dataset where some indication of a drop-off of signal

intensity was observed (Figures 4.2 to 4.4), indicating the data acquired were repeatable throughout each of the sample analysis runs.

In the NMR dataset, two samples, both from HCC patients with diabetes, were identified as outliers as their baseline was more variable than other samples (Figure 4.2 C), probably owing to extreme high glucose content of the urine as a result of diabetes. These spectra were removed from subsequent analyses due to their potential to bias the dataset, given the relatively large spectral window occupied by the multiple glucose protons.

For LCMS data, features were additionally scrutinised for their reproducibility through the use of the feature's relative standard deviation (RSD) among QC samples and correlation with the dilution factor of the diluted QC samples. In the HILIC dataset, 856/1201 features had RSD < 30% and 914/1201 had correlation with dilution factor > 0.8, resulting in 695 features that passed quality assessment filtering. In the RP dataset, 3129/3417 features had RSD of less than 30% and 3142/3417 had correlation with dilution factor of greater than 0.8, resulting in 2949 features that passed quality assessment filtering.

Table 4.2. Chemical shifts of metabolites annotated in the ^{1}H NMR dataset.

Compound	Chemical shifts and multiplicities*
Creatinine	3.05s, **4.05s**
D-Glucose	Various, **5.23d**
Hippuric acid	3.97s, 7.56t, 7.64t, **7.83d**
Trimethylamine-*N*-oxide	**3.28s**

*The chemical shift selected for peak integration highlighted in bold. d: doublet, s: singlet, t: triplet.

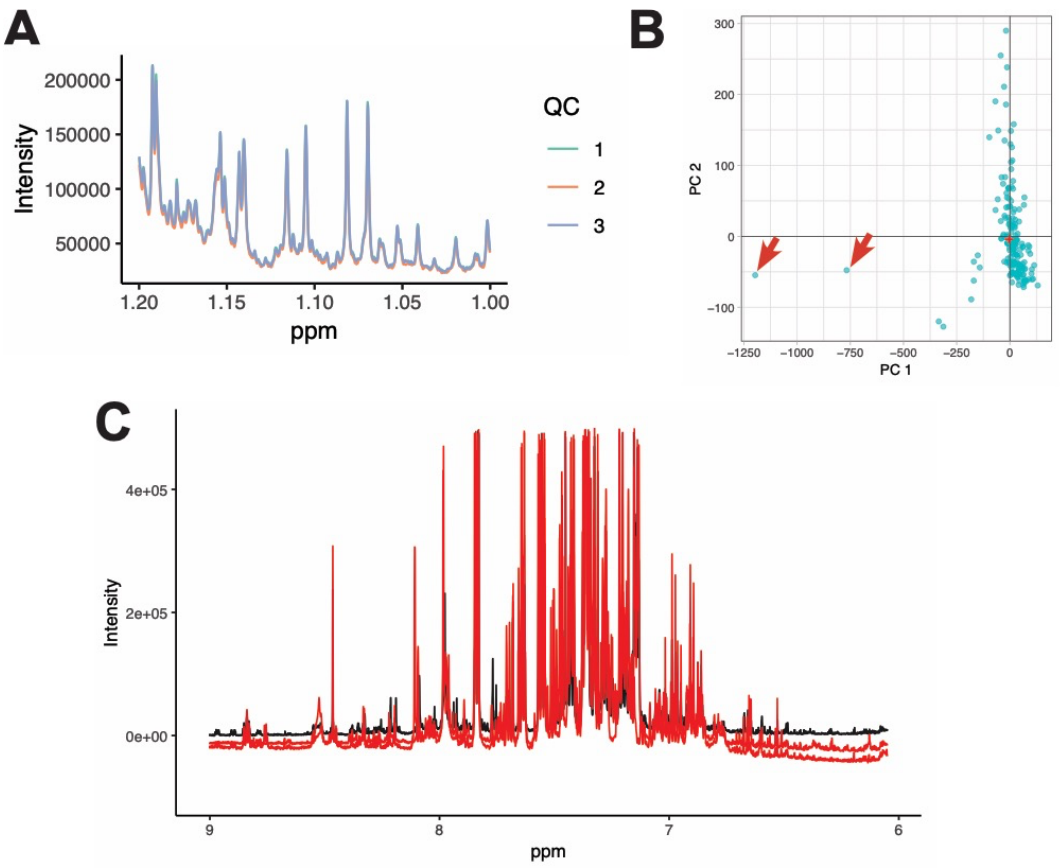

Figure 4.2. Quality assessment of the ^{1}H NMR data. (A) Overlay of quality control (QC) spectra. (B) Principal component analysis of samples (turquoise) and QC samples (red cross). Variance explained: PC1 17.1%, PC2 5.2%. (C) Spectra (red) of the two outliers (red arrows in (B)) with distorted baseline. A spectrum from QC (black) is shown as reference.

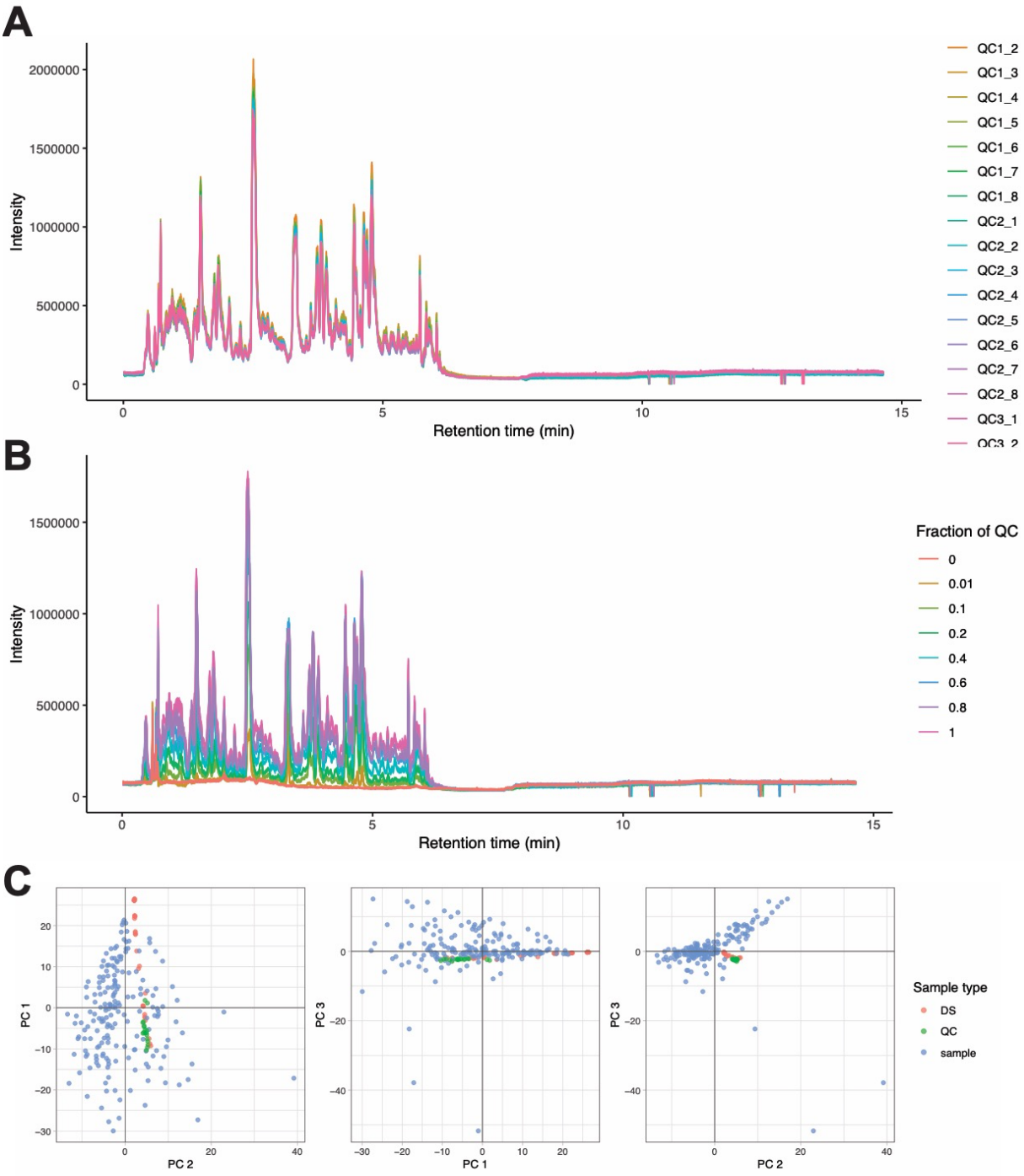

Figure 4.3. Quality assessment of the HILIC-LCMS data. Overlay of total ion chromatogram of (A) quality control (QC) samples run every 11 samples of study samples and (B) dilution of QC sample run at the beginning of the run. (C) Scores plot of principal component analysis co-mapping samples, QC and dilution series (DS). Variance explained: PC1 17%, PC2 5.1%, PC3 3.5%.

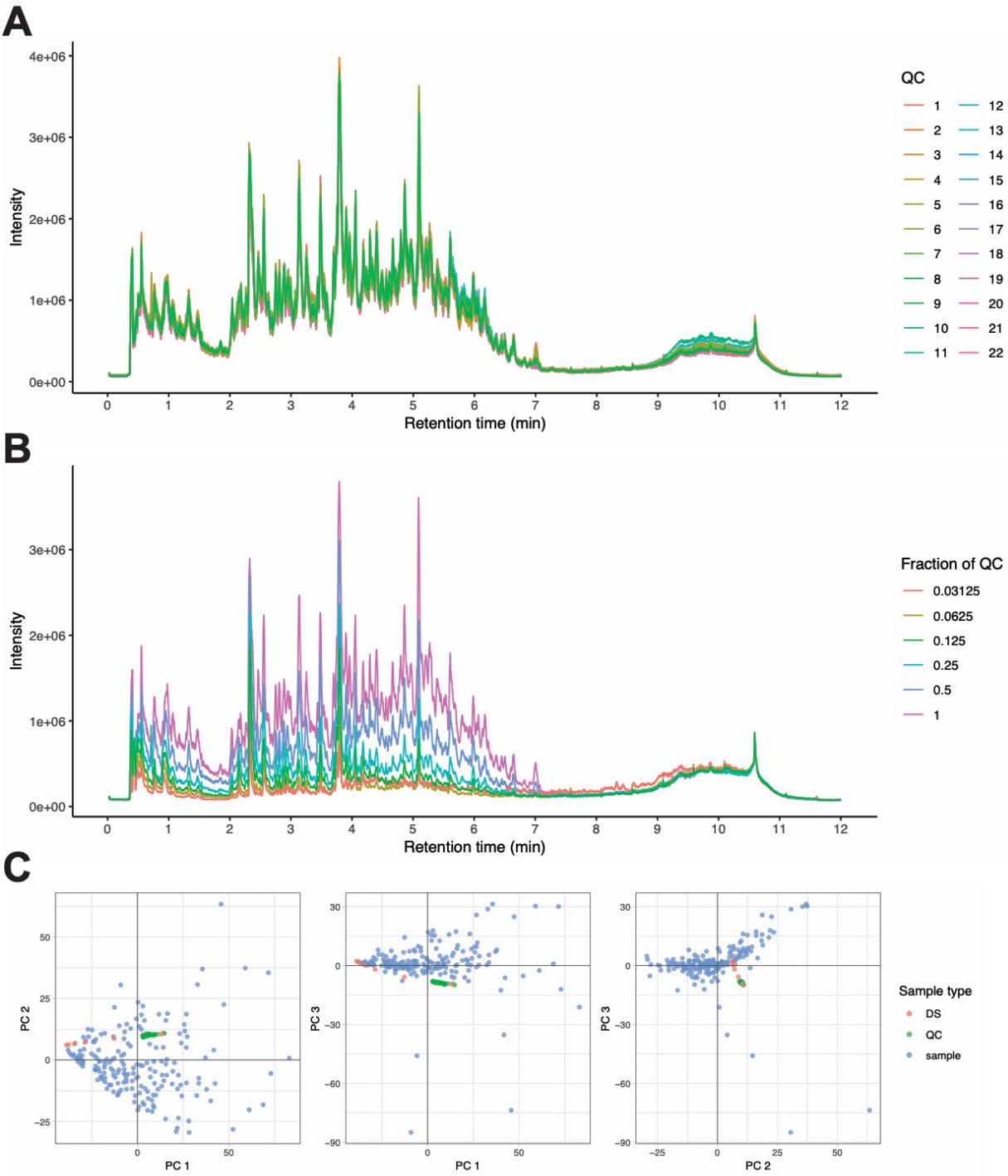

Figure 4.4. Quality assessment of the RP-LCMS data. Overlay of total ion chromatogram of (A) quality control (QC) samples run every 11 samples of study samples and (B) dilution of QC sample run at the beginning of the run. (C) Scores plot of principal component analysis co-mapping samples, QC and dilution series (DS). Variance explained: PC1 17.8%, PC2 5.6%, PC3 4.4%.

4.3.3. Metabolite identification

The 62 compounds with any of the metrics (number of times reported, vote and fold-change with or without weighting by risk of bias assessment and the final score, which was additionally weighted for concordance of reports) among the top 30 for HCC identified in Chapter 2 (Appendix A Table 6) were targeted for analysis in the current datasets. A total of 4, 13 and 17 metabolites were identified in NMR, HILIC-LCMS and RP-LCMS datasets, respectively (Table 4.2 and 3).

4.3.4. Statistical analysis

The candidate metabolites were tested for differences between groups (HCC vs cirrhosis and HCC compared to healthy control) using Wilcoxon's rank-sum test (Table 4.4, Figure 4.5-7) since the comparisons of interest were between single group pairs i.e. healthy vs HCC and between cirrhosis vs HCC.

Many of the metabolites showed statistical significance in the HCC vs healthy comparison. Metabolites annotated in more than one dataset that were significantly higher in HCC than in healthy control participants included D-Glucose, L-phenylalanine and pyroglutamic acid. Those found in more than one dataset and were significantly lower in HCC patients included hippuric acid, creatinine and L-xylonate. Other metabolites that were significantly higher in the HCC group included 5-hydroxyindoleacetic acid, adenosine, glycocholic acid, indoleacetic acid, L-cysteine in the RP dataset. Metabolites that were significantly lower in HCC compared to control were L-methionine in the HILIC dataset, 1,3-dimethyluric acid, L-carnitine and uric acid in the RP dataset. Acetyl-L-carnitine and alpha-hydroxyhippuric acid were found to be

significantly lower and pyridoxal significantly higher in the RP dataset but not in the HILIC dataset.

Table 4.3. Evidence for annotations made in LCMS data.

Compound name	Adduct	Theoretical m/z	Δppm	Actual m/z	RT (min)	cor(DS)	RSD	Co-eluting adduct	Evidence#	Fragment m/z	Evidence#
HILIC-LCMS											
Alpha-Hydroxyhippuric acid	$[M+H]^+$	196.060	3.47	196.061	0.921	0.877	7.251	$[M+Na]^+$	0.814	105.0337 (300%)	
Acetyl-L-carnitine	$[M+H]^+$ isotope*	205.123	22.14	205.128	4.449	0.970	6.801	$[M+Na]^+$	0.648		
Creatine	$[M+H]^+$	132.077	9.56	132.078	4.751	0.970	7.683	$[M+Na]^+$	Observed	90.0553 (7%)	
Creatinine	$[M+H]^+$	114.066	12.11	114.068	2.509	0.928	6.408	$[M+Na]^+$	0.537	86.0741 (8%)	
D-Glucose	$[M+NH_4]^+$	198.097	7.54	198.099	1.316	0.935	27.890	$[M+Na]^+$	0.696	145.0491 (300%)	
Hippuric acid	$[M+H]^+$	180.066	7.00	180.067	0.890	0.969	6.780	$[M+Na]^+$	0.920	105.0339 (20%)	0.989
L-Methionine	$[M+H]^+$	150.058	8.81	150.060	2.022	0.989	8.463	$[M+Na]^+$	0.978	133.031 (13%)	
L-Phenylalanine	$[M+H]^+$	166.086	7.22	166.087	3.537	0.991	6.905	$[M+Na]^+$	0.831	120.0806 (17%)	Observed
L-Xylonate	$[M+H]^+$	167.055	15.77	167.058	1.009	0.945	14.988	$[M+Na]^+$	Observed		
Pyridoxal	$[M+H]^+$	168.066	6.66	168.064	0.974	0.964	4.992	$[M+Na]^+$	Observed	129.0125 (54%)	
Pyroglutamic acid	$[M+H]^+$	130.050	10.18	130.051	1.490	0.989	6.934	$[M+Na]^+$	Observed		
Taurine	$[M+H]^+$	126.022	10.46	126.023	2.347	0.978	10.185	$[M+Na]^+$	0.958	108.0119 (3%)	Observed
Trimethylamine N-oxide	$[M+H]^+$	76.076	11.30	76.077	3.812	0.968	5.922	$[M+Na]^+$	Observed		
RP-LCMS data											
1,3-Dimethyluric acid	$[M+H]^+$	197.067	1.428	197.067	2.718	1.000	12.348			169.0708 (11%)	0.900
5-Hydroxyindoleacetic acid	$[M+H]^+$	192.066	1.500	192.066	2.370	1.000	11.519	$[M+NH_4]^+$	0.943	146.06 (12%)	Observed
Adenosine	$[M+H]^+$	268.104	1.174	268.104	1.998	1.000	19.291			136.0621 (8%)	0.808
Alpha-Hydroxyhippuric acid	$[M+H]^+$	196.060	0.133	196.060	3.144	1.000	10.070			105.0337 (300%)	
Acetyl-L-carnitine	$[M+H]^+$	204.123	1.950	204.123	0.805	1.000	6.945				
Creatinine	$[M+H]^+$	114.066	7.852	114.067	0.504	1.000	13.133	$[M+K]+$	0.260	86.0741 (8%)	
	$[M+H]^+$							$[M+Na]^+$	0.125		
D-Glucose	$[M+Na]^+$	203.053	0.699	203.053	0.474	1.000	8.072			145.0491 (300%)	
Glycocholic acid	$[M+Na]^+$	488.298	2.267	488.297	6.217	1.000	7.560	$[M+H]^+$	0.944	430.294 (18%)	Observed
Hippuric acid	$[M+Na]^+$	202.047	0.814	202.048	3.749	1.000	6.884	$[M+H]^+$	0.860	105.0339 (20%)	Observed
Indoleacetic acid	$[M+H]^+$	176.071	1.755	176.071	4.332	1.000	8.648	$[M+Na]^+$	0.978		
L-Carnitine	$[M+H]^+$	162.112	2.044	162.113	0.488	0.886	7.204			103.0358 (5%)	
L-Cysteine	$[M+H]^+$	122.027	5.918	122.028	1.213	1.000	20.158			151.9838 (5%)	
L-Phenylalanine	$[M+H]^+$	166.086	1.862	166.087	3.385	1.000	11.665			120.0806 (17%)	0.961
L-Xylonate	$[M+H]^+$	167.055	9.934	167.057	2.261	1.000	10.241				
Pyridoxal	$[M+H]^+$	168.066	11.954	168.064	1.850	0.943	5.193			129.0125 (54%)	
Pyroglutamic acid	$[M+H]^+$	130.050	7.116	130.051	4.151	1.000	6.585	$[M+NH_4]^+$	0.834		
Uric acid	$[M+H]^+$	169.036	3.225	169.036	0.923	1.000	11.359				

#Evidence of co-eluting adduct or fragment peaks, which may be observed in the mass spectrum in the extracted ion chromatograms or was also extracted as a feature, in which case, Pearson correlation coefficient with the parent feature across samples is shown.
*Only the isotopic peak, but not the parent ion was peak-picked by XCMS.
cor(DS): Spearman correlation coefficient of intensities detected dilution series of quality control (QC) sample and the dilution factor; RSD: relative standard deviation of intensities detected across quality control samples. RT: retention time.

Table 4.4. Statistical analysis of metabolites annotated.

Compound name	HCC vs Cirrhotic log_2(FC)	*p*	$p_{adjusted}$	HCC vs Healthy control log_2(FC)	*p*	$p_{adjusted}$	Model 1 OR (95% CI)	*p*	Model 2 OR (95% CI)	*p*
Alpha-fetoprotein							3.98 (1.91-10.54)	**0.001381**	3.97 (1.89-10.67)	**0.00168**
NMR										
Creatinine	-0.068	0.180	0.541	-0.199	**4.97E-02**	9.95E-02	1.04 (0.2-5.36)	0.963	0.51 (0.08-2.96)	0.452
D-Glucose	-0.062	0.964	0.964	0.268	**5.63E-08**	**1.31E-07**	1.8 (0.9-4.17)	0.124	1.17 (0.59-2.62)	0.664
Hippuric acid	0.065	0.657	0.657	-0.411	**2.97E-09**	**1.78E-08**	0.28 (0.04-1.27)	0.133	0.59 (0.1-2.66)	0.517
Trimethylamine-N-oxide	0.250	0.435	0.633	0.117	0.740	0.864	1.16 (0.33-4.18)	0.812	1.49 (0.38-6.29)	0.576
HILIC-LCMS										
Alpha-Hydroxyhippuric acid	0.085	0.412	0.434	0.022	0.768	0.209	5.09 (1-27.91)	0.054	4.35 (0.74-27.43)	0.107
Acetyl-L-carnitine	-0.010	0.695	0.556	-0.238	0.213	7.94E-02	0.96 (0.37-2.5)	0.931	1.08 (0.38-3.05)	0.883
Creatine	0.342	0.101	0.254	0.031	0.743	0.204	0.97 (0.39-2.3)	0.940	1.18 (0.48-3.04)	0.716
Creatinine	-0.092	0.096	0.253	-0.224	**4.16E-04**	**7.08E-04**	1.25 (0.62-2.63)	0.527	1.43 (0.7-2.93)	0.318
D-Glucose	0.524	0.230	0.346	1.106	**4.48E-04**	**7.25E-04**	1.32 (0.95-1.77)	0.076	1.16 (0.79-1.6)	0.410
Hippuric acid	-0.174	0.691	0.555	-0.724	**1.31E-06**	**8.93E-06**	0.47 (0.11-1.83)	0.285	1.08 (0.25-4.55)	0.917
L-Methionine	-0.032	0.960	0.626	-0.350	**6.64E-04**	**9.78E-04**	0.94 (0.15-5.93)	0.945	1.05 (0.16-7.16)	0.955
L-Phenylalanine	-0.189	0.781	0.583	0.301	**5.26E-03**	**4.59E-03**	1.92 (0.72-5.46)	0.204	1.31 (0.46-3.84)	0.611
L-Xylonate	0.065	0.353	0.409	-1.343	**1.66E-05**	**5.97E-05**	0.33 (0.15-0.68)	**3.79E-03**	0.55 (0.24-1.16)	0.148
Pyridoxal	0.059	0.451	0.450	-0.021	0.599	0.173	1.46 (0.26-8.65)	0.664	1.31 (0.2-8.79)	0.772
Pyroglutamic acid	0.115	0.486	0.463	0.296	**4.83E-02**	**2.38E-02**	1.39 (0.44-5.26)	0.591	1.53 (0.49-5.66)	0.482
Taurine	0.068	0.603	0.520	0.109	0.508	0.154	1.72 (0.98-3.11)	0.063	1.37 (0.73-2.6)	0.329
Trimethylamine N-oxide	0.151	0.358	0.411	-0.082	0.213	7.94E-02	0.78 (0.33-1.23)	0.382	0.95 (0.43-1.5)	0.846
RP-LCMS										
1,3-Dimethyluric acid	0.110	0.2298	0.3777	-0.723	**8.03E-04**	**9.84E-04**	0.49 (0.24-0.77)	**1.13E-02**	0.62 (0.35-0.95)	**4.68E-02**
5-Hydroxyindoleacetic acid	0.525	0.2996	0.4285	1.780	**4.42E-03**	**4.06E-03**	0.89 (0.49-1.58)	0.682	0.91 (0.48-1.71)	0.764
Adenosine	0.236	0.2160	0.3653	0.551	**0.0395**	**0.0251**	1.24 (0.71-2.15)	0.439	1.49 (0.8-2.73)	0.197
Alpha-Hydroxyhippuric acid	1.529	0.0882	0.2411	2.307	**6.11E-03**	**5.30E-03**	1.03 (0.82-1.28)	0.792	1.06 (0.84-1.32)	0.605
Acetyl-L-carnitine	0.179	0.7455	0.6437	-0.625	**2.70E-03**	**2.68E-03**	0.71 (0.35-1.4)	0.335	0.83 (0.4-1.67)	0.613
Creatinine	-0.164	0.1395	0.2988	-0.619	**2.86E-05**	**5.64E-05**	0.9 (0.5-1.58)	0.709	0.89 (0.48-1.6)	0.695
D-Glucose	-0.624	0.6613	0.6183	1.038	**1.06E-03**	**1.23E-03**	1.02 (0.79-1.29)	0.877	0.91 (0.64-1.22)	0.585
Glycocholic acid	0.197	0.5875	0.5870	4.077	**2.06E-14**	**4.94E-13**	2.72 (1.64-4.75)	**1.97E-04**	1.6 (0.85-3.16)	0.157
Hippuric acid	-0.110	0.1395	0.2988	-1.084	**7.65E-09**	**4.85E-08**	0.19 (0.05-0.59)	**6.73E-03**	0.39 (0.1-1.25)	0.136
Indoleacetic acid	0.079	0.7284	0.6391	0.496	**1.90E-03**	**2.00E-03**	0.86 (0.53-1.38)	0.540	0.86 (0.5-1.44)	0.576
L-Carnitine	-0.688	**0.0485**	0.1853	-0.710	**1.73E-03**	**1.84E-03**	0.61 (0.33-1.09)	0.103	0.67 (0.35-1.24)	0.213
L-Cysteine	0.236	0.1827	0.3391	0.310	**0.0115**	**9.05E-03**	2.38 (0.4-15.01)	0.345	3.2 (0.48-22.48)	0.229
L-Phenylalanine	0.111	0.0624	0.2051	0.061	0.0870	**0.0485**	14.73 (1.17-216.14)	**4.22E-02**	10.94 (0.69-208.13)	9.85E-02
L-Xylonate	-0.292	0.1347	0.2976	-1.091	**2.84E-07**	**1.06E-06**	0.24 (0.1-0.5)	**3.81E-04**	0.35 (0.14-0.77)	**1.29E-02**
Pyridoxal	0.237	**0.0441**	0.1784	0.602	**1.11E-04**	**1.81E-04**	1.18 (0.3-4.8)	0.815	0.95 (0.23-4.02)	0.947
Pyroglutamic acid	0.716	**3.26E-04**	**0.0137**	0.969	**4.45E-05**	**8.27E-05**	4.3 (1.58-12.75)	**6.01E-03**	4.65 (1.59-14.89)	**6.82E-03**
Uric acid	-0.155	0.1520	0.3117	-0.514	8.64E-04	**1.04E-03**	0.69 (0.2-2.21)	0.536	0.81 (0.23-2.76)	0.737

Model 1: Logistic regression of Presence of HCC ~ Metabolite Intensity + Sex + Age
Model 2: Logistic regression of Presence of HCC ~ Metabolite Intensity + Sex + Age + Child-Pugh score
P-values and adjusted p-values below 0.05 are highlighted in **bold**.
CI: confidence interval; log_2(FC): Log base-2 of median fold change of HCC over the study group compared; OR: odds ratio. $p_{adjusted}$: Adjusted p-value.

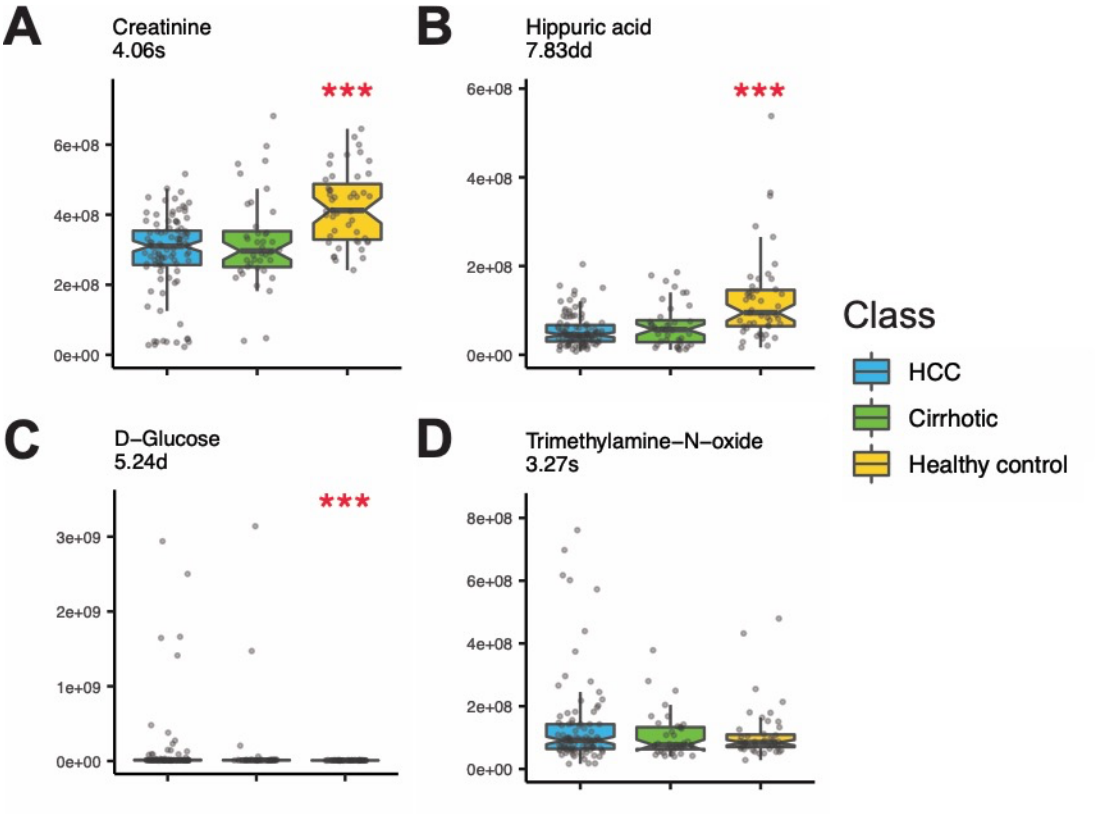

Figure 4.5. Intensities of metabolites found in NMR in each study group. (A-B) Metabolites with lower intensity in HCC. (C) Metabolite with higher intensity in HCC. (D) Metabolite with no significant difference found. The Wilcoxon rank sum test was used to establish significance for group comparisons.

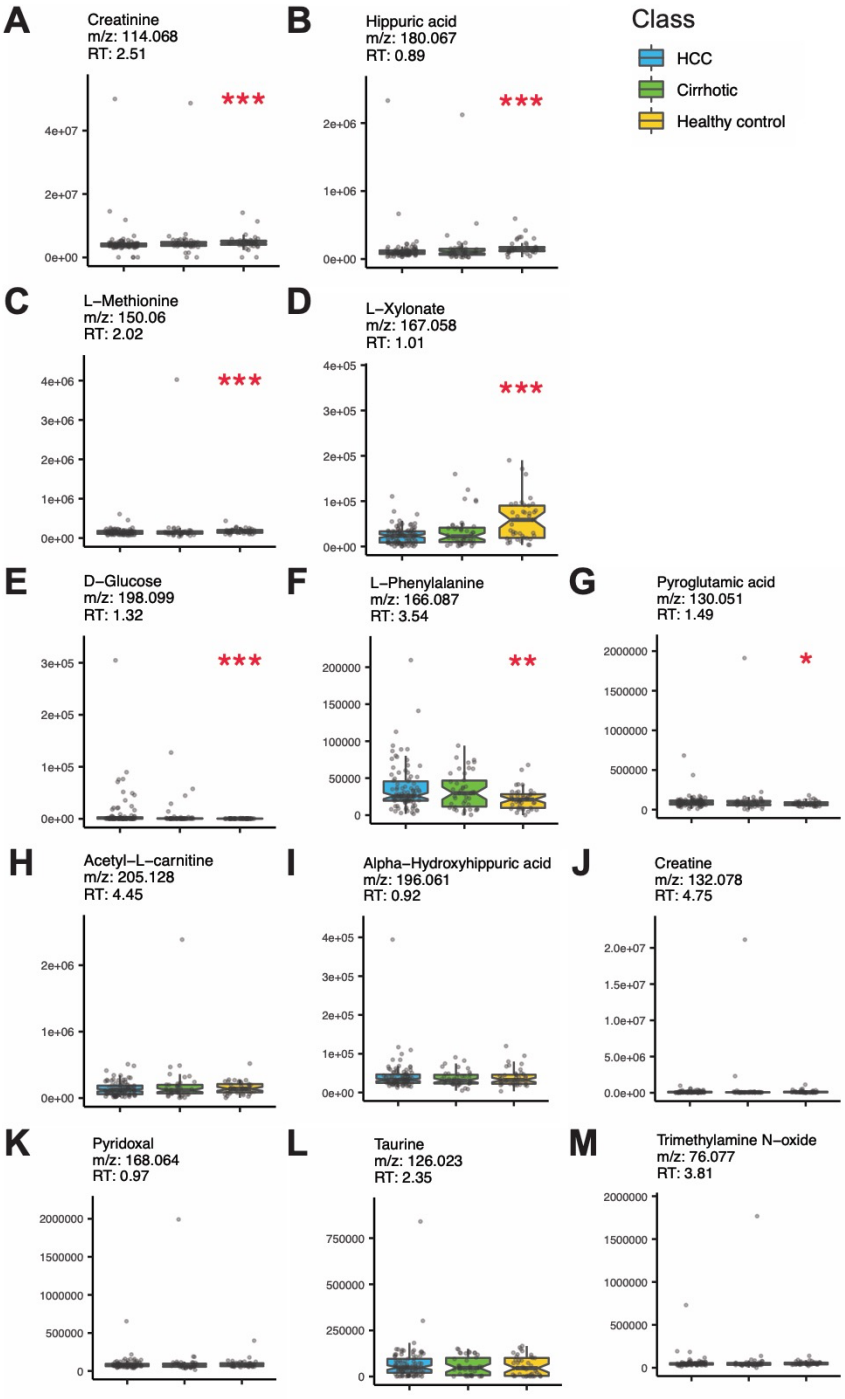

Figure 4.6. Relative intensities of metabolites found in HILIC-LCMS in each study group. (A-D) Metabolites with lower intensities in HCC. (E-G) Metabolites with higher intensities in HCC. (H-M) Metabolites with no significant difference found between HCC and control groups. ***: $q < 0.001$, **: $0.001 \leq q < 0.01$, *: $q < 0.05$. The Wilcoxon rank sum test was used to establish significance for group comparisons.

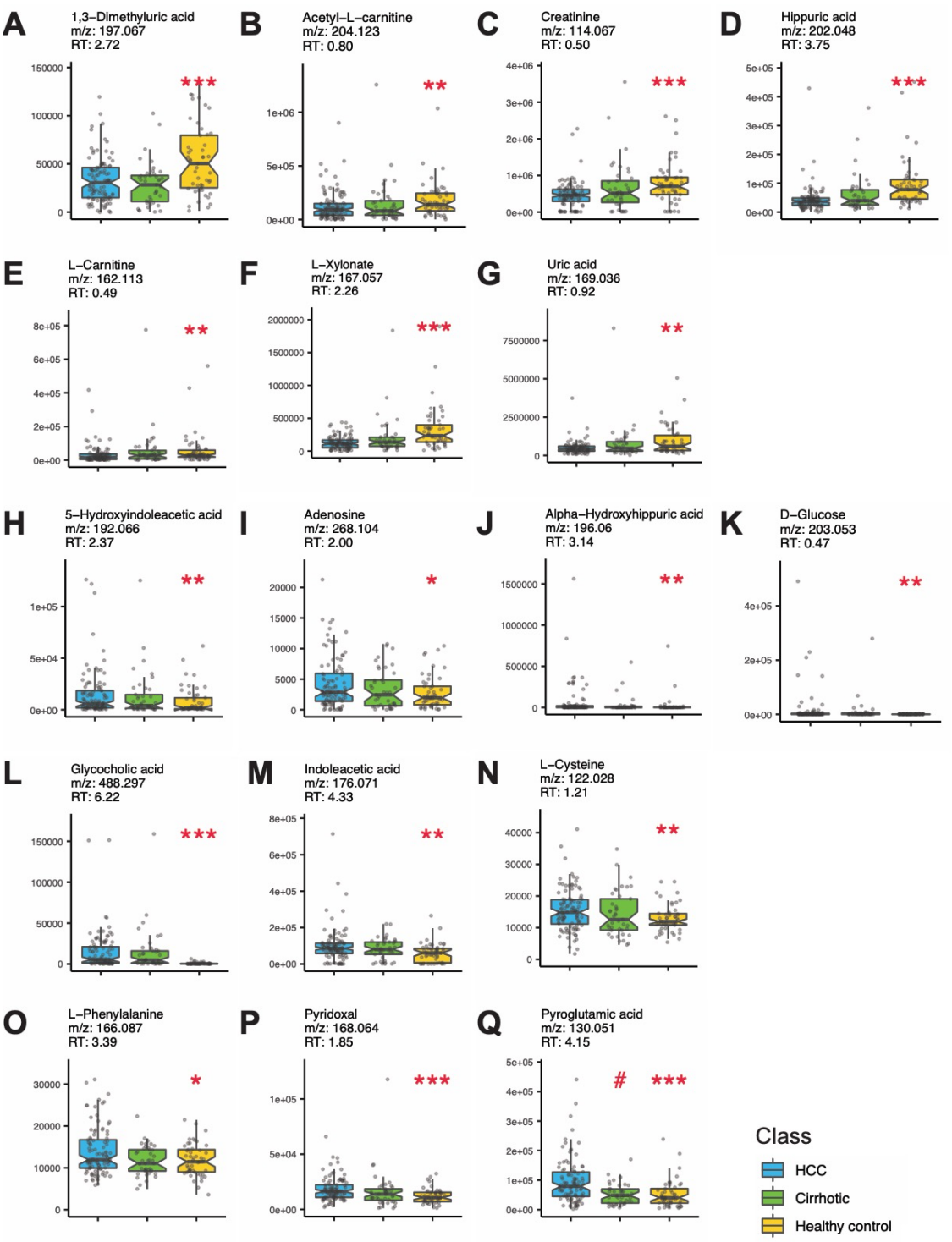

Figure 4.7. Relative intensities of metabolites found in RP-LCMS in each study group. (A-G) Metabolites with lower intensities in HCC. (H-Q) Metabolites with higher intensities in HCC. ***: $q < 0.001$, **: $0.001 \leq q < 0.01$, *: $q < 0.05$ for the comparison between HCC and the healthy control group; #: $q < 0.05$ for the comparison between HCC and the cirrhotic group. The Wilcoxon rank sum test was used to establish significance for group comparisons.

For the comparison between HCC and cirrhosis, only pyroglutamic acid in the RP dataset remained significant after accounting for multiple testing (q = 0.0137). It was found to be higher in HCC compared to cirrhotic (log_2FC = 0.716), and matching for directionality, it was also higher in HCC compared to healthy control (log_2FC = 0.969). Metabolites that showed marginal differences between HCC and cirrhotic participants with p values of less than 0.05 was pyridoxal, which was higher in HCC, and L-carnitine, which was lower in HCC, compared to individuals with cirrhosis.

From the inspection of the boxplots (Figures 4.4-6), relative intensities of many of the annotated metabolites that were altered in the HCC vs healthy comparison were already altered in the cirrhotic group. For example, creatinine and hippuric acid in the NMR data set had similar relative intensities between the HCC and cirrhotic group (Figure 4.4), suggesting that the levels of these metabolites may be altered as a result of liver impairment due to cirrhosis, rather than being specific to HCC.

As an alternative approach to determine the ability of the annotated metabolites to predict HCC, intensity values were first log-transformed to improve normality and logistic regression was used to model for the presence of HCC. In *Model* 1, age and sex were included as independent variables to account for the mismatch of sex and age between the study groups and also because these are independent risk factors of HCC. *Model 2* additionally adjusted for the effect of the severity of liver impairment by including Child-Pugh score as an independent variable. Child-Pugh score is a composite score based on serum total bilirubin, albumin, a measure of blood clotting (either using prothrombin time or the international normalised ratio) and the degree of ascites, and hepatic encephalopathy used to assess liver impairment in patients with

advanced liver disease (13). Given that many of the alterations observed in the spectral profiles of HCC patients may be due to liver impairment, it is thought that by incorporating it as an independent variable could remove its effect and help elucidate the association between metabolite intensities and the presence of HCC.

Serum alpha-fetoprotein (AFP) is the only established biomarker for HCC. To use AFP as a benchmark for assessing the performance of the annotated metabolites, models were also built using the same way as the metabolites investigated and AFP was found to be a significant predictor in both *Models 1 and 2* ($p < 0.002$, Table 4.4). Metabolites with p-values < 0.05 in *Model 1* are L-xylonate (in both datasets), 1,3-dimethyluric acid, hippuric acid, with an odds ratio (OR) less than 1, and glycocholic acid, L-phenylalanine and pyroglutamic acid with an OR of greater than 1. In *Model 2*, only 1,3-dimethyluric acid ($p = 0.0468$), xylonate ($p = 0.0129$) and pyroglutamic acid ($p = 0.00682$) in the RP dataset remained significant, suggesting that these metabolites were associated with the presence of HCC independent of sex, age and liver damage.

4.3.5. Comparison with reports from the literature

The aim of this Chapter was to validate metabolites reported in the literature. A comparison of the current findings against those reported in the literature gathered from the systematic review in Chapter 2 is provided in Table 4.4. In the comparison between HCC and healthy control groups, the direction of change observed in the current cohort was in agreement with the published literature for ten of the metabolites. This includes the top-ranking metabolites such as creatinine, hippuric acid, glycocholic acid, L-threonine and L-xylonate. However, trimethylamine-*N*-oxide, which was reported to be decreased in three separate publications, and ranked third in the list,

was not found to be significantly different in the current cohort. On the other hand, seven metabolites were found to be significantly different from the corresponding control cohort, but in the opposite direction to that reported in the literature. However, for each of these metabolites I found to contrast with the reported association with HCC, there was only one publication each that cited a statistically significant association. Such insufficient amount of evidence may explain the contradicting results. Finally, no significant difference was found between the HCC group and the cirrhosis group, indicating the previously reported difference could not be observed in the current cohort. Nevertheless, for some of the metabolites, such as L-xylonate (Figures 4.6D and 4.7F), and adenosine (Figure 4.7I), a trend could be observed from healthy control, to cirrhosis, to HCC. The lack of statistical difference observed may be related to additional alteration in the HCC group, but at a degree that did not reach significance. However, the alteration of these metabolites in the cirrhotic group suggests that their alteration may be related to liver impairment, and hence limit their specificity as an HCC marker.

Table 4.5. Comparison of findings of the current cohort to those of published results.

Compound name	Rank#	Identified in^	HCC vs Cirrhotic				HCC vs Healthy			
			Score#	nReport#	Vote#	Agree-ment^	Score#	nReport#	Vote#	Agree-ment^
Creatinine	1	NMR	-19.61	3	-3	-	-13.41	2	-2	o
		HILIC				-				o
		RP				-				o
Hippuric acid	2	NMR	-8.25	2	-2	-	-18.16	5	-4	o
		HILIC				-				o
		RP				-				o
Trimethylamine N-oxide	3	NMR	-4.13	1	-1	-	-16.40	3	-3	-
		HILIC				-				-
Glycocholic acid	5	RP					21.97	2	1	o
L-Carnitine	6	RP	7.98	2	2	-	2.45	1	1	x
L-Threonine	8	HILIC	2.45	1	1	-	12.73	3	3	o
L-Xylonate	9	HILIC	-4.13	1	-1	-	-10.32	2	-2	o
		RP				-				o
L-Cysteine	14	RP					-12.12	1	-1	x
Adenosine	22	RP	3.07	1	1	-	5.42	1	1	o
5-Hydroxyindoleacetic acid	23	RP					-4.13	1	-1	x
Alpha-Hydroxyhippuric acid	23	HILIC					-4.13	1	-1	-
		RP								x
1,3-Dimethyluric acid	27	RP	5.52	2	2	-				*
Pyridoxal	28	HILIC					7.94	1	1	-
		RP								o
Pyroglutamic acid	29	HILIC					-7.55	1	-1	x
		RP				*				x
Acetyl-L-carnitine	30	HILIC	2.45	1	1	-	2.45	1	1	-
		RP				-				x
Indoleacetic acid	30	RP	2.45	1	1	-	2.45	1	1	o
Creatine	38	HILIC	5.52	2	2	-	2.45	3	1	-
Uric acid	44	RP					5.50	1	1	x
D-Glucose	54	NMR					2.45	1	1	o
		HILIC								o
		RP								o
L-Methionine	54	HILIC	2.45	1	1	-				*
Taurine	63	HILIC					4.13	1	1	-
L-Phenylalanine	93	HILIC					2.67	1	1	o
		RP								o

#Data from the systematic review (see Chapter 2 for detail): Rank: final rank based on the total score from all three comparisons; Score: the final score from Chapter 2; nReport: Number of times the metabolite was reported to be discriminatory between study groups; Vote: The total vote from the reports (+1 given to a report of increase and -1 given to a report of decrease).
^Findings from the current cohort: Agreement: A circle ‘o’ indicates significant difference in the same direction as reports from the literature; A cross ‘x’ indicates significant difference in the opposite direction of that reported in the literature; A hyphen ‘-‘ indicates no statistical difference found, An asterisk ‘*’ indicates significant difference found with no previous reports.

4.4. Discussion

In an attempt to validate previously reported urine discriminatory metabolites for HCC, metabolites with the ranking in any of the metrics among top 30 from the systematic review were targeted for annotation in three datasets (^{1}H NMR, RP and HILIC LCMS) from the analysis of urine samples in a UK cohort. Most of the statistically significant alterations were found in the HCC vs healthy control comparison and with no difference observed in the HCC and cirrhosis comparison, suggesting that the altered levels of these metabolites are related to liver impairment, rather than being specific to HCC. Only pyroglutamic acid was found to be statistically different in both comparisons and was the metabolite with the strongest association with the presence of HCC after adjusting for the effects of age, sex and liver impairment. Comparing the current findings from reports from the literature, the alteration of ten of the metabolites in the HCC and healthy comparison were in agreement. However, there exists substantial disagreement as seven other metabolites were found to be altered in the opposite direction as those reported in the literature and none of the alterations in the HCC and cirrhosis comparison was observed.

Apart from the ten metabolites whose alteration in the HCC and healthy control volunteer comparison could be reproduced, the lack of agreement in other metabolites and most importantly in the HCC and cirrhotic comparison were alarming. This suggests that urinary metabolites that are the most probable candidates to be markers (based on metrics including the frequency of reports of alterations in the literature, the risk of bias of the reporting publication, absolute fold change values and agreement between reports) could not be reproduced. The disagreement may be related to the lack of sufficient, high-quality evidence in the literature. Firstly, urine was the sample

type with the fewest studies on this topic published to date (10 in total, Figure 2.2A). Secondly, from the risk of bias assessment, studies that analysed urine had the lowest average score compared to reports of other sample types (Section 2.3.2), suggesting that the few studies reporting on urine were more prone to risk of bias. Finally, due to the low numbers of studies, only few of the metabolites had been reported to be discriminatory more than once and all metabolites found to be altered in the opposite direction had been reported once only, indicating the lack of pre-existing confirmatory evidence. Altogether, the failure to reproduce previously reported findings highlight the need for large-scale, high-quality studies on urine samples, with a focus on the comparison between HCC and cirrhotic patients.

Normalisation is a means to account for different dilutions between samples, which is particularly important for urine as its concentration varies depending on water intake. Creatinine, the highest-ranking urine metabolite from the systematic review, is the breakdown product of muscle metabolism, which is proportional to a person's muscle mass and is often used as reference for normalising urine dilution (14). However, patients with liver disease are known to have a lowered baseline creatinine value in blood and the creatinine level may also be affected by renal dysfunction, which is often present in patients with liver disease (15). Therefore, not only is creatinine unlikely to be a specific marker for HCC, the utility of creatinine as a reference for normalising other compounds present in urine in patients with liver disease may be limited. Datasets from metabolic profiling benefit from the simultaneous measurement of many metabolites, which allows for normalisation using other means such as the probabilistic quotient method employed in the current datasets. However, these methods are not appropriate for targeted assays that only quantify a small number of

metabolites. The implication is that for future work on the development of a urine test for HCC, unless the marker is highly specific that its presence is indicative of HCC, creatinine is inappropriate as the reference for normalisation and that alternatives, such as urine osmolality should be considered.

Hippuric acid, the second highest ranking metabolite, is the glycine-conjugated product of benzoic acid or gut microbiota metabolism of polyphenolic compounds from diet (16). The majority of the glycine conjugation takes place in the liver and its level reflect hepatic function (17). In addition to patients with liver disease, it is also found to be lower in individuals who are obese (18) and is negatively associated with blood pressure (19). Given its non-specific nature to HCC and that its level is similar in the HCC and cirrhosis groups, it is unlikely to be a specific candidate marker for HCC.

Pyroglutamic acid was the only metabolite found to have significant change in both the HCC and healthy comparison and the HCC and cirrhotic comparison. Moreover, it was the only metabolite, among those studied, to be associated with the presence of HCC after adjusting for sex, age and liver impairment (Table 4.4). Pyroglutamic acid is a byproduct in the biosynthesis of glutathione (20) and its urinary levels are found to be increased in response to depletion of glutathione due to heightened oxidative stress (21). However, reports from tissue (22, 23) and blood (24-27) studies regarding its level in HCC compared to control were contradictory. Further verification is needed to evaluate its utility as a marker for HCC.

A limitation of the investigation is that all metabolite annotation was made through matching with database references and therefore only reached Level 2 in confidence

of annotation (28). However, given the stringent workflow used for metabolite annotation in the LCMS datasets, with accurate mass matching and identification of co-eluting adducts and fragments, mis-assignments were unlikely. Nevertheless, confirmation using spike-in experiments are necessary to ascertain the annotations.

In conclusion, the vast majority of alterations of urinary metabolites in HCC patients reported in the literature could not be reproduced in a UK cohort. Creatinine and hippuric acid, the two highest ranking metabolites, are unlikely to be markers specific for HCC given their altered levels present in patients with liver disease. Future work on the search for diagnostic urinary marker for HCC should return to the use of the discovery strategy to accrue evidence of alterations of other metabolites and should focus on the comparison between HCC and cirrhosis to increase the chances of identifying markers specific to HCC. Nevertheless, despite the apparent failed effort to validate the systematic collation of evidence from the literature on urine metabolites, similar endeavor should be made for datasets from blood samples. Given that there is a much larger volume of published evidence regarding discriminatory metabolites in blood on the topic, it is anticipated that the consensus from multiple studies should better represent the true alterations and as such, it is more likely to find concordant results in validation.

4.5. References

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Chapter 5 – Discovery of potential serum diagnostic marker for HCC using ^{1}H-NMR spectroscopy: comparative findings from a UK and a Nigerian cohort

Summary

With the aim to identify potential diagnostic markers for HCC that are valid across different populations, serum samples from two cohorts, one from the UK and another from Nigeria, were analysed using untargeted ^{1}H NMR spectroscopy. Most metabolic changes observed were related to impairment due liver disease rather than specific to tumorigenesis. Furthermore, there was marked difference in metabolic alterations between the UK and the Nigerian cohort, probably owing to difference due to a combination of genetics and lifestyle factors. A decreased glutamine-to-glutamate ratio was identified as a potential marker for delineating HCC from cirrhotic patients, with superior performance compared to AFP in the Nigerian cohort and similar performance as AFP in the UK cohort. However, its opposite direction of alteration in the comparison between HCC patients and healthy control individuals in the two cohorts (lower glutamine-to-glutamate ratio in HCC compared to controls in the UK cohort, but higher in the Nigerian cohort) necessitates further investigation to assess its utility as a marker for HCC.

5.1. Rationale

Hepatocellular carcinoma (HCC) is a late-stage manifestation of various liver diseases that have different prevalence in different populations and geographical regions. As a result, patients who develop HCC in different populations have different disease co-morbidities and general physiological characteristics depending on their underlying liver disease aetiology. In Western Europe, the most common causes of HCC are Hepatitis C infection and alcohol-related liver disease (1), with NAFLD/NASH manifesting as an increasingly common cause (2). In West Africa, due to high endemicity of HBV infection acquired at early age, HCC tends to develop earlier (3). Exposure to aflatoxin and iron overload syndrome are other main causes of HCC in this region (4, 5).

Additionally, available healthcare resources determine the presentation and treatment options available (6). In the UK, patients with high risk of developing HCC are followed on surveillance on a six-monthly basis (7), which allows for the earlier identification of HCC leading to better clinical outcomes. In contrast, due to a combination of limited healthcare resource, public confidence in Western medicine and preference for alternative medicine, patients are more likely to be diagnosed at a later stage in West Africa with larger tumours and poorer survival (5).

Diagnostic biomarkers for HCC have the greatest utility if they are applicable regardless of underlying aetiology and population. Having systematically reviewed the literature in Chapter 2 and attempted to validate findings from the systematic review in Chapter 4, this chapter aims to tackle the problem using a different approach – by comparing differences observed in two cohorts of different populations. The

advantage of this strategy is that common alterations observed in different cohorts can act as confirmation for each other and are more likely to be markers that have universal applicability.

This chapter presents results from NMR spectroscopic analysis of serum samples from a UK and a Nigerian cohort. Differences in relative metabolite concentrations were compared to identify coherent changes. Most metabolic alterations observed in HCC were not specific to carcinogenesis as they were also altered in cirrhotic groups. Nevertheless, glutamine-to-glutamate ratio was identified as a potential marker for delineating HCC from cirrhotic patients due to the common alteration observed in both cohorts, with additional confirmation obtained from a validation cohort from the Gambia.

5.2. Methods

5.2.1. Participant recruitment

For the Nigerian cohort, participants were recruited at Jos University Teaching Hospital (JUTH) in Plateau State, Nigeria as part of the **PR**evention **O**f **LI**ver **FI**brosis and **C**ancer in **A**frica (PROLIFICA) study, a European Framework 7-funded study (8). Diagnoses of HCC and cirrhosis were made using ultrasound scans, serum AFP, liver function tests and clinical findings as per local clinical practice. Additionally, chronic hepatitis B (CHB) carriers, with positive hepatitis B surface antigen (HBsAg) and healthy control volunteers with no known liver conditions (confirmed on ultrasound scan) and with negative HBsAg were recruited. A total of 303 participants, consisting of 81 HCC, 79 cirrhosis, 75 CHB patients and 68 healthy volunteers, were recruited. For each participant, a written consent was obtained and a serum sample was

collected. The study had been approved by the Ethics Committee at JUTH and Imperial College London. Samples were stored at -80ºC and were transported on dry ice until analysis. The cohort from the UK, consisting of a total of 199 participants (97 HCC, 38 cirrhotic and 64 healthy control) was recruited as described in Chapter 4.

An independent validation cohort from the Gambia, also part of the PROLIFICA study, was used to validate the performance of the potential discriminatory marker, glutamine-to-glutamate ratio, identified. Cohort recruitment is detailed in Lemoine, *et al.* (9). This was a cohort of 591 participants comprising of 67 HCC, 33 cirrhotic, 436 CHB patients and 55 healthy control volunteers. A heparin plasma sample was collected from each participant and a written consent was obtained. Storage, transport and analysis of samples were identical to those for the Nigerian cohort. Ethics approval was granted by the Government of the Gambia and MRC Gambia Joint Ethics Committee.

5.2.2. Data acquisition and processing

Samples were prepared and analysed according to published protocols (10). Samples were thawed at room temperature and centrifuged at 13,000 *g* at 4ºC for 10 minutes. The resulting supernatant was mixed with buffer (0.142M Na_2HPO_4, 2mM sodium azide, 0.08% TSP at pH 7.4) at a 1:1 ratio. A total volume of 600µL was prepared and transferred to 6mm SampleJet tube (Bruker BioSpin, Rheinstetten, Germany) for the UK cohort and 3mm SampleJet tubes of a mixture of 300µL was used instead for the Nigerian cohort due to limited sample volume. A pooled quality control (QC) sample was prepared by pooling equal volumes of 10µL from each sample. A QC was run every 47 study samples.

NMR experiments were conducted on Bruker 600MHz NMR spectrometer operating at the ^{1}H frequency of 600.13MHz at 310K. Three pulse sequences were used to acquire spectra for each sample as described (10). Firstly, a standard one-dimensional pulse sequence using the first increment of the nOe pulse sequence was used to achieve water suppression. In order to acquire further information on low-molecular-weight molecules, whose signals may have been obscured by those from macromolecules in the standard one-dimensional pulse sequence, a Carr-Purcell-Meiboom-Gill (CPMG) spin-echo sequence NMR experiment was employed using the parameters described by Dona, *et al.* (10). The third experiment used a 2-dimensional *J*-resolved pulse sequence to elucidate the number of peaks and the coupling constants in each multiplet in order to aid with metabolite identification.

The three pulse sequences are as follows:

Standard 1D: D[-90°- t_1-90°-t_m-90°-acquire FID]

CPMG: D [-90” - (τ - 180” - τ)$_n$ - acquire FID],

JRes: [D - 90” – t_1 - 180” - t_1 - collect FID for time t_2]

Where D = a delay to allow T_1 relaxation; t_1 = the interpulse delay; t_m = the mixing time; τ = a fixed delay to allow spectral editing via T_2 relaxation; *n* = a fixed loop (usually of 128 cycles) to give a total relaxation time of 2 *n* τ for the CPMG sequence.

Once acquired, CPMG spectral data were imported into R at resolution of 0.001ppm using in-house script. The spectra were aligned to the α-glucose doublet at 5.233 ppm. Regions corresponding to TSP and noise at both ends (< 0.6 ppm and ppm > 8.5 ppm) and water (4.52-4.86 ppm) were removed and the spectra were normalised using the probabilistic quotient method (11). Annotations were made using published and in-

house databases and was facilitated by the statistical algorithms STOCSY (12) and STORM (13) that calculated statistical correlations between structurally related peaks.

5.2.3. Statistical analysis

PCA with mean centering and pareto scaling was used to obtain an overview of the data and to identify experimentally or biologically aberrant samples. Hierarchical clustering of the Spearman correlation matrix of metabolite relative intensities was used to identify clusters of metabolites that showed strong correlations across individuals. Wilcoxon's rank-sum test was used for 2-way comparisons of relative intensities between groups. Multiple testing was accounted for using the Benjamini-Hochberg method (14). The threshold of $p_{\text{False discovery rate}}$ (pFDR) < 0.05 was considered statistically significant, accepting that there is a 5% chance of false discovery.

Logistic regression models were built to evaluate the ability of the potential marker to predict the presence of HCC. In these models, the presence of HCC was the binary response variable and metabolite intensity with or without other variables to be adjusted for (sex, age, the presence of cirrhosis, and/or Child-Pugh score) were used as predictor variables. Predictor significance was evaluated using the Wald test (15). A total of four models were built (Model 4 was only built for the UK cohort), shown below:

Model 1: Presence of HCC ~ marker intensity

Model 2: Presence of HCC ~ marker intensity + sex + age

Model 3: Presence of HCC ~ market intensity + sex + age + presence of cirrhosis

Model 4: Presence of HCC ~ market intensity + sex + age + Child-Pugh score

Additionally, receiver operating characteristic (ROC) curves were used to assess diagnostic accuracy. Statistically significant differences between ROC curves were determined by bootstrapping with 2,000 iterations. All data processing and analysis steps were performed in R (version 3.6) (16).

Table 5.1. Clinical characteristics of the UK cohort.

	HCC		Cirrhotic		Healthy control		
		n[a]		*n*[a]		*n*[a]	*p*-value[b]
n Total		97		38		64	
Demographic information							
Male sex, n (%)	80 (83%)	96	29 (76%)	38	29 (49%)	59	<0.001[c]
Ethnicity, white, n (%)	86 (89%)	97	29 (76%)	38	44 (69%)	64	0.007[c]
Age, year*	70 (62,78)	96	56 (49,63)	38	36 (30,48)	62	<0.001[d]
BMI, kg m^{-2}*	28.4 (25.8,32.3)	94	29.3 (25.8,35)	38	24.7 (22.2,26.8)	64	<0.001[d]
Co-morbidities							
Diabetes, n (%)	55 (57%)	97	10 (26%)	38	1 (2%)	64	
Hypertension, n (%)	40 (41%)	97	9 (24%)	38	4 (6%)	64	
Blood test results							
Alanine aminotransferase, IU L^{-1}	36 (26.5,58)	91	33 (26,55.25)	38			0.062
Alkaline phosphatase, IU L^{-1}	140 (91,175)	91	109 (77,124.75)	38			0.003
Albumin, g L^{-1}	34 (29,38)	94	35.5 (31.25,41.5)	38			0.667
Alpha-fetoprotein, ng L^{-1}	16 (3,192)	82	2.5 (2,4.25)	32			<0.001
Bilirubin, µmol L^{-1}	15.5 (9,30.25)	94	18.5 (10.25,27.75)	38			0.610
Creatinine, µmol L^{-1}	74 (64,97.5)	91	72 (61.5,88.25)	38			0.553
International normalised ratio	1.1 (1.1,1.225)	60	1.2 (1.1,1.4)	26			0.083
Prothrombin time, s	14.3 (12.6,15.9)	77	15 (13.5,16.55)	36			0.099
Urea, mmol L^{-1}	5.4 (4.3,7)	93	4.45 (3.6,5.8)	38			0.019
Liver condition							
Cirrhotic, n (%)	76 (78%)	97	38 (100%)	38			
Child-Pugh Stage, n		97		38			
A	56		22				
B	37		14				
C	4		2				
HCC tumour condition							
BCLC stage, n		97					
0	5						
A	20						
B	15						
C	39						
D	8						
Aetiology, n		97					
CHB	5						
CHC	9						
ARLD	23						
NAFLD/NASH	33						
Autoimmue-related	4						
Mixed	6						
Other	12						
Multiplicity, n		95					
Single	49						
Multiple	43						
Diffuse process	3						
Diameter of largest tumour, n		83					
≤ 5	56						
>5	27						
Metastasis, n	6	97					

Data shown as median (interquartile range) with the exception of *, for which mean (±1 stand deviation) is shown. [a]number of data points available. [b]*p*-values of Mann-Whitney *U* test, unless stated otherwise. [c]χ^2 test. [d]1-way analysis of variance.

Table 5.2. Clinical characteristics of the Nigerian cohort.

	HCC		Cirrhotic		CHB		Healthy control		
		n[a]		*n*[a]		*n*[a]		*n*[a]	*p*-value[b]
n		*81*		*79*		*75*		*68*	
Demographic information									
Male sex, n (%)	70 (86%)	*81*	57 (72%)	*79*	49 (66%)	74	24 (36%)	67	<0.001[c]
Age*, year	49.4 (57.3,41.6)	*81*	41.1 (47.9,34.3)	*79*	34.7 (41.3,28.2)	73	41.8 (48.3,35.3)	65	<0.001[d]
Clinical status									
Mass > 5cm, n (%)	31 (62%)	50							
Cirrhotic, n (%)	63 (78%)	81	79 (100%)	79					
HBsAg positive, n (%)	23 (34%)	67	46 (72%)	67					
HepC positive, n (%)	9 (14%)	65	3 (0.04%)	67					
Blood test results									
Alpha-fetoprotein, g L^{-1}	99.9 (32.9,392.275)	48	96.9 (10.975,402.25)	32	16 (9.1,58)	17	131.3 (89.45,173.15)	2	0.032
Alanine aminotransferase, IU L^{-1}	46 (23.5,75)	63	41 (18,92)	61	35 (20,53)	21	30 (21,40.5)	11	0.335
Albumin, g L^{-1}	30 (24,35.75)	62	28 (21,35.5)	59	36.5 (32.5,42)	20	28 (25,37.5)	11	0.011
Bilirubin, µmol L^{-1}	10.2 (10.2,29.5)	63	10.2 (10.2,53.125)	62	10.2 (10.175,10.2)	20	10.2 (10.2,10.2)	11	0.002
Creatinine, µmol L^{-1}	88 (69,127)	61	86.5 (68,107)	58	96 (80.5,113.5)	19	103 (76,108)	9	0.970
Haemoglobin, g dL^{-1}	10.5 (9.625,12.95)	58	11.1 (9,12.7)	57	13.7 (12.475,14.7)	20	11 (9.65,11.4)	11	0.001
Platelets, $x10^9$ L^{-1}	202 (156,293.25)	52	138 (100,184)	49	214 (160,261)	23	210 (138.75,225.5)	10	0.001
Prothrombin time, s	18.5 (15.25,23)	38	18 (15,28.6)	43	13.5 (12,15.75)	18	20 (14.5,27.75)	6	0.006
White blood cell, $x10^3$ μL^{-1}	7.52 (4.5,10.1)	57	6 (5.16,8.625)	52	5.1 (4.15,6.5)	23	7.5 (5.1,10.25)	11	0.189

Data shown as median (interquartile range) with the exception of *, for which mean (±1 stand deviation) is shown. [a]number of data points available. [b]*p*-values of Mann-Whitney *U* test, unless stated otherwise. [c]χ^2 test. [d]1-way analysis of variance.

5.3. Results and discussion

5.3.1. Cohort characteristics

Serum samples from a total of 199 participants (97 HCC, 38 cirrhotic and 64 healthy control) from the UK were analysed. The minor difference in the number of samples analysed in this Chapter and those presented in Chapter 4 were due to the differential availability of samples of the two biofluids. However, the cohort characteristics were largely similar in that HCC patients were of older age and with a greater proportion of male participants (Table 5.1).

For the Nigerian cohort, samples from a total of 303 participants were analysed, comprising of 81 HCC, 79 cirrhosis, 75 CHB patients and 68 healthy volunteers (Table 5.2). Similar to the UK cohort, because participants were recruited without matching

for sex and age, patients with liver disease were of older age and with a greater proportion of male progressively in groups of advancing liver disease (from CHB, cirrhosis to HCC).

The proportion of HCC patients with positive HBsAg (34%) was not as high as expected, suggesting that other risk factors of HCC, namely, exposure to aflatoxin, alcohol consumption and iron overload syndrome, may be other major underlying causes of HCC in this population (4, 5). In previous epidemiological studies conducted in sub-Saharan Africa, exposure to aflatoxin had an estimated population attributable risk (PAR) in HBsAg-negative individuals of 12%-20% (17) and alcohol consumption had a PAR of 12.8% for liver cancers overall (18). However, alcohol-related liver disease (ARLD) only accounts for a small proportion of liver disease patients in Nigeria (19). This suggests that iron overload related to alcohol consumption may play a more dominant role in the carcinogenesis of these patients. Iron overload is known to be common in rural sub-Saharan African populations through consumption of home-brewed alcohol with high contents of iron due to the use of ungalvanized iron barrels (4, 5, 19). This heightened exposure may also be compounded by genetic predisposition to iron overload due to polymorphism in the ferroportin gene (20). Given that consumption of locally brewed alcohol in Plateau State, Nigeria is especially popular (pers. comm., Dr Pantong Mark), this is also likely to be a prominent underlying cause of HCC among HBsAg-negative patients in this cohort.

As such, the major aetiologies of HCC in the Nigerian cohort are CHB, aflatoxin exposure and iron overload related to consumption of home-brewed alcohol. In contrast, in the UK cohort, NAFLD/NASH (*n* = 33), followed by ARLD (*n* = 23) were

the most common underlying causes of HCC, accounting for 58% of HCC cases, while CHB only accounted for 5% (*n* = 5) of the cases.

5.3.2. Overview of experimental data

The pooled QC samples co-mapped in the principal component analysis (PCA) scores plots indicated that the experiments were reproducible over the course of sample analysis for each of the cohorts (Figure 5.1).

The PCA scores plot of samples coloured according to study group (HCC, cirrhotic, pre-cirrhotic liver disease and healthy volunteers) displayed substantial overlap between the HCC group and the cirrhotic group (Figure 5.2). The healthy control group (and the CHB group for the Nigerian cohort) was visibly more closely clustered to one side of the second and first component for the Nigerian and UK cohort, respectively. This suggests that the overall ^{1}H NMR spectral pattern of HCC samples is more similar to those of the cirrhosis group, while spectra of healthy control volunteers (and CHB carriers in the Nigerian cohort) were more homogenous and dissimilar to those of HCC and cirrhotic patients. This implies that metabolic changes observed in HCC compared to healthy control were likely to be confounded by background metabolic changes that were already present in patients with cirrhosis, and that there were fewer metabolic changes unique to HCC.

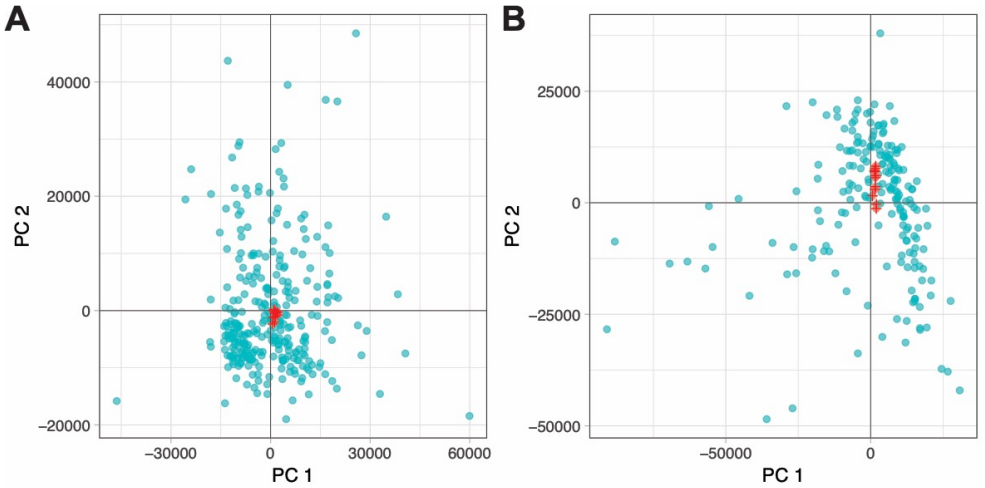

Figure 5.1. Principal component analysis (using mean-centered, Pareto-scaled data) scores plot of serum samples together with quality control samples. (A) Nigerian cohort. Variance explained: PC1 19.3%, PC2 17.9%. (B) UK cohort. Red cross: quality control, turquoise: study sample. Variance explained: PC1 29.9%, PC2 19.7%.

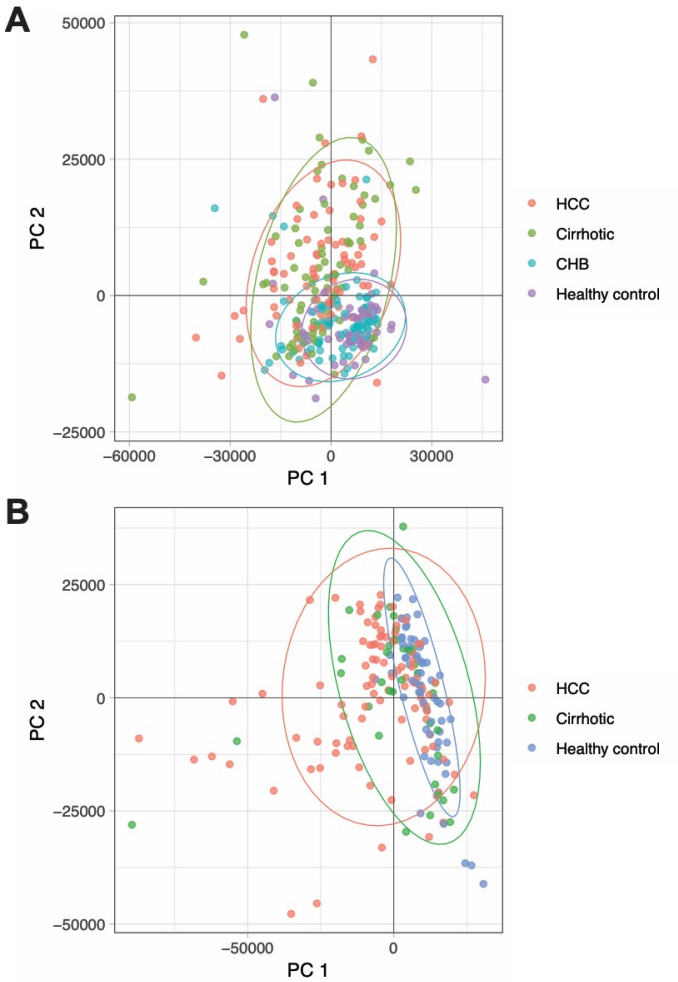

Figure 5.2. Principal component analysis scores plot (using mean-centered, Pareto-scaled data) of samples coloured according to study group. (A) Nigerian cohort. Variance explained: PC1 19.3%, PC2 18%. (B) UK cohort. 95% eclipses of multivariate t-distribution for each study group is displayed. Variance explained: PC1 30.3%, PC2 19.8%.

Table 5.3. Chemical shifts and evidence used for the assignment.

Compound	Chemical shifts and multiplicities*	Evidence
1-Methylhistidine	**7.04s**, 7.77s	1d-NMR, STOCSY
2-Hydroxybutyrate	**0.90t**, (1.65m), (1.71m), (3.97dd)	1d-NMR
2-Hydroxyisobutyrate	**1.35s**	1d-NMR
3-Hydroxybutyrate	(1.20d), (2.30m), 2.39m, **4.17m**	1d-NMR, STOCSY
3-Hydroxyisobutyrate	**1.07d**, (2.46m), (3.53m), (3.7m)	1d-NMR
Acetate	**1.92s**	1d-NMR
Acetoacetate	**2.27s**, (3.44s)	1d-NMR
Acetone	**2.22s**	1d-NMR
Alanine	**1.48d**, 3.78q	1d-NMR, STOCSY
Choline	3.19s, 3.52m, **4.06m**	1d-NMR, STOCSY
Citrate	2.54d, **2.66d**	1d-NMR, STOCSY
Creatine	3.05s, **3.92s**	1d-NMR, STOCSY
Creatinine	3.05s, **4.05s**	1d-NMR, STOCSY
Dimethylamine	**2.72s**	1d-NMR
Formate	**8.45s**	1d-NMR
Glutamate	(2.10m), **2.35m**, 3.77t	1d-NMR, STOCSY
Glutamine	2.14m, **2.43m**, 3.77t	1d-NMR, STOCSY
N-acetylglycoproteins	**2.03s**	1d-NMR
Isoleucine	**0.93t**, 1.00d, (1.26m), (1.48m), (3.68d)	1d-NMR, STORM
Lactate	1.32d, **4.11q**	1d-NMR, STOCSY
Leucine	**0.95t**, 1.71m, (3.73t)	1d-NMR, STORM
Lysine	(1.48m), **1.70m**, 1.91m, 3.03t, (3.76t)	1d-NMR, STOCSY
Methionine	**2.14s**, (2.16m), 2.64t, (3.86t)	1d-NMR, STOCSY
Methylamine	**2.50s**	1d-NMR
Phenylalanine	3.97, **7.32d**, 7.37m, 7.42m	1d-NMR, STORM
Pyruvate	**2.37s**	1d-NMR
Thiamine	2.52s, 2.55s, (3.18t), (3.89t), (5.46s), **8.06s**, (9.43s)	1d-NMR, STOCSY
Tyrosine	(3.06dd), (3.2dd), (3.94dd), **6.89d**, 7.19d	1d-NMR, STOCSY
Valine	0.98d, **1.03d**	1d-NMR, STORM

*The chemical shift selected for peak integration is highlighted in **bold**. Peaks not observed due to overlap with other signals are shown in (brackets). d: doublet, m: multiplet, q: quartet, s: singlet, t: triplet.

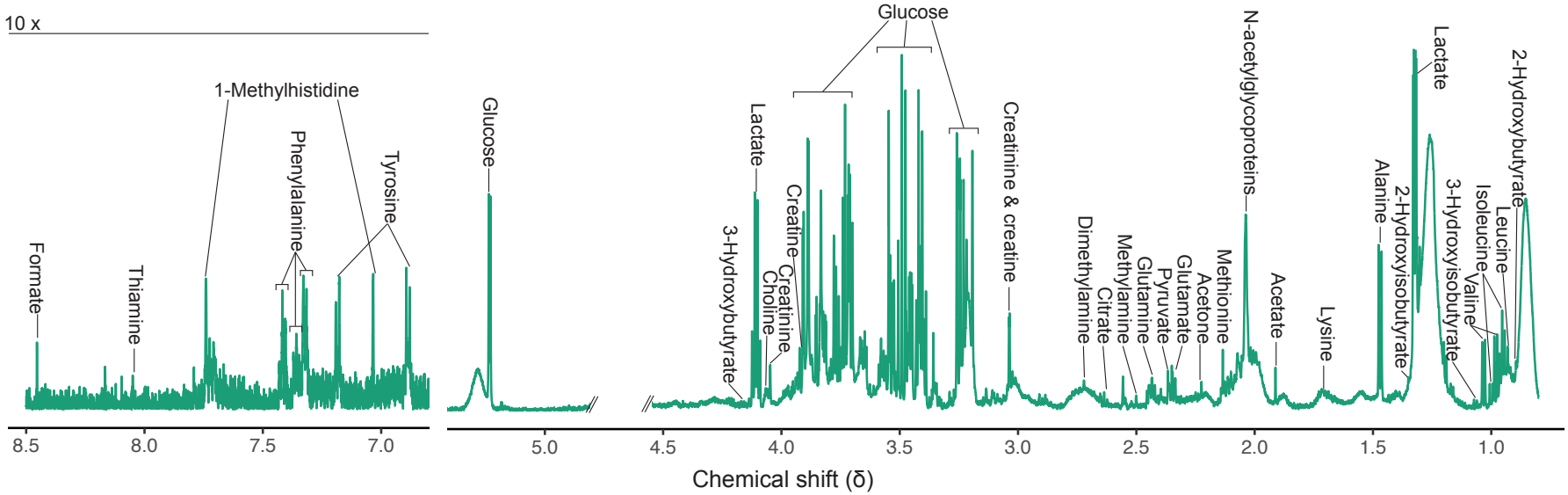

Figure 5.3. A spectrum of a quality control sample from the Nigerian study showing metabolite assignment.

Table 5.4. Median fold change and statistical test results of the assigned metabolites in the Nigerian cohort.

Compound name	HCC vs Cirrhotic			HCC vs CHB			HCC vs Healthy			Cirrhotic vs Healthy		
	$log_2(FC)^a$	*p*	*p*FDR[b]	$log_2(FC)^a$	*p*	*p*FDR[b]	$log_2(FC)^a$	*p*	*p*FDR[b]	$log_2(FC)^a$	*p*	*p*FDR[b]
1-Methylhistidine	-0.195	0.06	0.32	-0.585	**6.57E-11**	0.32	-0.875	**4.96E-11**	**1.49E-10**	-0.680	**2.56E-08**	**5.91E-08**
2-Hydroxybutyrate	-0.001	0.99	0.99	-0.110	**1.03E-04**	0.99	-0.164	**2.60E-07**	**6.49E-07**	-0.164	**1.30E-05**	**2.43E-05**
2-Hydroxyisobutyrate	-0.012	0.93	0.99	0.127	0.16	0.99	0.157	0.06	0.07	0.169	0.08	0.11
3-Hydroxybutyrate	-0.126	0.11	0.44	-0.480	**1.04E-06**	0.44	-0.586	**2.72E-12**	**1.02E-11**	-0.460	**3.92E-09**	**1.17E-08**
3-Hydroxyisobutyrate	-0.020	0.22	0.46	0.044	0.14	0.46	0.142	**8.84E-04**	**1.33E-03**	0.162	**2.77E-06**	**5.93E-06**
Acetate	-0.043	0.99	0.99	-0.327	**9.35E-04**	0.99	-0.733	**4.83E-11**	**1.49E-10**	-0.690	**2.68E-09**	**8.94E-09**
Acetoacetate	0.082	**0.047**	0.241	0.108	**0.016**	0.241	0.009	0.832	0.860	-0.073	0.052	0.074
Acetone	-0.001	0.60	0.82	0.224	**5.80E-04**	0.82	0.305	**1.02E-04**	**1.70E-04**	0.306	**4.75E-04**	**7.12E-04**
Alanine	-0.181	**6.73E-03**	**4.76E-02**	-0.489	**3.20E-13**	**4.76E-02**	-0.589	**1.28E-14**	**8.39E-14**	-0.409	**5.43E-09**	**1.48E-08**
Choline	-0.081	0.66	0.86	-1.481	**4.88E-05**	0.86	-1.577	**1.40E-14**	**8.39E-14**	-1.497	**1.36E-12**	**1.36E-11**
Citrate	-0.050	0.24	0.48	0.083	**2.04E-02**	0.48	0.046	0.41	0.43	0.096	0.06	0.08
Creatine	0.120	0.18	0.45	0.383	**2.32E-08**	0.45	0.391	**3.73E-07**	**8.62E-07**	0.271	**9.07E-05**	**1.60E-04**
Creatinine	-0.124	0.28	0.48	-0.068	0.66	0.48	-0.139	0.13	0.14	-0.015	0.89	0.93
Dimethylamine	-0.083	0.72	0.90	-0.137	**7.41E-03**	0.90	-0.112	**3.27E-02**	**4.08E-02**	-0.030	0.11	0.14
Formate	0.076	0.47	0.67	0.596	**6.11E-06**	0.67	0.647	**1.03E-07**	**2.80E-07**	0.572	**3.24E-04**	**5.11E-04**
Glutamate	0.305	**7.93E-03**	**4.76E-02**	-0.175	0.21	**4.76E-02**	-0.381	**1.85E-05**	**3.48E-05**	-0.686	**1.92E-09**	**7.21E-09**
Glutamine	-0.564	**1.15E-04**	**1.18E-03**	0.010	0.46	**1.18E-03**	0.238	**1.59E-03**	**2.27E-03**	0.802	**2.70E-10**	**1.16E-09**
***N*-acetylglycoproteins**	0.291	**9.21E-07**	**2.76E-05**	0.332	**1.41E-09**	**2.76E-05**	0.286	**5.24E-07**	**1.12E-06**	-0.005	0.90	0.93
Isoleucine	0.090	0.21	0.46	-0.056	**2.81E-02**	0.46	-0.155	**7.62E-07**	**1.52E-06**	-0.245	**8.24E-09**	**2.06E-08**
Lactate	0.010	0.82	0.97	-0.065	0.95	0.97	-0.093	0.98	0.98	-0.102	0.95	0.95
Leucine	0.121	0.31	0.49	-0.295	**7.40E-11**	0.49	-0.545	**2.70E-17**	**4.06E-16**	-0.666	**1.56E-14**	**2.34E-13**
Lysine	0.066	0.16	0.45	-0.119	**1.61E-04**	0.45	-0.363	**4.39E-13**	**2.20E-12**	-0.428	**2.26E-11**	**1.13E-10**
Methionine	-0.006	0.86	0.97	0.118	**1.15E-05**	0.97	0.078	**6.11E-03**	**8.33E-03**	0.084	0.11	0.14
Methylamine	-0.159	0.29	0.48	-0.411	0.72	0.48	-0.499	0.10	0.11	-0.340	0.39	0.45
Phenylalanine	0.047	0.40	0.60	0.275	**3.87E-06**	0.60	0.037	0.14	0.15	-0.009	0.74	0.83
Pyruvate	0.142	0.14	0.45	0.807	**3.82E-16**	0.45	0.623	**2.07E-12**	**8.88E-12**	0.481	**4.27E-06**	**8.54E-06**
Thiamine	-0.839	0.17	0.45	0.087	0.21	0.45	1.686	**1.16E-02**	**1.51E-02**	2.525	**1.27E-04**	**2.11E-04**
Tyrosine	0.051	0.27	0.48	0.133	**7.89E-03**	0.48	0.219	**4.14E-04**	**6.54E-04**	0.168	**9.79E-04**	**1.40E-03**
Valine	-0.034	0.12	0.44	-0.458	**2.65E-15**	0.44	-0.573	**4.98E-16**	**4.98E-15**	-0.539	**1.93E-11**	**1.13E-10**
Gln/Glu	-0.927	**1.18E-04**	**1.18E-03**	0.296	0.82	**1.18E-03**	0.594	**9.75E-05**	**1.70E-04**	1.521	**1.64E-11**	**1.13E-10**
BCAA/AAA	-0.091	0.87	0.97	-0.623	**6.07E-17**	0.97	-0.684	**3.03E-18**	**9.10E-17**	-0.593	**1.56E-14**	**2.34E-13**

[a]Log base-2 of median fold change of the first group over the second group in each comparison.
[b]Adjusted p-value using the Benjamini-Hochberg method. CHB: Chronic hepatitis B.
p and *p*FDR < 0.05 are highlighted in **bold.**

Table 5.5. Median fold change and statistical test results of the assigned metabolites in the UK cohort.

Compound name	HCC vs Cirrhotic			HCC vs Healthy			Cirrhotic vs Healthy		
	log_2(FC)[a]	*p*	*p*FDR[b]	log_2(FC)[a]	*p*	*p*FDR[b]	log_2(FC)[a]	*p*	*p*FDR[b]
1-Methylhistidine	-0.175	5.12E-02	1.69E-01	-0.220	**7.94E-03**	**1.40E-02**	-0.04	0.827	0.833
2-Hydroxybutyrate	0.066	6.13E-02	1.69E-01	-0.096	**4.97E-05**	**1.24E-04**	-0.16	**1.59E-06**	**5.29E-06**
2-Hydroxyisobutyrate	-0.006	7.23E-01	8.67E-01	-0.208	**1.51E-02**	**2.38E-02**	-0.20	0.095	0.135
3-Hydroxybutyrate	0.191	**6.32E-04**	**9.48E-03**	0.157	**7.34E-04**	**1.57E-03**	-0.03	0.621	0.665
3-Hydroxyisobutyrate	-0.013	1.58E-01	3.39E-01	0.007	7.44E-01	7.97E-01	0.02	0.321	0.385
Acetate	-0.066	6.91E-02	1.73E-01	0.041	8.13E-01	8.41E-01	0.11	**4.36E-02**	0.073
Acetoacetate	0.24	**1.09E-03**	**1.13E-02**	0.01	0.78	0.83	-0.23	**3.15E-04**	**8.14E-04**
Acetone	0.082	8.39E-01	8.99E-01	-0.262	**2.49E-02**	**3.55E-02**	-0.34	0.068	0.108
Alanine	0.018	9.75E-01	9.82E-01	-0.040	4.42E-01	4.91E-01	-0.06	0.559	0.621
Choline	0.018	8.28E-01	8.99E-01	-0.055	2.11E-01	2.63E-01	-0.07	0.408	0.471
Citrate	-0.078	8.16E-01	8.99E-01	0.279	**4.35E-04**	**1.00E-03**	0.36	**9.50E-04**	**2.19E-03**
Creatine	0.178	**3.52E-03**	**2.11E-02**	0.095	1.26E-01	1.64E-01	-0.08	0.099	0.135
Creatinine	0.040	6.16E-01	8.40E-01	-0.081	3.85E-01	4.62E-01	-0.12	0.192	0.250
Dimethylamine	-0.010	6.51E-01	8.49E-01	-0.272	**1.83E-10**	**7.85E-10**	-0.26	**1.00E-07**	**6.02E-07**
Formate	-0.054	7.01E-01	8.67E-01	0.680	**3.61E-12**	**2.16E-11**	0.73	**3.63E-09**	**2.72E-08**
Glutamate	0.185	**4.37E-03**	**2.19E-02**	0.470	**5.65E-22**	**1.70E-20**	0.29	**1.27E-12**	**3.80E-11**
Glutamine	-0.219	**2.14E-03**	**2.08E-02**	-0.184	**5.57E-03**	**1.04E-02**	0.03	0.311	0.385
***N*-acetylglycoproteins**	0.137	**2.77E-03**	**2.08E-02**	0.004	8.48E-01	8.48E-01	-0.13	**6.53E-04**	**1.63E-03**
Isoleucine	0.060	**1.01E-02**	**4.34E-02**	-0.060	**1.63E-02**	**2.44E-02**	-0.12	**1.59E-05**	**4.78E-05**
Lactate	-0.017	5.69E-01	8.36E-01	0.363	**4.96E-07**	**1.86E-06**	0.38	**1.04E-06**	**3.92E-06**
Leucine	0.106	**2.33E-02**	8.75E-02	-0.140	**3.09E-06**	**9.26E-06**	-0.25	**9.08E-07**	**3.89E-06**
Lysine	0.010	5.17E-01	8.31E-01	-0.040	**1.43E-02**	**2.38E-02**	-0.05	**6.60E-03**	**1.24E-02**
Methionine	-0.096	4.01E-01	7.08E-01	0.185	**2.20E-03**	**4.39E-03**	0.28	**1.51E-03**	**3.01E-03**
Methylamine	-0.014	9.82E-01	9.82E-01	0.102	**2.65E-02**	**3.61E-02**	0.12	0.082	0.123
Phenylalanine	0.110	2.81E-01	5.26E-01	0.329	**5.11E-16**	**5.11E-15**	0.22	**2.86E-07**	**1.43E-06**
Pyruvate	0.174	2.61E-01	5.23E-01	0.492	**6.97E-07**	**2.32E-06**	0.32	**1.44E-03**	**3.01E-03**
Thiamine	0.068	5.85E-01	8.36E-01	0.216	4.20E-01	4.85E-01	0.15	0.833	0.833
Tyrosine	-0.145	6.20E-02	1.69E-01	0.402	**1.64E-10**	**7.85E-10**	0.55	**8.65E-11**	**8.65E-10**
Valine	-0.002	5.26E-01	8.31E-01	-0.238	**1.34E-05**	**3.64E-05**	-0.24	**2.91E-04**	**7.93E-04**
Gln/Glu	-0.399	**1.91E-04**	**5.73E-03**	-0.655	**2.13E-14**	**1.60E-13**	-0.26	**1.12E-02**	**1.97E-02**
BCAA/AAA	0.162	1.41E-01	3.27E-01	-0.488	**2.69E-18**	**4.04E-17**	-0.65	**2.95E-12**	**4.42E-11**

[a]Log base-2 of median fold change of the first group over the second group in each comparison.
[b]Adjusted p-value using the Benjamini-Hochberg method.
p and *p*FDR < 0.05 are highlighted in **bold.**

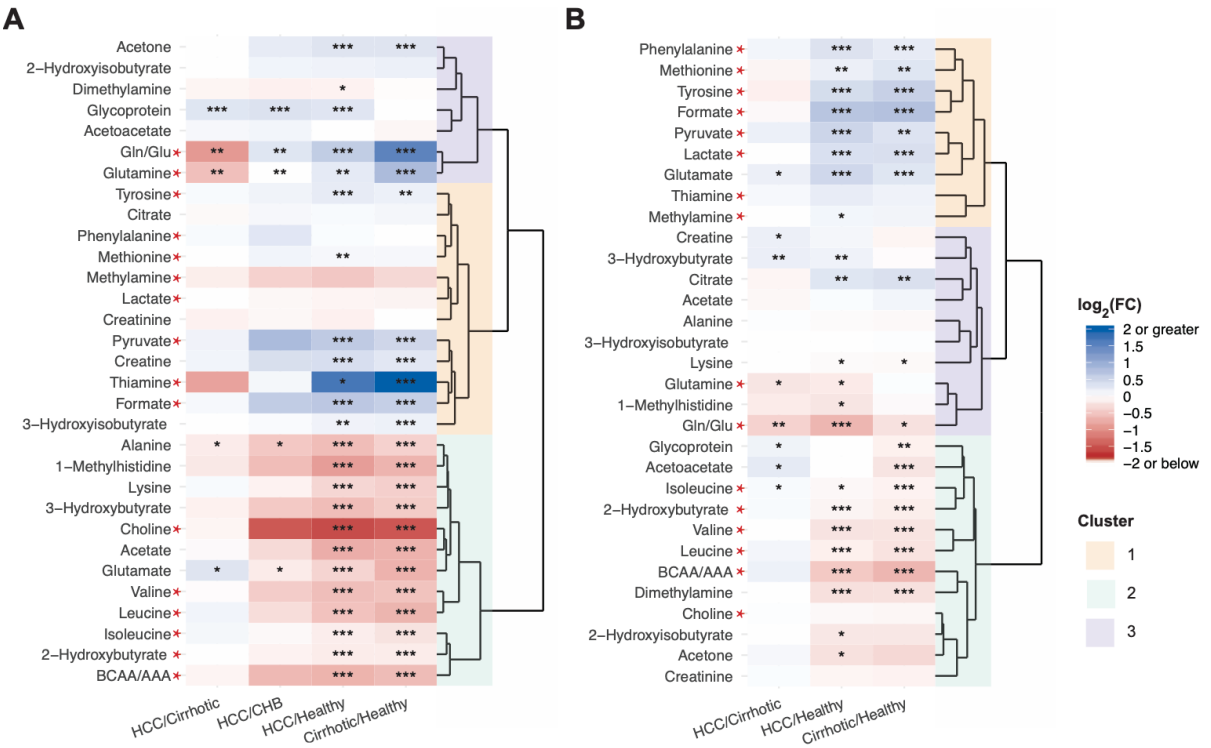

Figure 5.4. Median fold change between groups and statistical test significance of metabolites in each cohort. (A) Nigerian cohort. (B) UK cohort. Hierarchical clustering was used to cluster metabolites based on their correlation with each other and were arbitrarily assigned clusters 1, 2 and 3. Metabolites assigned to be in the same clusters in the two cohorts are indicated with a red asterisk. Within the heatmap, ***: pFDR < 0.001, **: pFDR <0.01, *: pFDR < 0.01 in the respective comparisons.

A total of 29 compounds were confidently annotated in the serum ^{1}H NMR spectrum (Table 5.3 & Figure 5.3). The annotated metabolites were quantified by taking the integrals of the peaks. For any metabolite with more than one chemical shift, the peak group with the highest intensity (for the best signal-to-noise ratio) and/or the least overlap with signals from other compounds was chosen (Table 5.3). In addition to these metabolites, two ratios: the glutamine (Gln) to glutamate (Glu) ratio, and the branched chain amino acids (BCAA) to aromatic amino acids (AAA) ratio, defined as (Isoleucine + Leucine + Valine) / (Phenylalanine + Tyrosine), were computed and analysed along with the other metabolites, based on their relevance further discussed below.

The difference in relative intensities between groups were summarised by taking the base-2 log of the median fold change and were tested for statistical significance using Wilcoxon's rank-sum test (Figure 5.4 and Tables 5.3 and 5.4 for the Nigerian and UK cohort, respectively). Overall, the Nigerian cohort had stronger differences between group (greater absolute fold change values and lower *p*FDR values), supporting the case that patients in the Nigerian cohort had more substantial metabolic changes owing to worse disease management compared to the UK cohort.

5.3.3. Hierarchical clustering revealed common and discrepant changes

In both cohorts, hierarchical clustering of the serum NMR peak integrals using Spearman correlation matrix classified metabolites into clusters under three major branches. These clusters were arbitrarily named Cluster 1, 2 and 3, based on similarity of metabolite membership in each cluster between the two cohorts (Figure 5.4). Cluster 1 consisted of metabolites that were downregulated in HCC and cirrhotic patients compared to healthy control participants. Common metabolites in Cluster 1 include BCAAs, 2-hydroxybutyrate and choline. Cluster 2 largely consisted of metabolites that were upregulated in HCC and cirrhotic patients compared to control individuals. Common metabolites in Cluster 2 included phenylalanine, tyrosine, methionine, formate, pyruvate, lactate, thiamine and methylamine. Cluster 3 consisted of metabolites that either had no significant difference between groups (e.g. lactate in the Nigerian cohort, or alanine in the UK cohort), or metabolites that were significant between HCC and cirrhosis. (e.g. glutamine and Gln/Glu ratio in both cohorts). Interestingly, Cluster 3 is the cluster with the highest proportion of metabolites that showed significant difference between HCC and cirrhosis with 3/5 in the Nigerian cohort and 4/7 in the UK cohort.

Despite the shared membership of many of the metabolites in corresponding clusters, many metabolites displayed dissimilarities in patterns of alteration between the two cohorts (Figure 5.4). Metabolites belonging the the same cluster may have significant difference in one cohort but not the other. For example, lactate was significant in two of the comparisons in the UK cohort but was not significant in any of the comparisons in the Nigerian cohort. The reverse was true for thiamine. In addition, some metabolites were classified into different clusters altogether. The most drastic example was glutamate. It was classified into Cluster 1, which contained metabolites with lower quantities in HCC and cirrhosis, in the UK cohort but into Cluster 2, which contained metabolites with higher quantities in HCC and cirrhosis, in the Nigerian cohort. Altogether, the results show that similar and dissimilar patterns of between-group differences exist in the two cohorts.

In previous studies that compared two cohorts of different ethnicities, different patterns of dysregulation had also been observed. Xiao, *et al.* (21) conducted targeted quantification of 15 metabolites in an American and Egyptian cohort comparing cirrhosis and HCC patients. Among others that had different patterns of alteration, two metabolites (both bile acids) which were similarly deregulated in both cohorts, were identified as putative biomarkers that together outperformed AFP. Di Poto, *et al.* (22) compared plasma GC-MS profiles of European Americans and African Americans with HCC and cirrhosis. Similar to the current study, some metabolites were significant in one ethnic group but not the other, with some displaying significant opposite directions of alteration.

This is the first study with two cohorts that also compared HCC and cirrhosis patients to healthy controls. Assuming that patients in Nigeria were of more advanced stage with poorer management, disease-related alterations of metabolites should show more extreme differences in the same direction (as seen in BCAAs). Therefore, background differences in the two population may be a more probable explanation for metabolites that showed different patterns of dysregulation.

Differences in co-morbidities may explain some of the differences observed in the two cohorts. Lactate is produced from glycolysis under anaerobic conditions. Obesity and diabetes are associated with higher fasting plasma lactate concentration (23) due to changes in glucose metabolism and transport (24). Although body weight data is lacking in the Nigerian cohort, the cohort is unlikely to have as high rates of diabetes and obesity as the UK cohort, while a large portion of cirrhosis and HCC patients in the UK cohort were diabetic and/or overweight with body mass index (BMI) > 25 (Table 5.1). This may explain the raised lactate observed in HCC and cirrhosis patients in the UK cohort but in not the Nigerian cohort, which is supported by the positive correlation of lactate levels to BMI (Spearman $\sigma = 0.257$, $p = 2.8 \times 10^{-4}$) and its association with diabetes status ($p = 7.6 \times 10^{-7}$, Wilcoxon's rank sum test), but not with cirrhosis ($p > 0.05$).

However, other dissimilarities between the two cohorts may not be as easily explained. Ketone body refers to three molecules, acetoacetate, acetone and 3-hydroxybutyrate, which are formed from fatty acid catabolism in the liver and can be used by other organs as an energy source. Acetoacetate and 3-hydroxybutyrate are the main ketone bodies, while acetone is a minor, spontaneous degradation product of acetoacetate.

Raised serum concentration of ketone bodies, known as ketosis, happens during elevated catabolic state and may also occur in diabetes and alcohol intoxication (25). The serum levels of these three metabolites displayed very dissimilar patterns of alteration in the two cohorts which has not been described previously (Figure 5.5).

Similarly, glutamine, glutamate and Gln/Glu ratio showed opposite directions of alteration in the two cohorts in the HCC and healthy control comparison (Figure 5.6). There are two possible scenarios to this observation. One is that the patient groups in the two cohorts display complete opposite directions of alteration, and the other being baseline difference among free living healthy individuals in the two countries. Assuming that patient groups were similar due to their common disease process, the latter explanation may be more probable. Circulating glutamine and glutamate levels can be affected by exercise levels (26) or intake from dietary sources (27). Verification using absolute quantification methods and further studies are necessary to aid the interpretation these different patterns of alterations in the two cohorts.

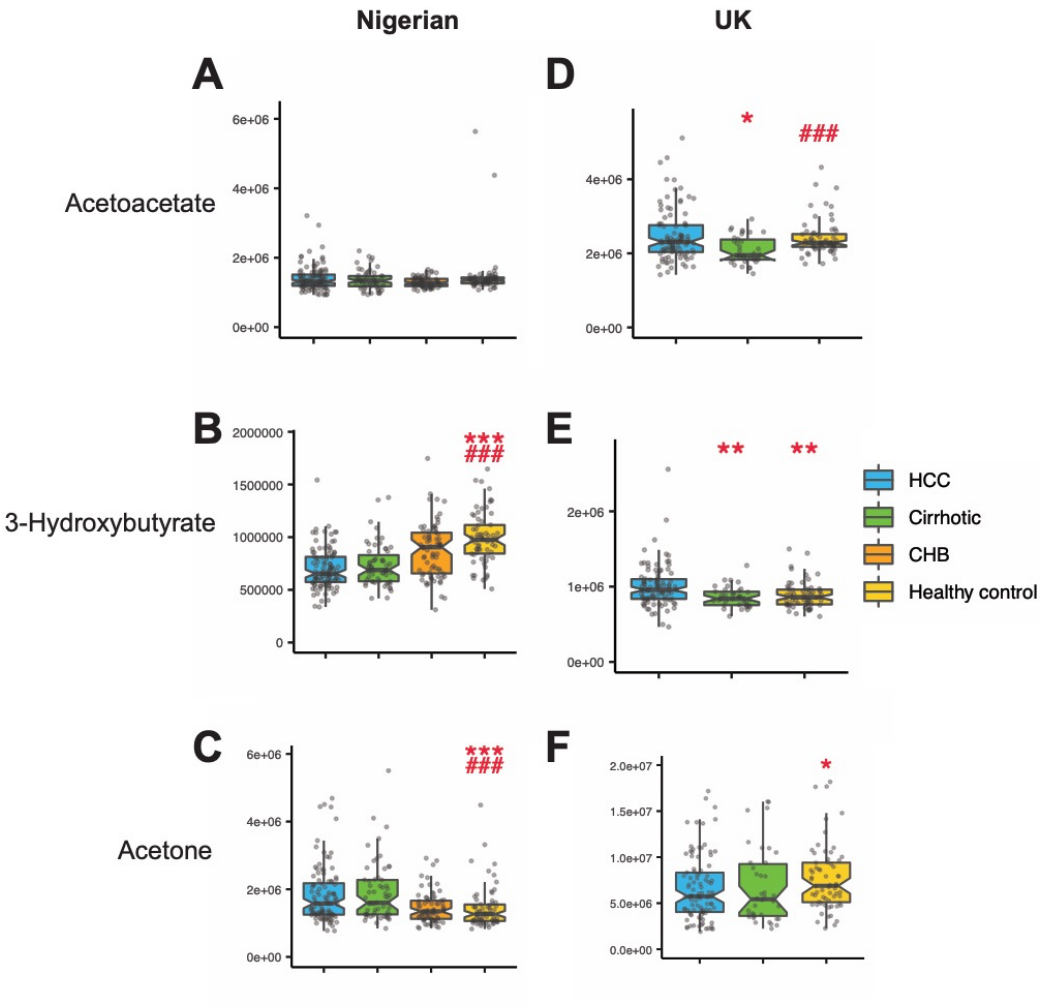

Figure 5.5. Relative intensities of ketone bodies. (A & D) Acetoacetate (B & E) 3-hydroxybutyrate, and (C & F) acetone for the Nigerian and the UK cohort respectively. CHB: Chronic hepatitis B. ***: pFDR < 0.001, **: pFDR <0.01, *: pFDR < 0.01 compared to HCC; ###: pFDR < 0.001, ##: pFDR <0.01, #: pFDR < 0.01 compared to cirrhotic patients.

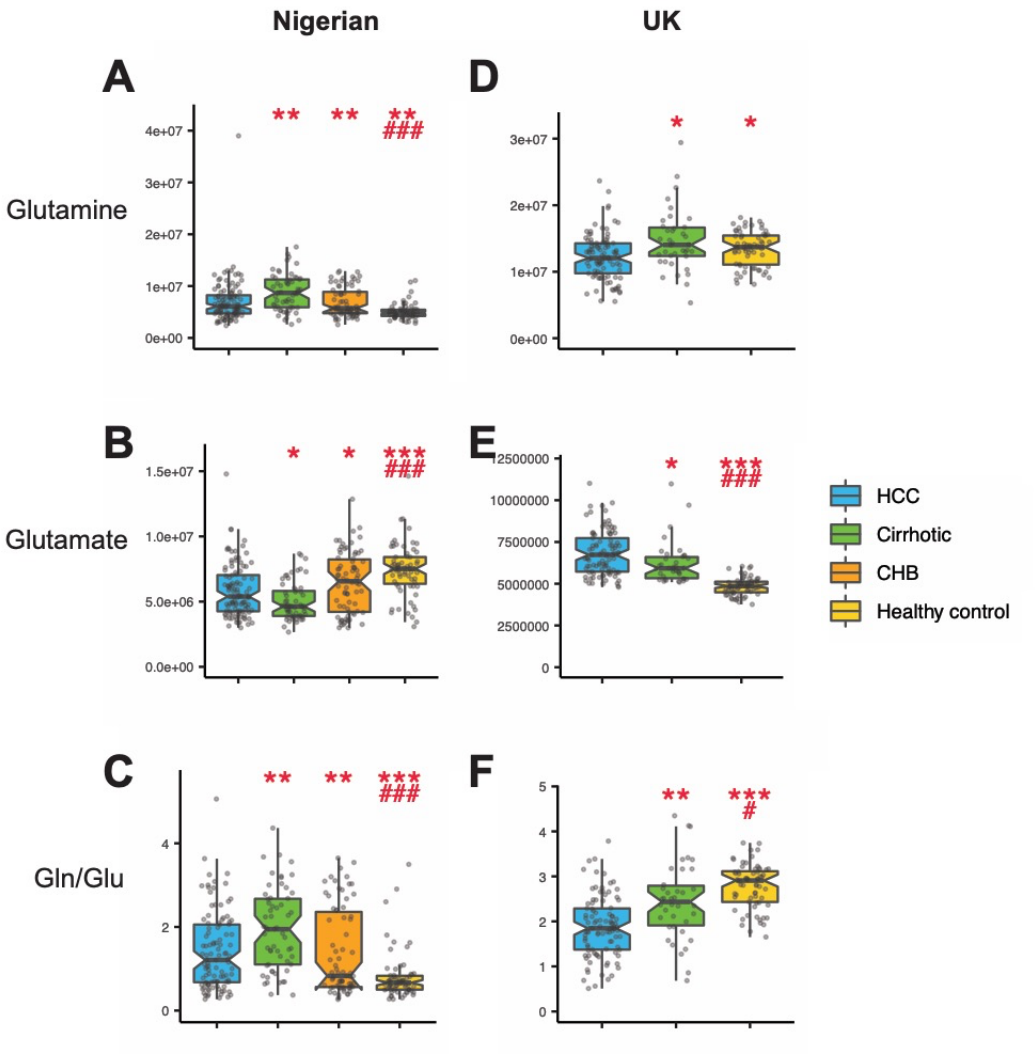

Figure 5.6. Relative intensities of glutamine (A & D), glutamate (B & E) and glutamine-to-glutamate (Gln/Glu) ratio (C & F) for the Nigerian and the UK cohort respectively. CHB: Chronic hepatitis B. ***: pFDR < 0.001, **: pFDR <0.01, *: pFDR < 0.01 compared to HCC; ###: pFDR < 0.001, ##: pFDR <0.01, #: pFDR < 0.01 compared to cirrhotic patients.

Table 5.6. Summary of changes observed in the two cohorts.

	HCC vs Cirrhotic	HCC vs Healthy	Cirrhotic vs Healthy
Increased in both cohorts	Glutamate *N*-acetylglycoproteins	Formate Methionine Pyruvate Tyrosine	Tyrosine Formate Pyruvate
Increased in one cohort	3-hydroxybutyrate Acetoacetate Creatine Isoleucine	3-hydroxyisobutyrate Creatine Citrate Lactate Methylamine *N*-acetylglycoproteins Phenylalanine Thiamine	3-Hydroxyisobutyrate Acetone Creatine Citrate Glutamine Lactate Methionine Phenylalanine Thiamine
Opposite change		3-hydroxybutyrate Acetone Glutamine Glutamate Gln/Glu ratio	Glutamate Gln/Glu ratio
Decreased in one cohort	Alanine	2-hydroxyisobutyrate Acetate Alanine Choline Dimethylamine	1-methyhistidine 3-Hydroxybutyrate Acetate Acetoacetate Alanine Choline Dimethylamine Lysine *N*-acetylglycoproteins
Decreased in both cohorts	Glutamine Gln/Glu ratio	1-methylhistidine 2-hydroxybutyrate Isoleucine Leucine Lysine Valine BCAA/AAA ratio	Isoleucine 2-Hydroxybutyrate Valine Leucine Lysine BCAA/AAA ratio

5.3.4. Common discriminatory metabolites between the two cohorts

Table 5.6 provides a summary of the changes observed in the two cohorts.

Metabolites that were significantly different in the HCC and Healthy comparison as well as the Cirrhotic and Healthy comparison but not the HCC and Cirrhotic comparison suggest that their difference was not specific to HCC and was more likely to be related to liver damage. Metabolites that fitted into this category included BCAAs (isoleucine, leucine and valine), 2-hydroxybutyrate and lysine, which were

downregulated in patients; and the AAA tyrosine, formate and pyruvate which were upregulated.

The BCAA/AAA ratio was significantly lower in both the HCC group and the cirrhosis group in the two cohorts. Moreover, in the UK cohort, the BCAA/AAA ratio was strongly negatively correlated to Child-Pugh score among patients (Spearman σ = -0.63, p = 2.8 x 10^{-16}). It has been well-established that the BCAA/AAA ratio, known as the Fischer ratio, is decreased in patients with advanced liver cirrhosis and that its level is negatively associated with disease progression (28). The metabolism of BCAAs is unique in that their initial catabolism does not occur in the liver but in skeletal muscle instead. The decrease in BCAAs in patients is thought to be due to increased catabolism in muscle while the increase of AAAs reflects the reduced capacity for the cirrhotic liver to catabolise them, leading to their accumulation in blood (29, 30). The accumulation of AAAs in blood leads to increased uptake in the brain, which disrupts neurotransmitter synthesis (29). The imbalance of these amino acids has been suggested to be a contributing factor to the development of hepatic encephalopathy in cirrhotic patients (30). On the other hand, BCAA plays important regulatory role in the metabolism of proteins, sugars and lipids and has been shown to be able to promote liver regeneration and suppress HCC cell proliferation *in vitro* (31). Therefore, the BCAA/AAA ratio represents a good indicator for assessing liver impairment and its simplified version of BCAA/tyrosine ratio has been suggested to have prognostic value for HCC in patients with cirrhosis (32). However, given its non-specific nature to HCC and that it was not found to be markedly changed in HCC patients compared to cirrhotic patients in the two cohorts currently studied, it does not serve as a useful

marker for HCC, but may add value as a marker of severity of liver impairment when used as part of a panel of biomarkers.

In the HCC vs Cirrhotic comparison, *N*-acetylglycoproteins, glutamine, glutamate and consequently, the Gln/Glu ratio showed consistent differences in the two cohorts.

The ^{1}H-NMR peak at 2.03 ppm is a non-specific signal from *N*-acetylated glycoproteins. Since the main contributors to the signal are various acute-phase proteins, such as alpha$_1$-acid glycoprotein, haptoglobin, etc., it has been proposed as a marker of systemic inflammation (33). Acute-phase proteins are a class of proteins mainly synthesised by hepatocytes and released into bloodstream in response to proinflammatory cytokines such as interleukin-6. Raised acute-phase proteins has been associated with poor prognosis in HCC (34) and has been investigated as a diagnostic marker for HCC with contradicting conclusions (35, 36).

Cancer cells are known to derive energy from glutamine in a process called glutaminolysis (37). Glutamine is first converted to glutamate, a reaction catabolised by glutaminase, which is then converted into 2-oxoglutarate to enter the tricarboxylic acid (TCA) cycle. In addition to being an energy source, glutamine also serves other critical functions for cancer cells as a precursor for the synthesis of amino acids, fatty acids, nucleotides and regulator of various cellular processes (38). During malignant transformation, HCC cells are found to switch from expressing the liver-specific glutaminase 2 (GLS2) to expressing the kidney-specific glutaminase 1 (GLS1) and high expression of GLS1 is associated with poor prognosis (39). Targeting GLS1 attenuates the stemness properties of HCC (40) and overexpression of GLS2 reduces

tumour growth (41). The increased demands of glutamine in HCC is facilitated by the upregulation of glutamine transporters ASCT2 (42). Together, HCC cells' reliance on glutamine through increased uptake and utilisation supports the lower glutamine and higher glutamate in serum found in HCC patients compared to cirrhotic patients. Further characterisation of healthy individuals in the two populations is needed in order to explain the opposing trend in comparisons to healthy control as discussed in Section 5.3.3.

5.3.5. Comparison with reports from the literature

Current findings were compared to reports from previous publications gathered for the systematic review in Chapter 2 (Table 5.7). Overall, many of these metabolites had contradictory reports in the literature. However, there was a greater portion of agreement between current findings and the literature, compared to data from urine in Chapter 4.

Metabolites were classified into categories based on their agreement with the consensus from the literature (Table 5.7). Of the 29 metabolites and two ratios investigated, in the HCC vs Healthy comparison, 11 were in agreement with the consensus of previous published records, six had opposite change from the published consensus, five were novel reports, two had contradictory reports in the literature and two were previously not reported differences. Of note, the two metabolites that have been reported the highest number of times were the AAAs tyrosine and phenylalanine. However, there have been contradicting reports in the HCC vs cirrhosis comparison.

Table 5.7. Comparison of current findings and previous reports.

Compound	HCC vs Cirrhosis										HCC vs Liver disease										HCC vs Healthy									
	Number of reports	Vote count	Reports of increase^	Reports of decrease^	Fincal score	Current findings# (Nigerian	Current findings# (UK)	Updated number of reports	Updated vote count	Category	Number of reports	Vote count	Reports of increase^	Reports of decrease^	Fincal score	Current findings# (Nigerian	Updated number of reports	Updated vote count	Category		Number of reports	Vote count	Reports of increase^	Reports of decrease^	Fincal score	Current findings# (Nigerian	Current findings# (UK)	Updated number of reports	Updated vote count	Category
1-Methylhistidine																					1	1	[36]		2.62	-1	-1	3	-1	D
2-Hydroxybutyrate	3	3	[43], [101], [48]		14.5			3	3	Nf											2	2	[101], [48]		13.6	-1	-1	4	0	D
2-Hydroxyisobutyrate																												-1	1	-1	Nv
3-Hydroxybutyrate	3	3	[63], [45], [101]		7.67		1	4	4	A1											4	4	[24,][36], [63], [101]		30.2	-1	1	5	3	C
3-Hydroxyisobutyrate																											1		1	1	Nv
Acetate	1	1	[72]		5.16			1	1	Nf											1	1	[42]		11.3	-1		2	0	D
Acetoacetate							1	1	1	Nv											2	0	[24]	[24]	0			2	0	Nf
Acetone																					1	1	[36]		2.62	1	-1	3	1	C
Alanine	2	2	[46], [45]		4.64	-1		3	1	D						-1	1	-1	Nv		3	1	[36], [46]	[87]	-0	-1		4	0	D
Choline	2	0	[43]	[69]	0			2	0	Cl	2	0	[93]	[31]	0		2	0	Nf		3	-1	[101]	[42], [69]	-5.2	-1		4	-2	A1
Citrate	3	1	[46], [101]	[73]	-0			3	1	Nf											3	3	[46], [42], [45]		20.9		1	4	4	A1
Creatine	3	1	[27], [101]	[45]	2.5		1	4	2	A1											1	-1		[85]	-5.9	1		2	0	D
Creatinine											1	-1	[93]		-6.4		1	-1	Nf		4	-2	[85]	[35], [24], [69]	-2.6			4	-2	Nf
Dimethylamine																										-1	-1	2	-2	Nv
Formate	1	-1		[63]	-3.6			1	-1	Nf											1	-1		[63]	-4.4	1	1	3	1	D
Glutamate	7	5	[43], [72], [45], [45], [73], [101]	[39]	22	1	1	9	7	A2	1	-1		[85]	-5.2	-1	2	-2	A1		6	2	[42], [45], [101], [87]	[60], [25]	9.47	-1	1	8	2	C
Glutamine	3	-1	[45]	[72], [101]	-2.7	-1	-1	5	-3	A2	1	1	[85]		5.35	1	2	2	A1		3	-3		[42], [101], [87]	-21	1	-1	5	-3	C
N-acetylglycoproteins	2	2	[72], [83]		9.8	1	1	4	4	A2						1	1	1	Nv		2	0	[83]	[42]	0	1		3	1	Cl
Isoleucine	2	2	[39], [73]		24.6		1	3	3	A1											5	-2	[36]	[33], [42], [87]	-7.2	-1	-1	7	-4	A2
Lactate	3	-1	[101]	[45], [73]	0.68			3	-1	Nf											4	4	[36], [35], [83], [101]		27.1		1	5	5	A1
Leucine	3	1	[46], [73]	[45]	3.19			3	1	Nf											8	0	[36], [50], [46], [60]	[35], [33], [42], [87]	0	-1	-1	10	-2	Cl
Lysine	1	1	[46]		3.61			1	1	Nf											4	-2	[46]	[35], [85], [87]	-5.7	-1	-1	6	-4	A2
Methionine	3	-3		[27], [101], [49]	-30			3	-3	Nf	3	1	[31], [85]	[49]	-1.1		3	1	D		6	4	[33], [46], [60], [85], [87]	[101]	19	1	1	8	6	A2
Methylamine																												1	1	1	Nv
Phenylalanine	6	0	[43], [46], [91]	[27], [63], [101]	0			6	0	Cl	1	1	[85]		5.35		1	1	Nf		13	9	[36], [33], [24], [50], [63], [46], [60], [42], [45], [104], [87]	[35], [91]	38.9		1	14	10	A1
Pyruvate																					3	1	[36], [35]	[67]	0.49	1	1	5	3	A2
Thiamine																											1	1	1	Nv
Tyrosine	4	0	[46], [101]	[27], [63]	0			4	0	Cl											11	7	[36], [33], [24], [50], [46], [42], [45], [85], [87]	[63], [60]	19.8	1	1	13	9	A2
Valine	5	2	[46], [39], [73]	[63]	9.1			5	2	Nf	1	1	[93]		7.65		1	1	Nf		5	-2	[46]	[33], [42], [87]	-6.2	-1	-1	7	-4	A2
Gln/Glu						-1	-1	2	2	Nv						1	1	1	Nv		1	1	[87]		10.5	1	-1	3	1	C
BCAA/AAA											1	1	[60]		3.35		1	1	Nf		3	-3		[33], [45], [87]	-14	-1	-1	5	-5	A2

Columns shaded in yellow show data from Chapter 2. Number of reports is the number of times a metabolite has been reported to be discriminatory. Vote count is the sum of reports of increase (+1) and decrease (-1). ^Reference numbers in Chapter 2.
Columns shaded in blue show vote counts from the current cohorts.
Columns shaded in green are an update of the number of report and vote count by including findings from the current cohorts. Category summarises the comparison of the current findings and previous reports: A1: Significant difference found in one cohort in the same direction as the consensus of published reports; A2: Significant difference found in two cohorts in the same direction as the consensus of published literature; C: contradictory findings in the two cohorts; Cl: contradictory findings in the literature; D: Significant difference found in the opposite direction as the consensus of published reports; Nf: Previously reported difference not found in the current cohorts; Nv: Novel difference not reported previously.

In the comparison between HCC and cirrhosis, all significant alterations found in one or both of the cohorts, with the exception of alanine, were in agreement with the consensus of the literature. However, there were also nine metabolites (e.g. methionine, which had the highest absolute final score) that have had reports of alteration but were not found in the current cohorts. Glutamate has been found to be higher in HCC compared to cirrhosis five times and was supported by the current data.

5.3.6. Selection of a potential biomarker

Ideally, a diagnostic biomarker for HCC should be able to discriminate patients with HCC to any other persons without HCC such that the intensity of the potential biomarker should be significantly changed in the same direction in the comparisons between HCC and each of the control groups. Additionally, a marker should be reproducible across cohorts of different genetic, geographical and aetiological backgrounds. However, there was no single metabolite that satisfied these criteria among the 28 annotated metabolites. Similarly, the two ratios investigated also failed to meet these criteria. For example, alanine was downregulated in HCC across all three comparisons in the Nigerian cohort, but no significant change was observed in the UK cohort. Since it is of greatest clinical value to identify patients with HCC among patients with advanced liver disease, subsequent analyses focused on the comparison between HCC and cirrhosis.

Common deregulated metabolites in the HCC vs Cirrhotic comparison in both cohorts are glutamine, glutamate and *N*-acetylglycoproteins. Since *N*-acetylglycoproteins represent acute-phase proteins that are indicative of an inflammatory response which is non-specific to HCC, it is therefore ruled out as a potential biomarker based on

biological considerations. Given the interesting observation of the antagonism of glutamine and glutamate observed in the two cohorts in the HCC vs cirrhotic comparison, the Gln/Glu ratio was selected to be further evaluated for its potential as a potential biomarker for HCC.

5.3.7. The evaluation of Gln/Glu ratio as a potential diagnostic biomarker

To evaluate the ability for Gln/Glu ratio to predict the presence of HCC, three logistic regression models were built (Table 5.8). *Model 1* was a crude model with only Gln/Glu as the predictor variable and the presence or absence of HCC as the response variable. *Model 2* included sex and age as covariates to adjust for these given that they were not well-matched between study groups and these are established independent risk factors of HCC. *Model 3* also adjusted for the presence of cirrhosis in addition to sex and age, as cirrhosis is a major confounder to metabolic changes observed in HCC patients. An additional *Model 4* was built for the UK cohort using Child-Pugh score in place of the presence of cirrhosis as an alternative to adjust for liver disease severity.

Gln/Glu ratio was a significant predictor in *Model 1*, the crude model for both cohorts (Ward test, $p < 0.001$). In Models 2 to 4, the Gln/Glu ratio remained a significant predictor ($p < 0.001$ for Nigerian models, and $p < 0.01$ for the UK models), demonstrating that it is able to predict the presence of HCC, even after adjusting for sex, age (*Model 2*) and the presence of cirrhosis (*Models 3* and *4*).

Table 5.8. Logistic regression models of glutamine-to-glutamate ratio as a predictor for the presence of tumour.

	Model 1		Model 2		Model 3		Model 4	
Cohort	**OR (95% CI)**	***p***	**OR (95% CI)**	***p***	**OR (95% CI)**	***p***	**OR (95% CI)**	***p***
Nigerian	0.5 (0.34-0.71)	0.000187	0.52 (0.35-0.76)	0.000878	0.41 (0.25-0.63)	0.000134	–	–
UK	0.35 (0.19-0.6)	0.000299	0.39 (0.2-0.71)	0.00354	0.4 (0.2-0.75)	0.00662	0.36 (0.18-0.68)	0.00245

Model 1: HCC (True/False) ~ Gln/Glu ratio
Model 2: HCC (True/False) ~ Gln/Glu ratio + Sex + Age
Model 3: HCC (True/False) ~ Gln/Glu ratio + Sex + Age + Cirrhosis (True/False)
Model 4: HCC (True/False) ~ Gln/Glu ratio + Sex + Age + Child-Pugh score
CI: confidence interval; OR: Odds ratio.

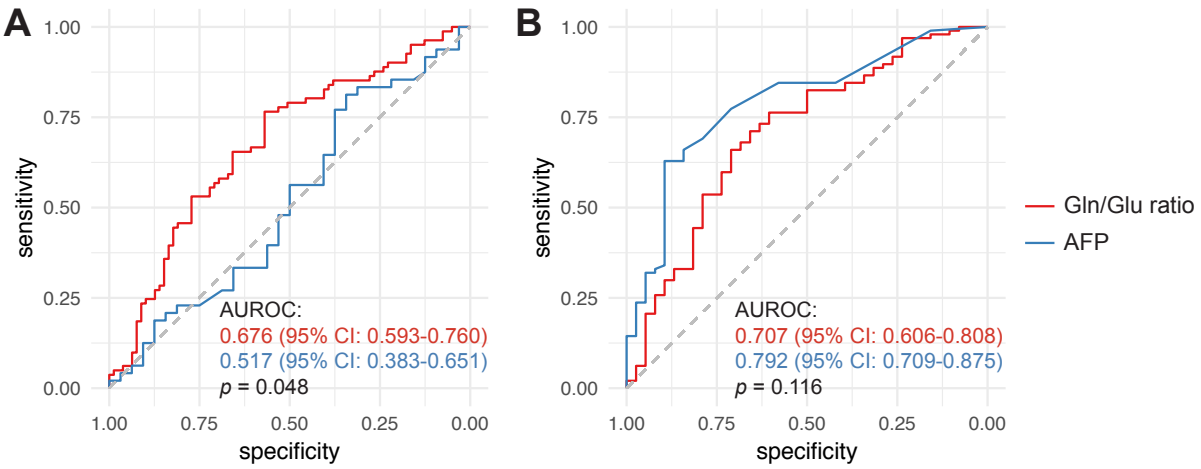

Figure 5.7. Receiver operating characteristic curves of glutamine-to-glutamate ratio and AFP. (A) Nigerian cohort. (B) UK cohort.

Table 5.9. Cutoff threshold, sensitivity & specificity of glutamine-to-glutamate ratio and AFP.

	Cutoff	Sensitivity	Specificity
Nigerian			
Gln/Glu	1.66*	0.77	0.57
AFP	20 g L^{-1}	0.81	0.34
AFP	200 g L^{-1}	0.40	0.53
UK			
Gln/Glu	2.08*	0.66	0.71
AFP	20 g L^{-1}	0.40	0.89
AFP	200 g L^{-1}	0.24	0.97

*The best cutoff was determined using ROC curve for the cutoff value with the highest sum of sensitivity and specificity.

ROC curves were used to assess the ability for Gln/Glu ratio to delineate HCC from cirrhotic patients and to benchmark its diagnostic utility against that of AFP's (Figure 5.6). In the Nigerian cohort, the area under ROC (AUROC) for Gln/Glu was 0.676 (95% CI: 0.593-0.760) which was significantly higher than that for AFP (AUROC = 0.517, 95% CI: 0.383-0.651; p = 0.048). In the UK cohort, AUROC for Gln/Glu was 0.707 (95% CI: 0.606-0.808), which was lower than that for AFP 0.792 (95% CI: 0.709-0.875) but was not significantly lower (p = 0.116). This suggests that Gln/Glu ratio had better diagnostic utility than AFP in the Nigerian cohort and was non-inferior to AFP in the UK cohort.

The estimated best cutoff value for Gln/Glu ratio for the Nigerian cohort was 1.66, which gave sensitivity of 0.765 and specificity of 0.570 (Table 5.9). The estimated best cutoff value was higher in the UK cohort at 2.08, which gave sensitivity of 0.660 and specificity of 0.711.

5.3.8. Validation of the diagnostic utility of Gln/Glu ratio

Finally, to assess the diagnostic utility of Gln/Glu ratio, an independent cohort from the Gambia was used. Cohort details are shown in Table 5.10 and experimental reproducibility is shown in Figure 5.8 (A). AUROC for Gln/Glu was 0.757 (95% CI: 0.661-0.853) which was similar (p = 0.944) to that of AFP (AUROC = 0.763, 95% CI: 0.636-0.890) (Figure 5.8 (B)). However, the absolute value of the ratio was much lower than those from the Nigerian and UK cohorts, with the best estimated cutoff at 0.404 achieving sensitivity of 0.657 and specificity of 0.848 (Table 5.9). In addition, of note, the levels of glutamine, glutamate and the Gln/Glu ratio across all study groups in this Gambian cohort were similar to that of the UK (Figure 5.8 (C-E)). Future validation

using absolute quantification methods and characterisation of its level in healthy individuals along with the disease groups are necessary to establish the utility of Gln/Glu ratio as a marker for HCC among patients with cirrhosis.

Table 5.10. Clinical characteristics of the Gambian validation cohort.

	HCC		Cirrhotic		CHB		Healthy control		
		n[a]		*n*[a]		*n*[a]		*n*[a]	*p*-value[b]
n		*67*		*33*		*436*		*55*	
Demographic information									
Male sex, n (%)	50 (79%)	*63*	19 (59%)	*32*	257 (60%)	*429*	25 (40%)	*55*	<0.001[c]
Age*, year	43 (35.25,50)	*62*	37 (31.5,40)	*31*	35 (31,44)	*428*	41 (33.5,59.5)	*55*	<0.001[d]
Clinical status									
Multiple mass, n (%)	29 (54%)	*54*							
Cirrhotic, n (%)	30 (61%)	*64*	33 (100%)	*33*					
HBsAg positive, n (%)	39 (89%)	*44*	18 (95%)	*19*					
Blood test results									
Alpha-fetoprotein, g L^{-1}	350 (118,6485)	*41*	28.72 (8.65,240.5)	*20*	6.12 (3.6,10)	*97*	3.95 (2.585,6.69)	*4*	7.9E-16
Alanine aminotransferase, IU L^{-1}	58 (36.25,111.75)	*62*	49.5 (27.5,115.5)	*32*	23 (18,31)	*431*	22 (18,28)	*54*	2.06E-25
Alkaline phosphatase, IU L^{-1}	320 (199,530)	*63*	228 (136,333.5)	*31*	86 (71,107)	*425*	91.5 (77.25,100)	*54*	1.58E-33
Albumin, g L^{-1}	32 (29,37)	*61*	30 (23.75,37)	*32*	42 (40,44)	*425*	41 (39,44)	*53*	3.09E-29
Aspartate aminotransferase, IU L^{-1}	196 (79,428.5)	*58*	144 (57.5,242)	*31*	30 (25,36)	*427*	25 (21,31)	*54*	1.93E-37
Bilirubin, µmol L^{-1}	29 (12.5,75)	*63*	37 (14,72.25)	*32*	9 (7,13)	*427*	10 (8,12.75)	*54*	7.89E-25
Creatinine, µmol L^{-1}	75.5 (58.75,95.5)	*46*	69 (54,82)	*29*	78 (63,91)	*361*	67 (59.75,83.25)	*48*	0.0652
Gamma-glutamyl transferase, IU L^{-1}	352 (152.5,616.5)	*58*	144.5 (83.75,199.25)	*32*	26 (20,36)	*401*	26 (20.75,32)	*52*	1.96E-33
Haemoglobin, g dL^{-1}	12.3 (9.95,13.975)	*62*	12.5 (11.5,13.7)	*33*	14.3 (12.9,15.65)	*423*	12.8 (11.8,13.8)	*54*	2.43E-14
Platelets, $x10^9$ L^{-1}	229 (164,338)	*62*	122 (72.6,172)	*33*	190 (150,238.5)	423	219.5 (180.25,258.5)	*54*	1.15E-08

Data shown as median (interquartile range) with the exception of *, for which mean (±1 stand deviation) is shown. [a]number of data points available. [b]*p*-values of Mann-Whitney *U* test, unless stated otherwise. [c]χ^2 test. [d]1-way analysis of variance.

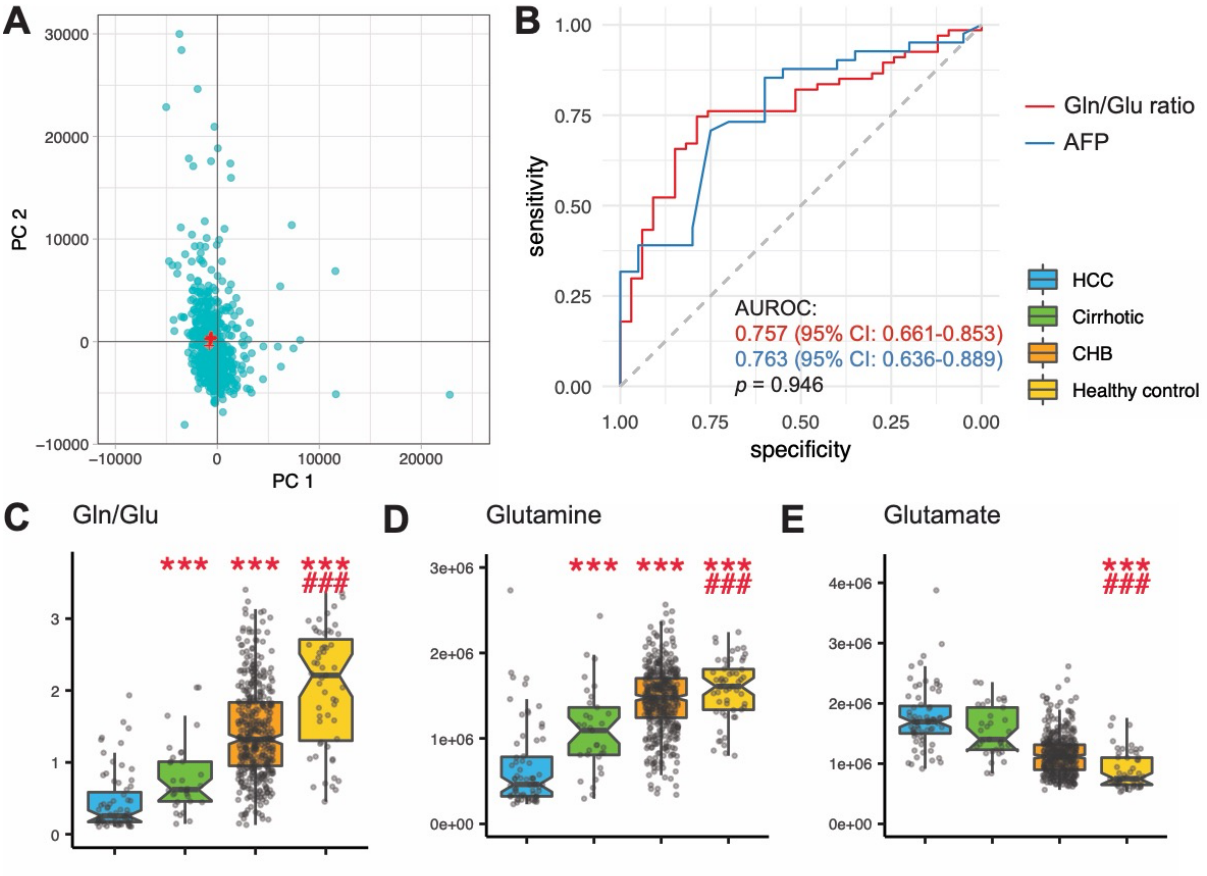

Figure 5.8. Validation of the glutamine-to-glutamate (Gln/Glu) ratio as a marker for delineating HCC from cirrhosis in a Gambian cohort. (A) Principal component analysis (using mean-centered, Pareto-scaled data) of samples together with quality control samples. Red cross: quality control, turquoise: study sample. Variance explained: PC1 43.5%, PC2 13.1%. (B) Receiver operating characteristic curves of glutamine-to-glutamate ratio and AFP. (C-E) Relative intensities of glutamine, glutamate and glutamine-to-glutamate (Gln/Glu) ratio respectively. CHB: Chronic hepatitis B. ***: pFDR < 0.001, **: pFDR <0.01, *: pFDR < 0.01 compared to HCC; ###: pFDR < 0.001, ##: pFDR <0.01, #: pFDR < 0.01 compared to cirrhotic patients.

The agreement in the change in Gln/Glu ratio observed across the three cohort investigated as well as the agreement in the levels of change in glutamine and glutamate with the consensus from the literature in the HCC and cirrhosis comparison supports the universal applicability of the Gln/Glu ratio (Table 5.7) as a potential marker for HCC. As discussed in Section 5.3.4, there is evidence supporting the biological plausibility that the decrease of glutamine and increase of glutamate are the

direct result of tumour activity. This includes the increased uptake of glutamine in tumour through upregulation of its transporter ASCT2 (42) and the established phenomenon of tumour cell's reliance on glutamine as an energy source and precursor for biosynthesis which has been demonstrated in HCC as well (38). Moreover, the use of Gln/Glu ratio as a biomarker has several benefits. Firstly, the incorporation of them in any potential diagnostic test should not require substantial technical development as they are amino acids for which there are readily available assays for quantification. Secondly, the use of a ratio does not require normalisation or any data manipulation which simplifies standardisation across testing labs. Finally, Gln/Glu ratio is likely to be specific as it has not been reported in other disease conditions. Therefore, given the biological plausibility, the current finding supports that glutamine-to-glutamate ratio should be followed up as one of the candidate markers in future validation studies.

5.4. Conclusion

In order to identify discriminatory metabolites in serum that are consistently altered in HCC patients in different populations, metabolic profiling was conducted in two independent cohorts from the UK and Nigeria using ^{1}H-NMR spectroscopy. Most metabolic alterations observed in HCC were also present in the cirrhotic groups, suggesting that they were more related to liver impairment rather than to carcinogenesis. Similar alterations observed in the two cohorts reflect common pathological processes (e.g. BCAA/AAA ratio in relation to liver impairment). Dissimilar alterations may be due to different co-morbidities (e.g. lactate related to diabetes in the UK cohort) while further investigation is needed to verify and elucidate other dissimilar patterns such as those observed in ketone bodies, and glutamine and glutamate in comparisons to healthy control. Focusing on the comparison of HCC to

cirrhotic patients, Gln/Glu ratio was identified as a novel marker for delineating HCC from cirrhotic patients based on the common alteration in the two cohorts and was validated using an independent cohort from the Gambia. However, its utility as a biomarker requires further validation in independent cohorts, especially to clarify its alteration in HCC compared to healthy individuals, using absolute quantification methods.

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Chapter 6 – General discussion

6.1. General interpretation of results

Identifying a reliable, non-invasive diagnostic biomarker for HCC is an unmet need in healthcare globally. This thesis presents an exploration to identify potential non-invasive biomarkers using multiple strategies.

In Chapter 2, a comprehensive systematic review of all published articles on this topic was conducted. A novel risk of bias metric was developed to assess the articles and a scoring system was implemented to rank reported discriminatory metabolites based on their frequency of being reported to be discriminatory, the fold change reported, the risk of bias of the reporting publication and the concordance of direction of change across the reports. This resulted in a ranked list for each of the sample types (tissue, blood and urine) identifying the most promising targets to take forward for validation. Key findings included the extreme heterogeneity of reported biomarkers in biofluids and tissues, with some metabolites being both negatively and positively associated with HCC in different studies. Also, the majority of studies were either based on small group sizes or failed to take into account the pre-existing metabolic consequences of cirrhosis. Nevertheless, the highest ranked potential discriminatory biomarkers of HCC included creatinine, hippuric acid and trimethylamine *N*-oxide in urine; several bile acids and lysophosphatidylcholines in blood; and glycerol 3-phosphate, malic acid and nicotinamide in tissue.

In Chapter 4, an attempt to validate the top-ranking urinary metabolites concluded from the systematic review was presented. Using three datasets (^{1}H NMR, reversed phase and HILIC UPLC-MS) of urine samples from a UK cohort, metabolites ranked

among top 30 in any of the metrics used in the systematic review were targeted to be annotated from the global profiles. Of the 62 metabolites targeted, 22 individual metabolites were confidently annotated. With this, it was possible to validate alterations of 10 metabolites in the HCC and healthy control comparison. However, no difference in the comparison between HCC and cirrhosis could be reproduced, highlighting the difficulty of identifying metabolites that could discriminate the two conditions. The lack of agreement between literature reports and the current findings may be due to the relatively small number of publications on urine markers. Nevertheless, similar efforts should be repeated using data from serum.

In Chapter 5, ^{1}H NMR data of serum samples from two cohorts (UK and Nigeria) with different genetic and aetiological background were compared to identify common alterations. Most of the alterations found between HCC and healthy control groups in both cohorts were already present in the comparison between cirrhosis and healthy control, suggesting that these alterations were related to liver impairment, rather than being specific to HCC. There was also a marked difference in the patterns of the metabolic expression of HCC between the two cohorts, possibly related to background differences between the two populations. Finally, the glutamine-to-glutamate (Gln/Glu) ratio, which was one of two common alterations found in the HCC and cirrhosis comparison in both cohorts (the other being a signal of acute-phase protein, which was deemed inappropriate as a marker as it was an indicator of inflammatory response), was identified as a potential marker for delineating the two. It was also validated in an independent cohort from the Gambia. However, absolute quantification of these two amino acids would be needed to establish the true cutoff value and also to clarify their concentration ranges in healthy volunteers.

The ideal biomarker should be specific to HCC such that its level is only altered in HCC patients but not in any of the control groups (Figure 6.1 (A)) and that this observation is reproducible across different cohorts. However, of the metabolites reported in the literature and those investigated in data generated for this thesis, none of the metabolites satisfied these criteria. Top-ranking urinary and blood metabolites in the systematic review either showed progressive alteration from healthy control, to cirrhosis to HCC (Figure 6.1 (B)) or displayed reversal in trend in the HCC group (the top three conjugated bile acids in blood) (Figure 6.1 (C)). Finally, pyroglutamic acid in urine, identified in Chapter 4 was uniquely raised in HCC but had contradictory reports in the literature and Gln/Glu ratio, the most promising marker identified in Chapter 5 has its levels in the healthy control group different in the Nigerian cohort compared to the UK and the Gambian validation cohort (Figure 6.1 (D)). A number of reasons may explain the challenge for identifying universal biomarkers for HCC, discussed below.

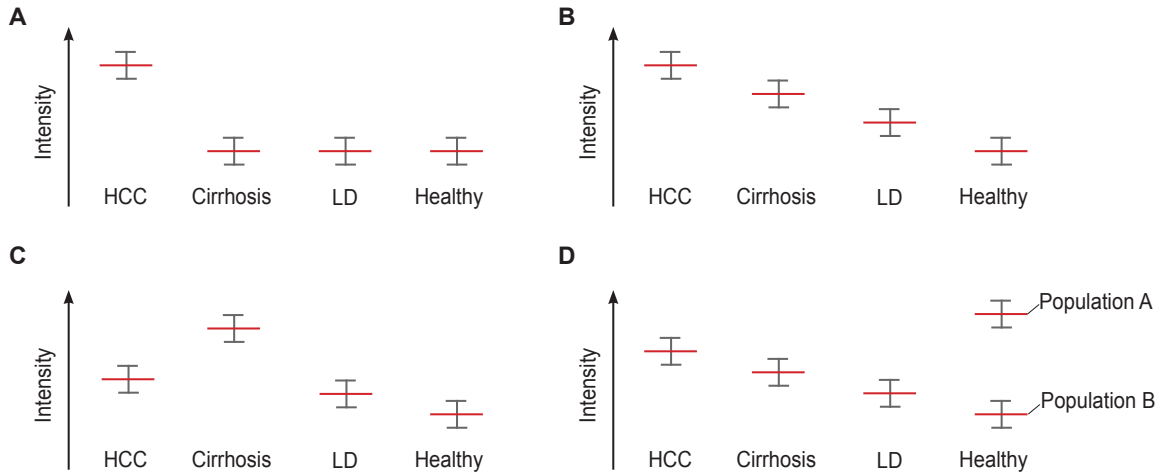

Figure 6.1. Trends across different study groups that a potential marker may display. (A) An ideal marker should be uniquely altered in the HCC group only. (B) Many metabolites showed progressive alteration across the study groups with progressively more severe liver disease. (C) Some even displayed reversal in trend between HCC and healthy and HCC and cirrhosis. (D) Some metabolites manifesting a different direction of change in different populations. A hypothetical case with the marker being higher in HCC than other study groups is displayed, it is also true for the reverse: markers that are lower in HCC than other study groups. LD: pre-cirrhotic liver disease.

6.2. Challenges faced by HCC biomarker discovery

The search for potential biomarkers in blood and urine was founded on the hypothesis that substantial metabolic reprogramming occurs during tumourigenesis and that such change leaves a detectable signature in blood or urine. However, the endeavour to search for potential discriminatory markers in this thesis has led to the identification of three issues that complicate the picture and are the main challenges faced by the search for biomarkers for HCC.

Background changes

The vast majority of HCC cases occur on a background of cirrhosis. Cirrhosis is characterised by the failure for the liver to perform its normal function and on its own is associated with drastic changes in the metabolism in the liver and the whole body (1). Metabonomic studies of patients with fibrosis and cirrhosis described alterations associated to the severity of liver cirrhosis (2).

In data generated in this project and from reports in the literature, many of the differences observed in HCC compared to healthy control volunteers were already present in the cirrhotic group, suggesting that these differences were more likely to be related to liver impairment due to cirrhosis and the underlying liver disease, rather than being specific to HCC. While some metabolites had similar levels in the HCC and cirrhotic groups, others displayed additional alterations in the HCC group. However, in such cases, it is difficult to determine whether the additional increase/decrease were due to HCC patients having more advanced liver disease or whether the development of HCC was indeed the cause of the additional alteration.

In addition to background liver disease, co-morbidities such as obesity and diabetes prevalent in patients with liver disease especially in the West, are associated with significant alterations in the metabolome (3, 4). These conditions are likely to complicate the effort to identify metabolic alterations specific to HCC, as seen in the association of lactate with diabetes in Chapter 5.

Heterogeneity

HCC is heterogeneous at multiple levels, which questions the plausibility of the existence of universal biomarkers. Firstly, HCC can be caused by a myriad of underlying liver diseases. Despite fibrosis and cirrhosis being the common pathway among some of these liver diseases to promote HCC development, each is associated with different mechanisms of pathogenesis (5).

Furthermore, heterogeneity is also present at the cellular and molecular level. Tumours exhibit diverse morphological appearances under histopathological examination (6). In recent genomics studies, the most common mutations only occur in 30% of cases (7). Depending on the combination of mutations present in the tumour, the alteration in the metabolic landscape differs (8, 9). Different sub-populations of cells within a tumour also display different metabolic phenotype (10).

Given the heterogeneity, the simplistic hypothesis of the existence of a single metabolite as the universal biomarker may not be realistic. Many studies proposed having a panel, rather than a single metabolite as the potential diagnostic test. However, this does not necessarily circumvent the issue of heterogeneity given each

marker metabolite may display heterogeneous patterns and thus it is necessary to demonstrate the value of each to be included in the panel, which involves mechanistic understanding. The more variables that are included in a diagnostic panel also imply more parameter estimations, and thus necessitate the need for larger cohorts to increase the statistical power.

Complex system

The metabolome consists of metabolites from endogenous metabolism, gut-host co-metabolism and those that are derived from diet, xenobiotics, genetic background and environmental exposure. Previous epidemiological metabonomic studies have identified differences in the metabolome of healthy individuals from different populations (11). This likely explains the difference in the patterns of alterations in cohorts from different populations as seen in the results drawn from different studies in Chapter 2 and the comparison of findings in Chapter 5.

Metabolites found in circulation and in urine are the combined result of metabolism in all organs in the body and many metabolites are involved in a multitude of pathways and biological functions. In the context of biomarker discovery for HCC, the implication is that it is difficult to delineate whether an alteration observed in biofluid is due to tumorigenesis itself, a secondary effect due to tumour development after its metabolic output having interacted with the metabolism of the rest of the body.

6.3. Lessons for future studies

Given the challenges faced by the research topic, future efforts should make use of a combination of different strategic approaches to maximise the chances of elucidating the right metabolite(s) as markers for HCC.

Study design

As most of the differences found between HCC and healthy volunteers are already present in patients with cirrhosis and are likely to be related to liver impairment, studies should always have cirrhosis as a control group and should focus on the comparison between HCC and cirrhosis, rather than the comparison between HCC and healthy control volunteers.

To allow for the establishment of whether alterations are different or consistent in HCC caused by different underlying aetiologies (and hence mechanism of pathogenesis), studies should consider setting specific targets for patients with different underlying liver diseases. For example, in the UK, equal numbers of HCC and cirrhosis patients due to alcohol-related liver disease, non-alcoholic fatty liver disease and chronic hepatitis C should be recruited.

Participants should also be recruited matching for sex and age with the following variables recorded: For all participants, height, weight, hepatitis B and C statuses, liver function tests, including alpha-fetoprotein; and for all patients, Child-Pugh score and staging for HCC for HCC patients. To avoid the influence due to food intake and to minimise variation due to circadian rhythm, morning fasted samples should be collected.

Analytical strategy

Metabonomic studies usually adopt either a targeted or untargeted approach. Targeted approach measures a limited number of metabolites, typically belonging to the same chemical or metabolic class. The low number of metabolites profiled limits targeted approach's utility in biomarker discovery as it does not allow high-throughput assessment of a large number of metabolites in a single assay. On the other hand, metabolite identification is a major bottleneck for yielding information from data generated by untargeted assays. For LCMS experiments, batch-to-batch variation in retention time and the dependence on chromatographic methods used add to the challenge of metabolite identification and comparison of results from different datasets.

Given that it is necessary to validate previously reported findings, future studies should consider a pseudo-targeted approach. In an experiment with a pseudo-targeted approach, experimental methods the same as an untargeted approach is used. However, investigators select metabolites that are of interest and are likely to be detected in the method prior to the experiment. A mixture of the chemical standards of the selected metabolites should be prepared and run along with the samples. With information from the standards mixture, data analysis is expedited as the metabolite identification step is bypassed.

Moreover, a pseudo-targeted approach confers additional benefit as the workflow of data analysis would be different from an untargeted approach. In typical analysis for data generated from untargeted assays, the initial step is to identify features that are discriminatory between conditions of interest and metabolite identification efforts are

only focused on these features. Consequently, only features found to be discriminatory are reported. However, to validate potential biomarkers, findings of no significant difference is as important as findings of significant difference as it informs the consistency of the discriminatory power of the potential marker.

Therefore, a pseudo-targeted approach would increase the amount of information yielded from untargeted profiling and allow rapid validation of any potential markers in a hypothesis-driven manner. Metabolites to be targeted may be those high-ranking ones concluded from the systematic review and may also be selected in an informed manner through review of the literature.

Data analysis strategy

To overcome the challenge of substantial background metabolic changes as a result of liver impairment, three strategies may be used.

Firstly, filtering for features that are discriminatory between HCC and healthy control, and between HCC and cirrhosis, but not between cirrhosis and healthy control would allow for the identification of alterations unique to HCC (Figure 6.2A).

Secondly, assuming that the potential marker is linear related to the progression of HCC, investigations could also correlate features to HCC stages or tumour size. By comparing the correlation of features to HCC stages and liver disease severity (e.g. Child-Pugh score), features that display strong correlation with the former but not the latter are more likely to be a specific marker of HCC (Figure 6.2B). However, given that liver disease severity is often associated with tumour burden, i.e. the two are not

completely independent variables, identifying features that are correlated to one but not the other is less likely, but this strategy should nevertheless be attempted in future datasets.

Finally, cirrhosis may be treated as a confounder in data modelling (Figure 6.2C). A confounder is defined as a factor that is both associated with the predictor (in this case, metabolite) and outcome (in this case, the presence of HCC). Cirrhosis fits well to the definition. In regression models, variables could be adjusted for by including them as addition predictor variables. As demonstrated in the modelling in Chapters 4 and 5, this may prove a useful way to identify association between potential marker and the presence of HCC with the effects of liver impairment removed.

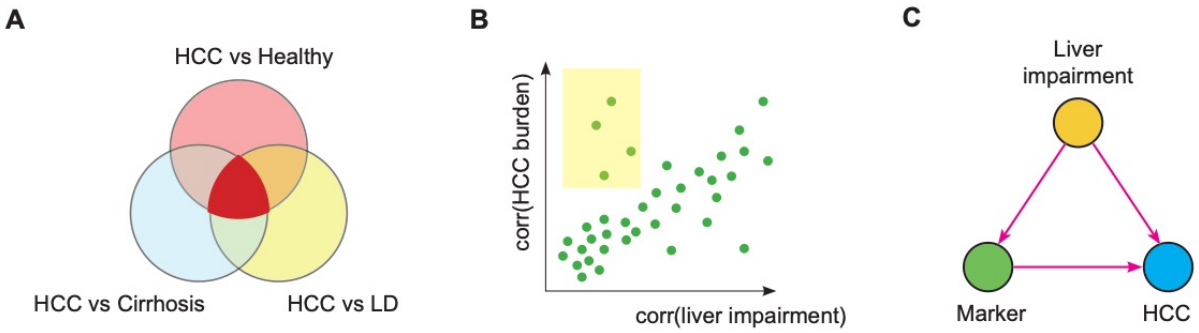

Figure 6.2. Strategies to identify markers specific to HCC, not markers of liver impairment. (A) Features may be filtered for their significance in all three comparisons (red filled area). (B) Features that show strong correlation with HCC burden that is not strongly associated with liver impairment (yellow shaded area) are more likely to be specific to HCC. (C) Liver impairment may also be treated as a confounder in regression models as one of the factors adjusted for.

Additional lines of evidence

In order to establish a metabolite as a potential marker of HCC, biological plausibility of it being a direct effect of HCC tumorigenesis has to be demonstrated. To this end, the sole use of exploratory analysis on biofluids is insufficient. Animal or *in vitro* studies establishing the secretion or uptake of the potential marker by the tumour would offer definitive evidence that alteration of the marker found in biofluids is indeed due to the metabolic activities of the tumour. Alternatively, parallel investigations of paired biofluid and tissue samples from patients would also be a promising method to demonstrate such causal relationship.

Minimal reporting standards and application of risk of bias assessment

The heterogeneity in study design and reporting standards seen among the published studies reviewed in Chapter 2 complicated the comparison of results between studies. Given the difference in disease patterns (such as underlying aetiology), the availability of analytical platforms and the need to explore alternative data analysis strategies illustrated above, it may not be feasible to strictly enforce a standardised design. Nevertheless, future efforts should aim to adhere to minimal reporting standards and fulfill basic requirements to minimise risk of bias. Despite numerous metabonomics studies having been published, there has yet been a checklist or tool to evaluate the risk of bias of studies of this kind. To this end, the novel tool developed to assess risks of bias in Chapter 2 addressed this gap and should be implemented by any future researchers as a checklist when preparing for reports and in the peer review process. Furthermore, this tool has wide applicability as it can also be implemented to metabonomic clinical studies on other diseases or conditions.

6.4. The way forward for a real-world solution

In summary, in order to move forwards in the search for novel non-invasive diagnostic test for HCC through metabonomic platforms, it is necessary to conduct studies with strict adherence to minimal reporting standards and risk of bias criteria with the above discussed considerations taken into account. Validation of previously reported potential marker is of equal importance as exploratory analysis to identify potentially new markers. For any metabolites deemed to have high potential, analytical methods that provide absolute quantification shall be used to establish cutoff values and for comparing results from different cohorts. Communication and coordinated efforts between research teams of different populations are encouraged to allow for comparison of findings. Only in this way that a real-world solution may be provided by the cumulation of research efforts.

6.5. References

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Appendix A – Supplementary Tables for Chapter 2

Appendix A Table 1. Search strategy on databases.

A. Medline		
Step	**Search**	**# Results**
1	Carcinoma, Hepatocellular/	77529
2	adult liver cancer*.mp. [mp=title, abstract, original title, name of substance word, subject heading word, floating sub-heading word, keyword heading word, organism supplementary concept word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier, synonyms]	7
3	hepatoma*.mp. [mp=title, abstract, original title, name of substance word, subject heading word, floating sub-heading word, keyword heading word, organism supplementary concept word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier, synonyms]	27961
4	hepatocarcinoma*.mp. [mp=title, abstract, original title, name of substance word, subject heading word, floating sub-heading word, keyword heading word, organism supplementary concept word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier, synonyms]	3870
5	primary liver carcinoma*.mp. [mp=title, abstract, original title, name of substance word, subject heading word, floating sub-heading word, keyword heading word, organism supplementary concept word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier, synonyms]	363
6	hepato-cellular carcinoma*.mp. [mp=title, abstract, original title, name of substance word, subject heading word, floating sub-heading word, keyword heading word, organism supplementary concept word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier, synonyms]	151
7	(liver? adj1 cell? adj1 carcinoma?).mp. [mp=title, abstract, original title, name of substance word, subject heading word, floating sub-heading word, keyword heading word, organism supplementary concept word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier, synonyms]	347
8	(hepatic? adj1 cell? adj1 carcinoma?).mp. [mp=title, abstract, original title, name of substance word, subject heading word, floating sub-heading word, keyword heading word, organism supplementary concept word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier, synonyms]	85

9	(hepatocellular adj1 carcinoma*).mp. [mp=title, abstract, original title, name of substance word, subject heading word, floating sub-heading word, keyword heading word, organism supplementary concept word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier, synonyms]	105853
10	1 or 2 or 3 or 4 or 5 or 6 or 7 or 8 or 9	122657
11	Metabolomics/	12099
12	metabo?omic?.mp. [mp=title, abstract, original title, name of substance word, subject heading word, floating sub-heading word, keyword heading word, organism supplementary concept word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier, synonyms]	24187
13	Metabolome/	7653
14	metabolome*.mp. [mp=title, abstract, original title, name of substance word, subject heading word, floating sub-heading word, keyword heading word, organism supplementary concept word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier, synonyms]	11433
15	metabotype*.mp. [mp=title, abstract, original title, name of substance word, subject heading word, floating sub-heading word, keyword heading word, organism supplementary concept word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier, synonyms]	181
16	metabolite*.mp. [mp=title, abstract, original title, name of substance word, subject heading word, floating sub-heading word, keyword heading word, organism supplementary concept word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier, synonyms]	250490
17	(metabolic adj1 profil*).mp. [mp=title, abstract, original title, name of substance word, subject heading word, floating sub-heading word, keyword heading word, organism supplementary concept word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier, synonyms]	12406
18	(metabolic adj1 signature*).mp. [mp=title, abstract, original title, name of substance word, subject heading word, floating sub-heading word, keyword heading word, organism supplementary concept word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier, synonyms]	828
19	(metabolic adj1 phenotyp*).mp. [mp=title, abstract, original title, name of substance word, subject heading word, floating sub-heading word, keyword heading word, organism supplementary concept word, protocol supplementary	3264

	concept word, rare disease supplementary concept word, unique identifier, synonyms]	
20	(metabolic adj1 panel*).mp. [mp=title, abstract, original title, name of substance word, subject heading word, floating sub-heading word, keyword heading word, organism supplementary concept word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier, synonyms]	288
21	11 or 12 or 13 or 14 or 15 or 16 or 17 or 18 or 19 or 20	272167
22	exp Plasma/	24550
23	plasma*.mp. [mp=title, abstract, original title, name of substance word, subject heading word, floating sub-heading word, keyword heading word, organism supplementary concept word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier, synonyms]	912169
24	exp Serum/	67452
25	serum*.mp. [mp=title, abstract, original title, name of substance word, subject heading word, floating sub-heading word, keyword heading word, organism supplementary concept word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier, synonyms]	1024403
26	Urine/	36510
27	collecting duct fluid*.mp. [mp=title, abstract, original title, name of substance word, subject heading word, floating sub-heading word, keyword heading word, organism supplementary concept word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier, synonyms]	18
28	exp Blood/	1059219
29	blood*.mp. [mp=title, abstract, original title, name of substance word, subject heading word, floating sub-heading word, keyword heading word, organism supplementary concept word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier, synonyms]	3600326
30	urine*.mp. [mp=title, abstract, original title, name of substance word, subject heading word, floating sub-heading word, keyword heading word, organism supplementary concept word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier, synonyms]	348532
31	exp Tissues/	1785249
32	tissue*.mp. [mp=title, abstract, original title, name of substance word, subject heading word, floating sub-heading word, keyword heading word, organism supplementary concept word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier, synonyms]	2022955

33	22 or 23 or 24 or 25 or 26 or 27 or 28 or 29 or 30 or 31 or 32	7510196
34	lipidomic*.mp. [mp=title, abstract, original title, name of substance word, subject heading word, floating sub-heading word, keyword heading word, organism supplementary concept word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier, synonyms]	3592
35	Lipids/	107114
36	34 or 35	109729
37	21 or 36	378523
38	10 and 33 and 37	976
39	38 not (exp animals/ not humans.sh.)	700

B. EMBASE

Step	Search	# Results
1	adult liver cancer*.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword, floating subheading word, candidate term word]	7
2	hepatoma*.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword, floating subheading word, candidate term word]	38232
3	hepatocarcinoma*.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword, floating subheading word, candidate term word]	5405
4	primary liver carcinoma*.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword, floating subheading word, candidate term word]	594
5	hepato-cellular carcinoma*.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword, floating subheading word, candidate term word]	364
6	exp liver cell carcinoma/	143917
7	(liver? adj1 cell? adj1 carcinoma?).mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword, floating subheading word, candidate term word]	144029
8	(hepatic? adj1 cell? adj1 carcinoma?).mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword, floating subheading word, candidate term word]	163
9	(hepatocellular adj1 carcinoma*).mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword, floating subheading word, candidate term word]	118183
10	1 or 2 or 3 or 4 or 5 or 6 or 7 or 8 or 9	184964

11	metabo?omic?.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword, floating subheading word, candidate term word]	34045
12	metabolome*.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword, floating subheading word, candidate term word]	10064
13	metabotype*.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword, floating subheading word, candidate term word]	232
14	exp metabolomics/	25044
15	exp metabolome/	7167
16	exp metabolite/	76056
17	metabolite*.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword, floating subheading word, candidate term word]	474463
18	(metabolic adj1 profil*).mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword, floating subheading word, candidate term word]	18133
19	(metabolic adj1 signature*).mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword, floating subheading word, candidate term word]	1256
20	(metabolic adj1 phenotyp*).mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword, floating subheading word, candidate term word]	4713
21	(metabolic adj1 panel*).mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword, floating subheading word, candidate term word]	1232
22	11 or 12 or 13 or 14 or 15 or 16 or 17 or 18 or 19 or 20 or 21	505724
23	exp Plasma/	210962
24	plasma*.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword, floating subheading word, candidate term word]	1297510
25	exp Serum/	287057
26	serum*.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword, floating subheading word, candidate term word]	1457474
27	Urine/	190727
28	collecting duct fluid*.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug	25

	manufacturer, device trade name, keyword, floating subheading word, candidate term word]	
29	exp Blood/	2531834
30	blood*.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword, floating subheading word, candidate term word]	4959483
31	urine*.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword, floating subheading word, candidate term word]	544268
32	exp Tissues/	4686556
33	tissue*.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword, floating subheading word, candidate term word]	4281805
34	23 or 24 or 25 or 26 or 27 or 28 or 29 or 30 or 31 or 32 or 33	11285487
35	exp lipidome/	960
36	exp lipidomics/	3522
37	lipid/ec [Endogenous Compound]	50250
38	lipidomic*.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword, floating subheading word, candidate term word]	5799
39	35 or 36 or 37 or 38	55774
40	22 or 39	555774
41	10 and 34 and 40	2537
42	41 not ((exp animal/ or nonhuman/) not exp human/)	1879

Appendix A Table 2. PRISMA-DTA checklists. (Reference no. 13)
A. PRISMA-DTA checklist. (see Note)

Section/topic	#	PRISMA-DTA Checklist Item	Reported in section
TITLE / ABSTRACT			
Title	1	Identify the report as a systematic review (+/- meta-analysis) of diagnostic test accuracy (DTA) studies.	Title
Abstract	2	Abstract: See PRISMA-DTA for abstracts.	–
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of what is already known.	2.1.
Clinical role of index test	D1	State the scientific and clinical background, including the intended use and clinical role of the index test, and if applicable, the rationale for minimally acceptable test accuracy (or minimum difference in accuracy for comparative design).	2.1.
Objectives	4	Provide an explicit statement of question(s) being addressed in terms of participants, index test(s), and target condition(s).	2.1.
METHODS			
Protocol and registration	5	Indicate if a review protocol exists, if and where it can be accessed (e.g., Web address), and, if available, provide registration information including registration number.	2.2.1.
Eligibility criteria	6	Specify study characteristics (participants, setting, index test(s), reference standard(s), target condition(s), and study design) and report characteristics (e.g., years considered, language, publication status) used as criteria for eligibility, giving rationale.	2.2.1.
Information sources	7	Describe all information sources (e.g., databases with dates of coverage, contact with study authors to identify additional studies) in the search and date last searched.	2.2.1.
Search	8	Present full search strategies for all electronic databases and other sources searched, including any limits used, such that they could be repeated.	Appendix A Table 1
Study selection	9	State the process for selecting studies (i.e., screening, eligibility, included in systematic review, and, if applicable, included in the meta-analysis).	2.2.2.
Data collection	10	Describe method of data extraction from reports (e.g., piloted forms, independently, in	2.2.2.

process		duplicate) and any processes for obtaining and confirming data from investigators.	
Definitions for data extraction	11	Provide definitions used in data extraction and classifications of target condition(s), index test(s), reference standard(s) and other characteristics (e.g. study design, clinical setting).	2.2.2.
Risk of bias and applicability	12	Describe methods used for assessing risk of bias in individual studies and concerns regarding the applicability to the review question.	2.2.3.
Diagnostic accuracy measures	13	State the principal diagnostic accuracy measure(s) reported (e.g. sensitivity, specificity) and state the unit of assessment (e.g. per-patient, per-lesion).	N / A
Synthesis of results	14	Describe methods of handling data, combining results of studies and describing variability between studies. This could include, but is not limited to: a) handling of multiple definitions of target condition. b) handling of multiple thresholds of test positivity, c) handling multiple index test readers, d) handling of indeterminate test results, e) grouping and comparing tests, f) handling of different reference standards	2.2.4.

Section/topic	#	PRISMA-DTA Checklist Item	Reported on page #
Meta-analysis	D2	Report the statistical methods used for meta-analyses, if performed.	N / A
Additional analyses	16	Describe methods of additional analyses (e.g., sensitivity or subgroup analyses, meta-regression), if done, indicating which were pre-specified.	N / A
RESULTS			
Study selection	17	Provide numbers of studies screened, assessed for eligibility, included in the review (and included in meta-analysis, if applicable) with reasons for exclusions at each stage, ideally with a flow diagram.	2.3.1.
Study characteristics	18	For each included study provide citations and present key characteristics including: a) participant characteristics (presentation, prior testing), b) clinical setting, c) study design, d) target condition definition, e) index test, f) reference standard, g) sample size, h) funding sources	Appendix A Table 3
Risk of bias and applicability	19	Present evaluation of risk of bias and concerns regarding applicability for each study.	2.3.2.
Results of individual studies	20	For each analysis in each study (e.g. unique combination of index test, reference standard, and positivity threshold) report 2x2 data (TP, FP, FN, TN) with estimates of diagnostic accuracy and confidence intervals, ideally with a forest or receiver operator characteristic (ROC) plot.	N / A
Synthesis of results	21	Describe test accuracy, including variability; if meta-analysis was done, include results and confidence intervals.	2.3.3.
Additional analysis	23	Give results of additional analyses, if done (e.g., sensitivity or subgroup analyses, meta-regression; analysis of index test: failure rates, proportion of inconclusive results, adverse events).	N / A
DISCUSSION			
Summary of evidence	24	Summarize the main findings including the strength of evidence.	2.4.
Limitations	25	Discuss limitations from included studies (e.g. risk of bias and concerns regarding applicability) and from the review process (e.g.	2.4.

		incomplete retrieval of identified research).	
Conclusions	26	Provide a general interpretation of the results in the context of other evidence. Discuss implications for future research and clinical practice (e.g. the intended use and clinical role of the index test).	2.5.
FUNDING			
Funding	27	For the systematic review, describe the sources of funding and other support and the role of the funders.	Funding

Note: The PRISMA-DTA checklist was used as a guide for the systematic review. Since the topic of research is not mature enough for the assessment of diagnostic test

Appendix A Table 3. Details of included studies.

Publication	Ref	Main cohort[#]	Validation cohort[#]	Country	HCC aetiology	Condition of sample collection	Matched control groups (sex / age)	Analytical technology	Funding sources
I. TISSUE									
Abel, et al. (2009)	[22]	13 / 13	–	South Africa	Mixed	necropsy or resection, snap frozen	yes / yes	GC-MS & biochemical methods	The Cancer Association of South Africa (CANSA) and the South African Department of Trade and Industry
Beyoglu, et al. (2013)	[28]	31 / 31	–	France	N.S.	N.S.	N.S.	GC-MS	Supported in part by National Institutes of Health/National Institute of Allergy and Infectious Diseases grant U19 AI067773-07/08 (to J.R.I.); Bernerische und Schweizerische Krebsliga, Sasella Foundation, and the Hassan Badawi Foundation Against Liver Cancer (to J.R.I., J.F.D.); this work was also the PAIR-CHC project NoFLIC (funded by INCa and Association pour la recherche contre le Cancer, ARC), the Reseau national CRB Foie and BioIntelligence (OSEO).
Budhu, et al. (2013)	[30]	30 / 30	–	China	>50% CHB	N.S.	yes / yes	LC-MS & GC-MS	The Intramural Research Program of the Center for Cancer Research, the US National Cancer Institute
Darpolor, et al. (2014)	[38]	11 / – / 11 / 5	–	USA	N.S.	resection, snap frozen	N.S.	NMR	The National Center for Research Resources and the National Center for Advancing Translational Sciences
Eggens, et al. (1988)	[41]	5 / 5 / 5h	–	Sweden	N.S.	biopsy	yes / yes	GC, LC & TLC	The Centrala Forsoksdjursnamnden, the Swedish Cancer Society and the Swedish Medical Research Council.
Krautbauer, et al. (2016)	[52]	21 / 21	–	Germany	Mixed	N.S.	yes / yes	DI-MS	The‚ÄúStiftung f√º r Pathobiochemie undMolekulare Diagnostik‚Äù the German Research Foundation
Li, et al. (2017)	[57]	20 / 20	–	China	>50% CHB	resection, snap frozen	yes / yes	LC-MS & MALDI-MS	National Natural Science Foundation of China and Beijing Key Laboratory Special Fund
Liu, et al. (2013)	[62]	10 / 10	–	China	100% CHB	washed with saline and dried on filter paper	yes / yes	LC-MS	N.S.
Lu, et al. (2019)	[65]	80 / 80	–	China	>50% CHB	resection	yes / yes	LC-MS	Science and Technology R&D fund of Shenzhen and Petrel Project of The Affiliated Tumor Hospital, Harbin Medical University
Morita, et al. (2013)	[70]	37 / 37	–	Japan	>50% CHC	resection, snap frozen	yes / yes	MALDI-MS	LipidMachinery and Ministry of Health, Labour and Welfare, Japan
Solinas, et al. (2014)	[86]	25 / 25	–	Italy	>50% CHC	biopsy, snap frozen	N.S.	MAS-NMR	N.S.
Tang, et al. (2018)	[89]	28 / 18 / 23a	33 / 33	China	>50% CHB	resection, frozen at -80ºC	N.S.	CE-MS	The National Key R&D Program of China and the Key Foundation of the National Natural Science Foundation of China (21435006; to G. Xu). This study is also supported by Hundred Talents Program of CAS (to Y. Liu) and Innovative Research Grant to Scientific Research Center for Translational Medicine at Dalian Institute of Chemical Physics.
Teilhet, et al. (2017)	[90]	28 / 28	–	France	Mixed	resection, snap frozen	yes / yes	NMR	Supported by the Program Canc√©rop√¥le Lyon Auvergne Rh√¥ne Alpes,‚ÄîOncostarter ,‚ÄúMECASTEN,‚Äù.
Wang, et al. (2008)	[92]	29 / 29 / 3h	–	China	N.S.	resection, frozen at -80ºC	yes / yes	LC-MS	Supported by (1) the foundation (No.20425516) for Distinguished Young Scholars from National Nat-ural Science Foundation of China, and the Knowledge InnovationProgram of the Chinese Academy of Sciences (KSCX2-SW-329,KGCX2-SW-213); (2) National Key Basic Research Program (973)of China Grant (2004CB518704, to D.F. Wan); and (3) Shanghai Sci-ence & Technology Committee Shanghai Pujiang Project 06PJ14069(to X.H. He).
Yang, et al. (2007)	[98]	17 / 14	–	China	N.S.	biopsy, snap frozen	yes / yes	MAS-NMR	The National Natural Science Foundation of China, National Basic Research Program of China and the Chinese Academy of Sciences.

II. TISSUE & BLOOD SERUM / PLASMA									
Han, et al. (2019)	[48]	T: 30 / 30; S: 30 / 30 / – / 30	–	China	>50% CHB	T: resection, snap frozen; S: fasted	yes / no	LC-MS	Supported in part by the National Natural Science Foundation of China (No. 81472284 and 81672699).
Huang, et al. (2013)	[49]	T: 50 / 50; S: 139 / 78 / 81b / –	S: 22 / 25 / – / –	China	>50% CHB	T: resection, snap frozen; S: fasted	no / no	S: LC-MS & CE-MS; T: LC-MS	State Key Science &Technology Project for Infectious Diseases (grant nos. 2012ZX10002-009 and2008ZX10002-019), the key foundation (grant no. 21175132), and the creativeresearch group project (grant nos. 81221061 and 21021004) from the NationalNatural Science Foundation of China.
Lu, et al. (2016)	[66]	T: 50 / 50; S: 24 / – / – / 24	S: 18 / 20 / – / 20	China	>50% CHB	T: resection; S: fasted	yes / yes	LC-MS & GC-MS	Partially supported by the grant of Natural ScienceFund of The Science and Technology Commission of Shanghai, China(no. 12ZR1404300), Singapore Medical Research Council (grant no.NMRC/1242/2009), the NUS secondment Funds (C.N. Ong), and the NUSEnvironmental Research Institute (NERI).
Lu, et al. (2018)	[68]	T: 50 / 50; S: 50 / – / – / 24	S: 18 / 20 / – / 20	China	>50% CHB	T: resection; S: fasted	yes / yes	LC-MS	Natural Science Fund of The Science and Technology Commission of Shanghai, China, Science and Technology Commission of Shanghai Municipality, NUS secondment Funds and NUS Environmental Research Institute.
Skill, et al. (2011)	[84]	T: 6 / 6; S: 6 / – / – / 4	–	USA	N.S.	T: resection; S: N.S.	no / no	TLC & ELISA	N.S.
III. BLOOD SERUM / PLASMA									
Ahaneku, et al. (1992)	[23]	15 / 12 / – / 20	–	Nigeria	N.S.	overnight fasted	N.S.	Biochemical methods	N.S.
Assi, et al. (2015)	[24]	114* / – / – / 222	–	10 Western Europe countries	N.S.	fasted	yes / yes	NMR	The French National Cancer Institute and the Université de Lyon.
Assi, et al. (2018)	[25]	147* / – / – / 147	–	10 Western Europe countries	Mixed	N.S.	yes / yes	LC-MS	NA was financially supported by the Universit√© Claude Bernard Lyon I through a doctoral fellowship awarded by the EDISS (Ecole Doctorale InterDisci-plinaire Sciences Sant√©) doctoral school to complete her PhD work. NA also holds a grant from the Fondation de France (FdF) supporting her postdoc-toral research (grant number: 00069254). The data on the EPIC-Hepatobiliary data set was generated through support from the French National Cancer In-stitute (L‚Äôô Institut National du Cancer; INCA) (grant number 2009‚Äì139; PI:MJ). The work undertaken by DCT reported in this publication was supported by the National Institutes of Health under award number P01 CA196559. RT is a co-principal investigator of the EPIC-Oxford cohort whose work is supported by Cancer Research UK under grant number C8221/A19170.

Banales, et al. (2018)	[26]	20 / – / – / 20	14 / – / – / 15	Spain & Poland	>50% CHC	N.S.	no / no	LC-MS	Supported by CIBERehd (EHD15PI05), AECC Scientific Foundation (2017/2020), ‚ÄúFondo de Investigaciones Sanitarias‚Äù (Carlos III Health Institute: PI15/01132 and PI18/01075 to J.M.B., PI16/00598 to J.J.G.M., PI16/01845 to B.S., PI16/01126 to M.A.A., PI16/00090 to J.M.), Spanish Ministry of Economy, Industry and Competitiveness (SAF2016-75197-R to R.I.R.M., SAF2017-87301-R to M.L.M.-C.), Miguel Servet Programme (CON14/00129 to J.M.B.), ‚ÄúDiputaci√≥n Foral de Gipuzkoa‚Äù (DFG15/010, DFG16/004 to J.M.B.), ‚ÄúBasque Foundation for Innovation and Health Research: EiTB Maratoia‚Äù (BIO15/CA/016/BD to J.M.B., BIO15/CA/016/BD to M.L.M.-C.), Basque Country Department of Health (2013111173 to L.B., 2017111010 to J.M.B., 2013111114 to M.L.M.-C.), the Andalusian Government (‚ÄúConsejer√≠a de Econom√≠a, Innovaci√≥n, Ciencia y Empleo‚Äù: CTS-6264 and ‚ÄúConsejer√≠a de Salud ‚Äù: PI-0198-2016 to J.M.), Junta de Castilla y Le√≥n, Spain (SA063P17 to J.J.G.M.), European Comission Horizon 2020 project (SEP-210503876; ESCALON to J.M.B.), and Proyecto Hepacare, Fundaci√≥n La Caixa (to M.A.A.).
Baniasadi, et al. (2013)	[27]	30 / 22 / – / –	–	USA	100% CHC	overnight fasted	N.S.	LC-MS	Supported by the NIH (1R21CA133770), the Oncological Sciences Center in Discovery Park andthe Purdue University Center for Cancer Research.
Bowers, et al. (2014)	[29]	37 / 21 / – / –	–	USA	100% CHC	fasted	N.S.	LC-MS	Support for this work by the NIH (1R21CA133770) as well as additional support from the Purdue University Center for Cancer Research and the Oncological Sciences Center in Discovery Park.
Butler, et al. (2013)	[31]	297* / – / 39b / 592	–	China	>50% CHB	non-fasted	yes / yes	LC-MS	Supported by the United States National Cancer Instituteat the NIH (grant numbers R01 CA43092 and R01 CA144034).
Chen, et al. (2011)	[32]	41 / – / – / 38	–	China	N.S.		N.S. / no	LC-MS	Supported by The National Basic Research Program (2007CB512905), National Natural Science Fund (30571664), and The State S&T Projects of 11th Five Year(2008ZX10002-007).
Chen, et al. (2013)	[33]	29 / – / – / 30	–	China	N.S.	N.S.	N.S. / yes	LC-MS	Supported by the national basic researchprogram of China (Grant 2012CB518303), the State KeyScience & Technology Project for Infectious Diseases (Grants2012ZX10002011 and 2012ZX10002009), the foundation(Grant 21175132) and the creative research group project(Grant 21021004) from the National Natural ScienceFoundation of China.
Chen, et al. (2013)	[34]	30 / 30 / 30b / 30	–	China	100% CHB	overnight fasted	N.S.	LC-MS	Supported by the State Key Science &Technology Project for Infectious Diseases (2012ZX10002-011)and the foundation (No. 21175132) and the creative researchgroup project (No. 21021004) from National Natural ScienceFoundation of China.
Chen, et al. (2016)	[36]	24 / – / – / 60	–	China	100% CHB	overnight fasted	N.S.	NMR	Supported by the National Natural Science Foundation of China (81272581), Science Research Foundation of Ministry of Health & United Fujian Provincial Health and Education Project for Tackling the Key Research (WKJ-FJ-05), the Fundamental Research Funds for Xiamen University (201412G012), the Project of Natural Science Foundation of Fujian Province (2015J01541), and the Project funding for the training of young talents in the health system of Fujian Province (2014-ZQN-JC-42).
Di Poto, et al. (2017)	[39]	63 / 65 / – / –	–	USA	Mixed		yes / yes	GC-MS	Supported by U01CA185188 (awarded to H.W. Ressom).
Di Poto, et al. (2018)	[40]	30 / 39 / – / –	–	USA	>50% CHC	N.S.	N.S.	GC-MS	The National Cancer Institute of the National Institutes of Health

Fages, et al. (2015)	[42]	114* / – / – / 222	–	10 Western Europe countries	Mixed		yes / yes	NMR	This work was supported by the French National Cancer Institute (L‚ÄôInstitutNational du Cancer; INCA; grant number 2009-139; PI: M. Jenab). AF receivedfinancial support (BDI fellowship) from the CentreNational de la Recherche Scientifique (CNRS) and Bruker Biospin. Thecoordination of EPIC is financially supported by the European Commission(DG-SANCO) and the International Agency for Research on Cancer. Thenational cohorts are supported by Danish Cancer Society (Denmark); LigueContre le Cancer, Institut Gustave Roussy, Mutuelle G√©n√©rale de l‚ÄôEducationNationale, and Institut National de la Sant√© et de la Recherche M√©dicale(INSERM) (France); Deutsche Krebshilfe, Deutsches Krebsforschungszentrum(DKFZ), and Federal Ministry of Education and Research (Germany); HellenicHealth Foundation (Greece); Italian Association for Research on Cancer(AIRC), National Research Council, Associazione Italiana per la Ricerca sulCancro-AIRC-Italy, and AIRE-ONLUS Ragusa, AVIS Ragusa, Sicilian Government(Italy); Dutch Ministry of Public Health, Welfare and Sports (VWS), NetherlandsCancer Registry (NKR), LK Research Funds, Dutch Prevention Funds, DutchZON (Zorg Onderzoek Nederland), World Cancer Research Fund (WCRF), andStatistics Netherlands (the Netherlands); European Research Council (ERC;grant number ERC-2009-AdG 232997) and Nordforsk, and Nordic Center ofExcellence Programme on Food, Nutrition and Health (Norway); HealthResearch Fund (FIS), Regional Governments of Andaluc√≠a, Asturias, BasqueCountry, Murcia (No. 6236) and Navarra, and ISCIII RETIC (RD06/0020) (Spain);Swedish Cancer Society, Swedish Scientific Council, and Regional Governmentof Sk√•ne and V√§sterbotten (Sweden); Cancer Research UK, Medical ResearchCouncil, Stroke Association, British Heart Foundation, Department of Health,Food Standards Agency, and Wellcome Trust (UK).
Fitian, et al. (2014)	[43]	30 / 27 / – / 30	–	USA	100% CHC	overnight fasted	yes / yes	LC-MS & GC-MS	supported by the NIH KL2 University of Florida Clinical Translational Science Scholar Award (R.C.), NIH/NCRR award UL1RR029890 (D.R.N., R.C.) and NIH/NCI award K24CA139570 (D.R.N).
Gao, et al. (2009)	[44]	39 / 36 / – / 63	–	China	N.S.		N.S.	NMR	Supported by ChinaPostdoctoral Science Foundation (20070410188), Knowledge Innova-tion Program of the Chinese Academy of Sciences (SIMM0709QN-07),and National Natural Science Foundation of China (20705037 and30470351).
Gao, et al. (2015)	[45]	26 / 34 / 32b / 40	10 / 18 / 16b / 21	China	100% CHB	overnight fasted	yes / yes	GC-MS	The Ministry of Science and Technology of China and the National Natural Science Foundation of China.
Gong, et al. (2017)	[46]	51 / 49 / – / 39	–	China	100% CHB	overnight fasted	N.S.	LC-MS & GC-MS	The Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences and the Key Lab of Training, Monitoring and Intervention of Aquatic Sports of General Administration of Sport of China, Jiangxi Normal University.
Grammatikos, et al. (2016)	[47]	122 / 127 / – / –	–	Germany	Mixed	N.S.	yes / yes	LC-MS	Supported by the German Research Foundation DFG (FOG784, PF361/7
Jee, et al. (2018)	[50]	75* / – / – / 134	–	Korea	Mixed	>12 hour fasted	yes / yes	LC-MS	Funded through grants from the Korean Health Technology R&D Project, Ministry of Health & Welfare, Republic of Korea (HI14C2686010115 and HI14C2686), and the Bio-Synergy Research Project (NRF-2012M3A9C4048762) of the Ministry of Science, ICT and Future Planning through the National Research Foundation of Korea, Republic of Korea.
Kawasaki, et al. (1988)	[51]	5 / 11 / 6h / 12	–	Japan	N.S.	overnight fasted	N.S.	LC	Supported in part by a grant for scientific research (60570321) from the Ministry of Education, Science and Culture, Japan.

Krautbauer, et al. (2017)	[53]	21 / 41 / – / 22	–	Germany	N.S.	N.S.	no / no	DI-MS	This study was supported by the Stiftung f√ºr Pathobiochemie und Molekulare Diagnostik and partly by the German Research Foundation (BU 1141/13-1).
Li, et al. (2013)	[55]	20 / – / – / 20	–	China	N.S.	N.S.	N.S.	LC-MS	Supported by grants of the State Key Science & Technology Project for Infectious Diseases (2012ZX10002011, 2012ZX10002009), the foundation and creative research group project (No. 21175132 and No. 21021004) from the NSFC.
Li, et al. (2017)	[56]	80 / – / – / 20	–	China	N.S.	fasted	N.S.	LC-MS	N.S.
Lin, et al. (2011)	[59]	28 / 28 / 26b / 30	–	China	N.S.	N.S.	N.S.	GC-MS	Supported by the State Key Science &Technology Project for Infectious Diseases (2008ZX10002-019,2008ZX10002-017) from State Ministry of Science & Technol-ogy of China, and the foundation (No. 20835006) fromNational Natural Science Foundation of China.
Lin, et al. (2014)	[60]	30 / 30 / 30b / 30	–	China	N.S.		N.S.	LC-MS	Supported by the State Key Science & Technology Project for Infectious Diseases (2012ZX10002011), the Sino-German Center for Research Promotion (GZ753), National Natural Science Foundation of China (21375011).
Liu, et al. (2014)	[61]	43 / 42 / – / 18	28 / 26 / – / 8	China	>50% CHB		N.S.	LC-MS & NMR	Grant sponsor: National Natural Science Foundation of China; Grant number:81273472; Grant sponsor: Science and Technology Commission of Shanghai, China; Grant number:12401900802
Liu, et al. (2017)	[63]	66 / – / – / 82	–	China	Mixed	N.S.	no / no	GC-MS	Supported by the National Nature Science Foundation of China (No. 81000968; No. 81101540; No. 81101637; No. 81172273; No. 81272388; No. 81301820; No. 81472673), Doctoral Fund of Ministry of Education of China (20120071110058), and The National Clinical Key Special Subject of China.
Lu, et al. (2015)	[64]	47 / – / 48b / 48	220 / – / – / 224	China	>50% CHB	>12 hour fasted	no / no	LC-MS	Supported by grant from Open Project of State Key Laboratory ofUrban Water Resource and Environment of Harbin Institute ofTechnology (No. ES201115).
Lu, et al. (2015)	[67]	36 / – / – / 19	10 / – / – / 5	China	100% CHB	overnight fasted	yes / yes	LC-MS & GC-MS	Supported in part by the grant of Natural Science Fund of The Science and Technology Commission of Shanghai, China (No. 12ZR1404300), Singapore Medical Research Council (No. NMRC/1242/2009), the NUS secondment Funds to CNO and the NUS Environmental Research Institute (NERI).
Luo, et al. (2017)	[69]	361 / 167 / – / 191	155 / 143 / 150b / 99	China	N.S.	overnight fasted	yes / yes	LC-MS	Supported by the National Key Research and Development Program of China (2017YFC0906900), the projects (No. 21375127) and key project (No. 21435006) from the National Natural Science Foundation of China and the National Grand Project (2012ZX10002,Äê011) of Science and Technology of China.
Muir, et al. (2013)	[71]	15 / 15 / – / –	–	Korea	N.S.		N.S.	GC-MS	The National Institutes of Health.
Nahon, et al. (2012)	[72]	22 / 62 / – / –	11 / 31 / – / –	France	Alcohol-related	overnight fasted	no / no	NMR	Paris 13 University.
Nezami Ranjbar, et al. (2015)	[73]	40 / 49 / – / –	–	Egypt	100% CHC	N.S.	yes / yes	GC-MS	The National Institutes of Health.
Passos-Castilho, et al. (2015)	[75]	25 / 15 / 25c / –	–	Brazil	100% CHC	N.S.	N.S.	MALDI-MS	Funda√ß√£o de Amparo √† Pesquisa do Estado de S√£o Paulo ,Äì FAPESP (2013/03701-0)
Passos-Castilho, et al. (2015)	[76]	32 / 30 / 25b / 34	–	Brazil	100% CHB	N.S.	yes / yes	LC-MS	The Fleury SA Group supported this work and AMPC received a doctorate scholarship from Funda√ß√£o de Amparo √† Pesquisa do Estado de S√£o Paulo,ÄìFAPESP (no. 2013/03701-0)
Patterson, et al. (2011)	[77]	20 / 7 / – / 6	–	Switzerland	Mixed	N.S.	no / no	LC-MS & GC-MS	The NIH National Cancer Institute Intramural Research Program, and Bernerische undSchweizerische Krebsliga, Sasella Foundation, and the Hassan Badawi Founda-tion Against Liver Cancer.
Ressom, et al. (2012)	[78]	78 / 184 / – / –	10 / 10 / – / –	USA	Mixed	N.S.	N.S.	LC-MS	The National Institutes of Health.

Shariff, et al. (2017)	[83]	53 / 26 / – / 19	–	Nigeria & Egypt	Mixed	fasted blood	yes / yes	NMR	Supported by project grants from the Associations of Physicians of Great Britain and Ireland. MIFS and NGL were supported by personal grants from the Royal College of Physicians of London, the University of London and the Trustees of the London Clinic, London, UK. MMEC is supported by a Fellowship from the Sir Halley Stewart Trust (Cambridge, UnitedKingdom). MMEC and SDT-R hold grants from the United Kingdom Medical Research Council. AIG was supported by a doctorate grant from the Egyptian Ministry of Higher Education.
Soga, et al. (2011)	[85]	19 / 10 / 24c / 53	13 / 8 / 11c / 4	Japan	100% CHC	N.S.	N.S.	LC-MS & CE-MS	Supported by Health and Labour Sciences Research Grants ‚ÄúResearch on Biological Markers for New Drug Development‚Äù (T.S.) and ‚ÄúResearch on Risk of Chemical Substances‚Äù (T.S.). Additional support was obtained through grants from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) for a Global COE Program entitled ‚ÄúHuman Metabolomic Systems Biology‚Äù in Life Sciences (T.S., M.T. and M.S.) and the ERATO Gas Biology Project (M.S.), as well as research funds from the Yamagata Prefectural Government and City of Tsuruoka.
Stepien, et al. (2016)	[87]	102* / – / – / 183	–	10 Western Europe countries	Mixed	N.S.	yes / yes	LC-MS	Grant sponsor:French National Cancer Institute (L‚ÄôInstitut National du Cancer; INCA);Grant number:2009‚Äì139;Grant sponsor:European Research Council (ERC);Grant number:ERC-2009-AdG 232997;Grant sponsor:Regional Governments of Andaluc ƒ±a, Asturias,Basque Country, Murcia;Grant number:6236;Grant sponsor:ISCIII RETIC;Grant number:RD06/0020;Grant sponsors:EuropeanCommission (DG-SANCO); International Agency for Research on Cancer; Danish Cancer Society (Denmark); Ligue Contre le Cancer;Institut Gustave Roussy; Mutuelle G en erale de l‚ÄôEducation Nationale; and Institut National de la Sant e et de la Recherche M edicale(INSERM) (France); Deutsche Krebshilfe, Deutsches Krebsforschungszentrum (DKFZ); and Federal Ministry of Education and Research(Germany); Stavros Niarchos Foundation; Hellenic Health Foundation; and Ministry of Health and Social Solidarity (Greece); ItalianAssociation for Research on Cancer (AIRC); National Research Council; and AIRE-ONLUS Ragusa, AVIS Ragusa, Sicilian Government(Italy); Dutch Ministry of Public Health, Welfare and Sports (VWS); Netherlands Cancer Registry (NKR); LK Research Funds; DutchPrevention Funds; Dutch ZON (Zorg Onderzoek Nederland); World Cancer Research Fund (WCRF); and Statistics Netherlands (theNetherlands); Nordforsk; and Nordic Center of Excellence Programme on Food, Nutrition and Health (Norway); Health Research Fund(FIS); Navarra; Catalan Institute of Oncology. (Spain); Swedish Cancer Society; Swedish Scientific Council; and Regional Government ofSk√•ne and V,Ç¨asterbotten (Sweden); Cancer Research UK; Medical Research Council; Stroke Association; British Heart Foundation;Department of Health; Food Standards Agency; and Wellcome Trust (UK)
Tan, et al. (2012)	[88]	262 / 76 / 74b / –	–	China	100% CHB	N.S.	yes / yes	LC-MS	Supported by the State Key Science andTechnology Project for Infectious Diseases (2008ZX10002-017,2008ZX10002-019) and China International Science and TechnologyCooperation Program (2009DFA41250) from State Ministry of Scienceand Technology of China, and the key foundation (No. 20835006) andthe creative research group project (No.30921006, 21021004) fromNational Natural Science Foundation of China.

Wang, et al. (2012)	[91]	23 / 28 / – / 70	59 / 20 / – / 20	China	100% CHB	overnight fasted	N.S.	LC-MS	Supported by the Natural Science Foundation ofChina (30901190, 81121002), the Major National S&T Projectfor Infectious Disease (2012ZX10002-007), the NationalProgram on Key Basic Research Project (2009CB522406 and2007CB513003), and the Health Bureau of Zhejiang ProvinceFoundation (2008QN010).
Wei, et al. (2012)	[93]	40 / – / 22c / –	–	USA	100% CHC	N.S.	N.S.	NMR	Funding from the National Cancer Institute, (1R21CA133770) and the Purdue Research Foundation
Xiao, et al. (2012)	[95]	40 / 49 / – / –	–	Egypt	100% CHC	N.S.	yes / yes	LC-MS	Supported by the National Institutes of Health(NIH) Grant R21CA153176 awarded to H.W.R. The UPLC-QTOF MS data presented in the manuscript were generatedthrough the Proteomics and Metabolomics Shared Resource atthe Lombardi Comprehensive Cancer Center, supported byNIH/NCI grant P30-CA051008.
Xiao, et al. (2014)	[96]	96 / 103 / – / –	–	Egypt	100% CHC	N.S.	yes / yes	LC-MS	upported by NCI grant R21CA153176 (to H.W.Ressom). The quantitation data were generated at Georgetown LombardiProteomics and Metabolomics Shared Resource supported by NIH/NCIgrant P30-CA051008.
Xue, et al. (2008)	[97]	20 / – / – / 20	–	China	N.S.	pre-therapeutic treatment	yes / N.S.	GC-MS	Financially supported by The National HighTechnology Research and Development Program of China863 Project (No. 2006AA02Z4C5) and the National NaturalScience Foundation of China (Nos. 30600739 & 30772505).
Yin, et al. (2009)	[99]	24 / 25 / – / 25	–	China	N.S.	N.S.	yes / yes	LC-MS	Supported by the National Key Projectof Scientific and Technical Supporting Programs(2006038079037) and the National Grand Scientific andTechnological Project of China (2008ZX10002-019 and2008ZX10002-017) and the foundation (No. 20675082) fromNational Natural Science Foundation of China.
Zeng, et al. (2014)	[100]	22 / 25 / – / 30	50 / 25 / – / 31	China	>50% CHB	N.S.	yes / yes	CE-MS	Supported by the State Key Science &Technology Project for Infectious Diseases (2012ZX10002-011,2012ZX10002-009); the foundation (no. 21175132); and thecreative research group project (no. 21321064) from NationalNatural Science Foundation of China, Program of ShanghaiMunicipal Commision of Health (XBR2013090).
Zeng, et al. (2015)	[101]	22 / 25 / – / –	50 / 25 / – / –	China	>50% CHB	fasted	N.S.	CE-MS	Supported by the State Key Science & Technology Project for Infectious Diseases (2012ZX10002-011) and the foundations (No.21205114, No. 21375011, No. 81172727 & No. 81301472) from National Natural Science Foundation of China.
Zhang, et al. (2015)	[102]	11 / – / 22b / –	–	China	100% CHB	N.S.	N.S.	LC-MS	Supported by the State Key Science & Technology Project for Infectious Diseases (2012ZX10002011), National Natural Science Foundation of China (21375011), and the Sino-German Center for Research Promotion (GZ 753).
Zhang, et al. (2018)	[103]	75 / 20 / – / 20	–	China	100% CHB	overnight fasted	N.S.	LC-MS	Supported by the General Project of Application Infrastructure and Cutting-Edge Technology Research Programs, Tianjin (grant no. 13JCYBJC22100), and the Program Project of Health and Family Planning Commission Technology Fund, Tianjin (grant no. 2014KY01).
Zhou, et al. (2012)	[104]	69 / 28 / – / 31	–	China	>50% CHB	fasted	N.S. / no	LC-MS	Supported by the State Key Science &Technology Project for Infectious Diseases (2012ZX10002011)and the Key Foundation (No.s 20835006, 21175132) and theCreative Research Group Project (No. 21021004) fromNational Natural Science Foundation of China.
Zhou, et al. (2012)	[105]	30 / 30 / 30b / 30	–	China	N.S.	fasted	yes / yes	LC-MS	Supported by the State Key Science and Technology Project for Infectious Diseases (2008ZX10002- 017 and 2008ZX10002-019) from State Ministry of Science and Technology of China, and the key foundation (no. 20835006) and the creative research group project (no. 21021004) from National Natural Science Foundation of China.

IV. BLOOD SERUM / PLASMA & URINE

Chen, et al. (2011)	[35]	82 / – / 24a / 71	–	China	>50% CHB	overnight fasted	N.S.	S: LC-MS & GC-MS; U: LC-MS	National Basic Research Program of China, the National Science and Technology Major Project, the Natural ScienceFoundation of Shanghai and the National ScienceFoundation of China.
V. URINE									
Cox, et al. (2016)	[37]	42 / 47 / 46b / 7	–	Bangladesh	>50% CHB	morning, mid-stream	N.S.	NMR	United Kingdom NIHR Biomedical Facility at Imperial College London for infrastructure support.
Ladep, et al. (2014)	[54]	63 / 32 / 107n / 88	141 / 56 / 178n / 88	Nigeria	>50% CHB	non-fasted	N.S.	NMR	The European Union Framework 7, the Trustees of the London Clinic (London, UK), the British Medical Research Council and the Halley Stewart Foundation (Cambridge, United Kingdom).
Liang, et al. (2016)	[58]	25 / – / – / 12	15 / – / – / 10	China	N.S.	N.S.	yes / yes	LC-MS	The Key Program of Natural Science Foundation of State.
Osman, et al. (2017)	[74]	55 / 40 / – / 45	–	Egypt	N.S.	morning	yes / yes	GC-MS	Clinical Biochemistry Department, National Liver Institute, Menoufia University.
Shao, et al. (2015)	[79]	33 / 27 / – / 26	33 / 21 / – / –	China	N.S.	fasted	yes / yes	LC-MS	The State Key Science &Technology Project for Infectious Diseases and the National Natural Science Foundation of China.
Shariff, et al. (2010)	[80]	18 / 10 / – / 14	–	Nigeria	>50% CHB	random	yes / yes	NMR	NIHR Biomedical ResearchFacility provided infrastructure support.
Shariff, et al. (2011)	[81]	16 / 14 / – / 17	–	Egypt	100% CHC	random	no / no	NMR	The AlanMorement Memorial Fund from the Imperial College London Healthcare Trustees (London, U.K.), and the Broad Medical Research Program, CA. M.I.F.S. was supported by a scholarshipfrom the London Clinic, London, U.K., A.I.G. was supported by ascholarship from the Egyptian Government, the Higher Education Funding Council for England and the British Liver Trust and a charitable donation from Mr. and Mrs. Barry Winter.
Shariff, et al. (2016)	[82]	13 / 25 / – / –	–	UK	Mixed	random, non-fasted	yes / yes	NMR	The Association of Physicians of Great Britain and Ireland, the Royal College of Physicians of London, the University of London, the Trustees of the London Clinic (London, UK) and the Sir Halley Stewart Trust (Cambridge, UK).
Wu, et al. (2009)	[94]	20 / – / – / 20	–	China	N.S.	morning, whole	yes / N.S.	GC-MS	The National Basic Research Program of China, Ministry of Health, China National Key Projects for Infectious Diseases and National Nature Science Foundation of China.

[#]Number of participants in each study group (HCC / cirrhosis / non-cirrhotic liver disease / healthy control) with the type of non-cirrhotic liver disease indicated by a: benign tumour, b: chronic hepatitic B, c: chronic hepatitis C, h: chronic active hepatitis, and n: non-cirrhotic liver disease.
*Prospective (samples were collected before the diagnosis of HCC)
CE: capillary electrophoresis; DI: direct injection; GC: gas chromatography; LC: liquid chromatography; MALDI: matrix-assisted laser desorption/ionisation; MAS: magic angle spin; MS: mass spectrometry; NMR: nuclear magnetic resonance; N. S.: not stated.

Appendix A Table 4. Top 30 metabolites reported to be discriminatory in tumour compared to non-tumour tissue according to the final weighted score and the intermediate steps.

			Overall ranking					Tumour tissue vs matched non-tumour tissue							
Compound name	HMDB identifier	LMSD identifier	Rank: Number of reports	Rank: Vote count	Rank: log2(Fold change)	Rank: log2(Fold change), RoB weighted	Rank: Final weighted score	Reports of upregulation*	Reports of downregulation*	Number of reports	Discordance penalty[a]	Vote count[b]	log_2(Fold change)[c]	log_2(Fold change), RoB weighted[d]	Final weighted score[e]
Glycerol 3-phosphate	HMDB0000126		6	4	1	1	1		[28],[30],[49],[89],[48]	5	1.00	-5	-7.41	-56.67	-56.67
Malic acid	HMDB0000744		1	1	2	2	2		[28],[30],[49],[68],[89],[48]	6	1.00	-6	-5.95	-48.92	-48.92
Niacinamide	HMDB0001406		1	1	4	3	3		[30],[49],[49],[68],[89],[48]	6	1.00	-6	-5.28	-42.92	-42.92
Glycerophosphocholine	HMDB0000086	LMGP01010000	1	1	3	4	4		[30],[49],[62],[68],[89],[48]	6	1.00	-6	-5.51	-42.57	-42.57
O-Phosphoethanolamine	HMDB0000224		6	4	7	5	5	[30],[49],[68],[89],[90]		5	1.00	5	4.93	39.72	39.72
Xanthosine	HMDB0000299		23	17	5	6	6		[30],[49],[48]	3	1.00	-3	-5.25	-37.11	-37.11
3-Methylglutarylcarnitine	HMDB0000552		6	4	8	7	7		[30],[49],[68],[48],[65]	5	1.00	-5	-4.77	-35.89	-35.89
Uric acid	HMDB0000289		11	8	10	8	8		[30],[49],[68],[89]	4	1.00	-4	-4.36	-35.37	-35.37
LPC(18:2)	HMDB0061700		6	4	6	9	9		[30],[49],[49],[62],[52]	5	1.00	-5	-5.24	-33.95	-33.95
Fumaric acid	HMDB0000134		11	8	12	10	10		[30],[49],[68],[89]	4	1.00	-4	-4.06	-33.13	-33.13
5'-Methylthioadenosine	HMDB0001173		23	17	9	11	11	[30],[49],[48]		3	1.00	3	4.75	32.48	32.48
Glycocholic acid	HMDB0000138	LMST05030001	11	8	11	12	12		[30],[49],[68],[48]	4	1.00	-4	-4.29	-29.26	-29.26
Glycochenodeoxycholic acid		LMST05030008	11	8	14	13	13		[69],[30],[49],[62]	4	1.00	-4	-3.83	-29.10	-29.10
myo-Inositol	HMDB0000211		11	8	15	14	14		[28],[30],[68],[48]	4	1.00	-4	-3.54	-29.02	-29.02
LPC(16:1)			23	17	20	15	15	[68],[48],[52]		3	1.00	3	3.18	28.58	28.58
Glycodeoxycholic acid	HMDB0000631	LMST05030006	23	17	31	16	16		[69],[49],[68]	3	1.00	-3	-2.43	-25.14	-25.14
C3:0 carnitine; Propionylcarnitine	HMDB0000824	LMFA07070105	11	8	21	17	17		[30],[49],[68],[65]	4	1.00	-4	-3.15	-24.75	-24.75
L-Glutamine	HMDB0000641		11	8	19	18	18	[49],[90],[98],[98]		4	1.00	4	3.19	24.65	24.65
D-Glucose	HMDB0000122		23	17	17	20	19		[28],[30],[68]	3	1.00	-3	-3.46	-22.75	-22.75
C16:1	HMDB0003229		11	8	23	21	20	[30],[49],[48],[65]		4	1.00	4	2.86	21.35	21.35
C18:2n6,9; Linoleic acid	HMDB0000673	LMFA01030120	11	8	25	22	21		[28],[30],[68],[90]	4	1.00	-4	-2.71	-21.29	-21.29
C4-OH carnitine; Hydroxybutyrylcarnitine	HMDB0013127	LMFA07070071	23	17	31	23	22		[49],[68],[65]	3	1.00	-3	-2.43	-21.09	-21.09
C18:0 carnitine; Stearoylcarnitine	HMDB0000848	LMFA07070008	23	17	18	24	23	[30],[68],[65]		3	1.00	3	3.23	20.99	20.99
Uridine diphosphate-N-acetylglucosamine	HMDB0000290		121	95	44	25	24	[89]		1	1.00	1	2.14	19.27	19.27
Adenosine 3',5'-diphosphate	HMDB0000061		121	95	45	26	25		[89]	1	1.00	-1	-2.12	-19.08	-19.08
LPC(20:4)			23	17	31	27	26		[49],[62],[52]	3	1.00	-3	-2.43	-18.65	-18.65
LPC(22:6)			23	17	31	27	26		[49],[62],[52]	3	1.00	-3	-2.43	-18.65	-18.65
beta-Glycerophosphoric acid	HMDB0002520		23	17	24	30	28		[28],[30],[49]	3	1.00	-3	-2.77	-18.39	-18.39
Betaine	HMDB0000043		23	17	37	31	29		[30],[49],[89]	3	1.00	-3	-2.26	-18.34	-18.34
L-Carnitine	HMDB0000062		23	17	47	32	30		[49],[89],[65]	3	1.00	-3	-2.10	-18.05	-18.05
C16:0 carnitine; Palmitoylcarnitine	HMDB0000222	LMFA07070004	23	17	22	33	31	[30],[68],[48]		3	1.00	3	2.96	18.05	18.05
Xanthine	HMDB0000292		23	17	36	37	34		[30],[49],[48]	3	1.00	-3	-2.26	-17.72	-17.72
C5-DC carnitine; Glutarylcarnitine	HMDB0013130	LMFA07070066	23	17	26	39	36		[30],[49],[65]	3	1.00	-3	-2.65	-17.09	-17.09
Phosphorylcholine	HMDB0001565		23	17	28	40	37	[30],[49],[90]		3	1.00	3	2.57	16.84	16.84
PG(18:2(9Z,12Z)/18:2(9Z,12Z))		LMGP04010956	52	39	30	45	41		[57],[66]	2	1.00	-2	-2.50	-15.68	-15.68
S-Adenosylhomocysteine	HMDB0000939		23	17	35	46	42		[30],[49],[48]	3	1.00	-3	-2.28	-15.42	-15.42
PE(18:0/20:3)			121	95	13	47	43	[57]		1	1.00	1	3.86	15.42	15.42
L-Lactic acid	HMDB0000190		23	17	75	59	54	[30],[68],[90]		3	1.00	3	1.65	14.30	14.30
C4:0 carnitine; Butyrylcarnitine	HMDB0002013	LMFA07070054	121	95	16	61	56	[30]		1	1.00	1	3.50	14.00	14.00

Adenosine monophosphate	HMDB0000045		23	17	47	62	57		[30],[49],[48]	3	1.00	-3	-2.10	-14.00	-14.00
N-Acetylneuraminic acid	HMDB0000230		23	17	57	65	60		[30],[49],[89]	3	1.00	-3	-1.90	-13.64	-13.64
Uridine	HMDB0000296		23	17	94	73	68		[30],[89],[48]	3	1.00	-3	-1.60	-12.97	-12.97
LPE(18:1)		LMGP02050004	23	17	62	88	81	[30],[30],[48]		3	1.00	3	1.85	10.53	10.53
Ophthalmic acid	HMDB0005765		121	95	29	91	84	[30]		1	1.00	1	2.56	10.22	10.22
L-Tryptophan	HMDB0000929		11	39	101	29	90	[69],[49],[48]	[30]	4	0.50	2	1.53	18.43	9.22
L-Phenylalanine	HMDB0000159		1	39	61	19	141	[69],[49],[68],[90]	[30],[62]	6	0.33	2	1.86	23.73	7.91
C6:0 carnitine; Hexanoylcarnitine	HMDB0000705	LMFA07070070	11	39	444	75	203	[30]	[49],[68],[65]	4	0.50	-2	-0.37	-12.83	-6.42
gamma-Aminobutyric acid	HMDB0000112		23	95	27	41	278	[49]	[30],[89]	3	0.33	-1	-2.59	-16.46	-5.49
L-Threonine	HMDB0000167		23	95	117	57	294	[49],[68]	[30]	3	0.33	1	1.40	14.47	4.82
L-Leucine	HMDB0000687		23	95	148	83	311	[49],[90]	[30]	3	0.33	1	1.05	11.77	3.92
L-Valine	HMDB0000883		6	95	140	34	322	[49],[68],[90]	[30],[86]	5	0.20	1	1.10	17.93	3.59
LPC(18:0)			23	95	176	292	385	[68]	[49],[62]	3	0.33	-1	-0.81	-4.90	-1.63
PE	HMDB0060501	LMGP02010000	23	95	176	341	453	[98]	[22],[52]	3	0.33	-1	-0.81	-3.27	-1.09
Tiglylcarnitine	HMDB0002366	LMFA07070108	23	95	152	374	456	[68]	[30],[65]	3	0.33	-1	-1.00	-2.41	-0.80
Glycine	HMDB0000123		23	95	401	389	458	[68]	[30],[90]	3	0.33	-1	-0.70	-2.12	-0.71
Choline	HMDB0000097		1	459	470	396	459	[84],[86],[90]	[30],[89],[92]	6	0.00	0	0.00	-1.98	0.00
C2:0 carnitine; Acetyl-L-carnitine	HMDB0000201	LMFA07070050	11	459	476	318	459	[30],[49]	[68],[65]	4	0.00	0	0.00	-4.07	0.00

*Reference number in main text of publication reporting significant change

[a]A penalty for contradicting direction of change (See Methods for detail of calculation)

[b]Total vote (+1 given to report of upregulation in HCC, -1 for downregulation)

[c]Reported or estimated log2(fold change)

[d]Log_2(fold change) weighted by score from risk of bias assessment

[e]Weighted log_2(fold change) with discordance penalty applied

Appendix A Table 5. Top 30 metabolites reported to be discriminatory in blood (plasma or serum) according to the final weighted score and the intermediate steps.

			Overall ranking					HCC vs Cirrhosis								HCC vs non-cirrhotic liver disease								HCC vs Healthy							
Compound name	HMDB identifier	LMSD identifier	Rank: Number of reports	Rank: Vote count	Rank: log2(Fold change)	Rank: log2(Fold change), RoB weighted	Rank: Final weighted score	Reports of upregulation*	Reports of downregulation*	Number of reports	Discordance penalty[a]	Vote count[b]	log2(Fold change)[c]	log2(Fold change), RoB weighted[d]	Final weighted score[e]	Reports of upregulation*	Reports of downregulation*	Number of reports	Discordance penalty[a]	Vote count[b]	log2(Fold change)[c]	log2(Fold change), RoB weighted[d]	Final weighted score[e]	Reports of upregulation*	Reports of downregulation*	Number of reports	Discordance penalty[a]	Vote count[b]	log2(Fold change)[c]	log2(Fold change), RoB weighted[d]	Final weighted score[e]
Glycocholic acid	HMDB0000138	LMST05030001	2	21	1	1	1		[29], [69], [78], [96], [96], [48]	7	1.00	-7	-4.24	-42.40	-42.40			1	0	0	0	0	0	[35], [50], [64], [46], [69], [55], [105], [104], [26], [48], [99]		11	1.00	11	24.29	184.46	184.46
Taurocholic acid	HMDB0000036	LMST05040001	33	34	2	2	2		[69], [78]	2	1.00	-2	-1.84	-22.04	-22.04									[35], [64], [69], [99], [99]		5	1.00	5	15.27	127.54	127.54
Taurochenodeoxycholic acid	HMDB0000951	LMST05040005	26	78	3	3	3		[78], [95], [96]	3	1.00	-3	-1.51	-16.37	-16.37	[103]		1	1	1	0.67	6.69	6.69	[64], [69], [69], [99]		4	1.00	4	10.75	112.31	112.31
Gluconic acid	HMDB0000625		89	34	9	4	4	[45], [101]		2	1.00	2	1.16	8.61	8.61									[101]		1	1.00	1	6.40	70.40	70.40
LPC(18:0)		LMGP01050026	4	7	4	9	5	[78], [91], [95], [48]	[63]	5	0.60	3	2.27	26.72	16.03										[33], [33], [34], [63], [69], [77], [91], [104], [48], [99]	10	1.00	-10	-12.02	-89.66	-89.66
LPC(18:1)			12	2	5	6	6	[48]	[63], [77]	3	0.33	-1	-0.85	-6.65	-2.22										[33], [33], [50], [63], [69], [77], [104], [48]	8	1.00	-8	-8.68	-68.03	-68.03
Phenylalanyl-Tryptophan	HMDB0029006		89	34	21	10	7		[43], [69]	2	1.00	-2	-1.57	-19.13	-19.13										[69]	1	1.00	-1	-3.32	-39.86	-39.86
Hypoxanthine	HMDB0000157		26	78	43	12	8	[43], [69], [101]		3	1.00	3	4.25	49.39	49.39									[36], [101]		[85], [99], [99]	5	0.20	-1	-1.43	5.66	1.13
LPC(16:0)		LMGP01050018	2	3	10	16	9	[78], [91], [95], [48]	[63], [63]	7	0.29	2	1.79	23.36	6.68									[46]		[33], [33], [34], [50], [63], [63], [69], [77], [91], [104], [48]	12	0.83	-10	-8.63	-65.58	-54.65
L-Phenylalanine	HMDB0000159		1	1	6	8	10	[43], [46], [91]	[27], [63], [101]	7	0.00	0	0.28	2.20	0.00	[85]		1	1	1	0.67	5.35	5.35	[36], [33], [24], [50], [63], [46], [60], [42], [45], [104], [87]	[35], [91]	13	0.69	9	8.29	56.14	38.87
LPC(22:6)			19	11	16	15	11	[78]	[77]	2	0.00	0	0.00	2.59	0.00		[34]	2	0.5	-1	-0.66	-1.97	-0.98		[34], [50], [69], [104], [66]	5	1.00	-5	-4.68	-43.18	-43.18
C8:0 carnitine; O-octanoylcarnitine	HMDB0013324	LMFA07070095	53	16	19	17	12																		[33], [64], [64], [69], [105]	5	1.00	-5	-5.15	-41.08	-41.08
C10:0 carnitine; Decanoylcarnitine	HMDB0000651	LMFA07070059	26	3	12	18	13		[63], [96]	2	1.00	-2	-0.54	-3.78	-3.78										[33], [67], [63], [64], [69], [105]	6	1.00	-6	-5.33	-37.30	-37.30
LPC(20:5)			19	3	27	19	14		[69], [48]	2	1.00	-2	-0.70	-7.63	-7.63			1	0	0	0	0	0	[50], [69], [77], [105], [66], [48]	6	1.00	-6	-3.78	-32.88	-32.88
Dihydrocholesterol		LMST01010077	261	177	35	20	15		[69]	1	1.00	-1	-3.32	-39.86	-39.86																
C18:2n6,9; Linoleic acid	HMDB0000673	LMFA01030120	33	11	17	14	16	[63], [43], [39]		3	1.00	3	2.61	27.78	27.78									[67], [63], [46]		4	0.75	3	2.62	15.69	11.77
LPC(18:3)			44	11	22	21	17																		[50], [69], [77], [66], [99], [99]	6	1.00	-6	-4.87	-38.44	-38.44
LPC(14:0)		LMGP01050012	17	3	14	22	18		[77]	1	1.00	-1	-0.51	-3.60	-3.60			2	0	0	0	0	0		[33], [105], [105], [67], [50], [69], [77]	7	1.00	-7	-4.85	-33.51	-33.51
Biliverdin	HMDB0001008		136	78	29	24	19	[77]		1	1.00	1	0.52	3.10	3.10									[104]		1	1.00	1	3.55	31.95	31.95
Chenodeoxycholic acid	HMDB0000518	LMST04010032	44	78	7	7	20		[48], [48]	2	1.00	-2	-2.47	-23.42	-23.42	[103]		1	1	1	0.67	6.69	6.69	[48]	[64], [48]	3	0.33	-1	-7.23	-53.56	-17.85
3-Hydroxybutyric acid	HMDB0000357		44	11	25	26	21	[63], [45], [101]		3	1.00	3	1.31	7.67	7.67									[36], [63], [101]		3	1.00	3	3.38	26.72	26.72

Bilirubin	HMDB0000054		53	34	18	27	22	[77]	[63]	2	0.00	0	0.00	-0.51	0.00									[63], [104], [99]		3	1.00	3	5.19	33.65	33.65
Trimethylamine N-oxide	HMDB0000925		136	78	38	28	23		[101]	1	1.00	-1	-1.64	-18.08	-18.08										[101]	1	1.00	-1	-1.29	-14.15	-14.15
Hippuric acid	HMDB0000714		136	78	47	29	24																		[69], [101]	2	1.00	-2	-2.79	-31.64	-31.64
Glycoursodeoxycholic acid	HMDB0000708	LMST05030016	136	78	48	31	25																	[50], [69]		2	1.00	2	2.76	30.50	30.50
C18:2(9E,12E) carnitine; Linoelaidyl carnitine		LMFA07070078	136	78	54	32	26		[95], [96]	2	1.00	-2	-2.58	-30.19	-30.19																
15S-HETE	HMDB0003876	LMFA03060001	89	34	41	36	27	[43], [46]		2	1.00	2	2.00	22.92	22.92									[46]		1	1.00	1	0.87	6.10	6.10
myo-Inositol	HMDB0000211		53	177	81	101	28	[69]		1	1.00	1	2.38	28.54	28.54									[24], [24]	[67], [69]	4	0.00	0	-0.32	-13.31	0.00
2-Hydroxybutyric acid	HMDB0000008		53	16	51	37	29	[43], [101], [48]		3	1.00	3	1.28	14.51	14.51									[101], [48]		2	1.00	2	1.33	13.61	13.61
3-(Pyrazol-1-yl)-L-alanine			136	78	70	38	30																		[69]	1	1.00	-1	-2.32	-27.86	-27.86
Fatty amide C20:1			261	177	70	38	30		[69]	1	1.00	-1	-1.32	-15.86	-15.86										[69]	1	1.00	-1	-1.00	-12.00	-12.00
L-Lactic acid	HMDB0000190		33	34	52	35	32	[101]	[45], [73]	3	0.33	-1	-0.64	2.03	0.68									[36], [35], [83], [101]		4	1.00	4	3.23	27.07	27.07
LPE(18:2)			75	34	46	30	33		[69]	2	0.50	-1	-0.51	-6.17	-3.09										[69], [104]	2	1.00	-2	-2.29	-24.61	-24.61
LPC(20:3)			44	16	32	42	36		[77]	1	1.00	-1	-0.51	-3.09	-3.09			1	0	0	0	0	0		[50], [69], [77], [105]	4	1.00	-4	-2.95	-24.32	-24.32
C16:1(9Z) carnitine; O-palmitoleoylcarnitine	HMDB0013207	LMFA07070097	75	21	31	44	38									[105]		1	1	1	0.67	4.68	4.68	[67], [64], [105]		3	1.00	3	3.19	22.31	22.31
L-Glutamic acid	HMDB0000148		6	11	20	13	39	[43], [72], [45], [45], [73], [101]	[39]	7	0.71	5	3.83	30.84	22.03		[85]	1	1	-1	-0.66	-5.24	-5.24	[42], [45], [101], [87]	[60], [25]	6	0.33	2	1.76	28.42	9.47
C10:1 carnitine; Decenoylcarnitine			53	21	33	46	40			1	0.00	0	0.00	0.00	0.00										[33], [64], [69], [105]	4	1.00	-4	-3.43	-26.21	-26.21
C16:1	HMDB0003229		53	34	28	48	42											2	0	0	0	0	0	[46], [60], [105]		3	1.00	3	4.25	24.67	24.67
LPC(18:2)		LMGP0105	8	7	13	33	43	[102]	[63]	3	0.00	0	0.00	1.04	0.00									[69]	[33], [34], [33], [50], [63], [77], [104], [99]	9	0.78	-7	-5.67	-31.12	-24.21
LPC(20:4)		LMGP01050121	19	7	30	64	46	[29]	[77], [96]	3	0.33	-1	0.45	4.15	1.38										[33], [34], [33], [50], [77], [104]	6	1.00	-6	-4.33	-24.22	-24.22
Glycodeoxycholic acid	HMDB0000631	LMST05030006	17	21	34	53	47		[78], [95], [96], [96], [96], [96], [96]	7	1.00	-7	-3.05	-31.29	-31.29									[46], [77], [104]		3	1.00	3	6.40	53.24	53.24
Phenylalanyl-Serine	HMDB0029004		89	34	73	56	50		[43], [48]	2	1.00	-2	-1.57	-15.45	-15.45										[48]	1	1.00	-1	-0.69	-6.21	-6.21
Betaine	HMDB0000043		44	177	15	11	51		[101], [49]	3	0.67	-2	-4.16	-42.79	-28.53	[85]	[49]	2	0	0	-2.06	-21.92	0	[85]		1	1.00	1	0.87	6.97	6.97
L-Tryptophan	HMDB0000929		19	16	24	25	52		[101]	2	0.50	-1	-0.94	-10.38	-5.19			1	0	0	0	0	0	[69]	[35], [33], [101], [105], [104]	6	0.67	-4	-3.78	-24.41	-16.27
C18:1; Oleic acid	HMDB0000207	LMFA01030002	53	21	23	57	53	[46]		1	1.00	1	0.52	3.10	3.10			1	0	0	0	0	0	[67], [46], [99]		3	1.00	3	4.35	18.29	18.29
Citric acid	HMDB0000094		44	21	64	59	55	[46], [101]	[73]	3	0.33	1	-0.22	-0.05	-0.02									[46], [42], [45]		3	1.00	3	2.62	20.92	20.92
L-Tyrosine	HMDB0000158		4	7	40	34	61	[46], [101]	[27], [63]	4	0.00	0	-0.13	-1.13	0.00									[36], [33], [24], [50], [46], [42], [45], [85], [87]	[63], [60]	11	0.64	7	3.05	31.08	19.78
L-Glutamine	HMDB0000641		33	34	135	49	64	[45]	[72], [101]	3	0.33	-1	-0.36	-8.06	-2.69	[85]		1	1	1	0.67	5.35	5.35		[42], [101], [87]	3	1.00	-3	-1.73	-21.19	-21.19
Glycochenodeoxycholic acid		LMST05030008	7	546	11	45	67	[91]	[69], [96], [96], [96], [96]	6	0.67	-4	-1.33	-14.97	-9.98			1	0	0	0	0	0	[35], [64], [55], [105], [99]	[91]	6	0.67	4	7.30	41.95	27.96
Isobutyric acid	HMDB0001873		75	21	49	79	72	[63], [101]		2	1.00	2	1.01	9.06	9.06									[36], [63]		2	1.00	2	1.74	7.85	7.85
L-Arginine	HMDB0000517		53	34	101	65	89		[69], [101]	2	1.00	-2	-1.15	-13.41	-13.41									[46]	[67], [101]	3	0.33	-1	-0.60	-6.56	-2.19
LPC(16:1)			75	21	57	98	90																		[50], [69], [99], [99]	4	1.00	-4	-2.50	-15.30	-15.30
C20:4; Arachidonic acid	HMDB0001043	LMFA01030001	8	34	8	5	93	[43], [46]		3	0.67	2	0.81	7.49	5.00			2	0	0	0	0	0	[50], [46], [105], [104]	[35], [60], [45]	7	0.14	1	7.50	70.35	10.05
C18:2 carnitine			53	21	58	106	98			1	0.00	0	0.00	0.00	0.00	[105]		1	1	1	0.67	4.68	4.68	[33], [105], [104]		3	1.00	3	1.81	9.35	9.35
6-Methylnicotinic acid			261	177	26	108	100																		[99]	1	1.00	-1	-4.64	-13.93	-13.93
1-Methyladenosine	HMDB0003331		75	21	185	113	103	[27], [43], [48]		3	1.00	3	1.02	10.59	10.59									[48]		1	1.00	1	0.33	3.00	3.00
Phytosphingosine	HMDB0004610	LMSP01030001	75	21	36	115	105		[63]	1	1.00	-1	-0.51	-4.12	-4.12										[63], [99], [99]	3	1.00	-3	-2.71	-9.11	-9.11
Succinic acid	HMDB0000254		53	21	37	95	107	[46]		1	1.00	1	0.52	3.61	3.61									[36], [46], [45]		4	0.75	3	2.62	12.21	9.15
L-Carnitine	HMDB0000062		12	16	68	94	108	[63], [46], [105], [48]		4	1.00	4	1.77	13.39	13.39		[49]	1	1	-1	-0.25	-2.22	-2.22	[35], [46], [48], [99]	[63], [105]	6	0.33	2	0.81	4.67	1.56

L-Methionine	HMDB0000696		8	78	227	348	116	[27], [101], [49]	3	1.00	-3	-3.07	-30.36	-30.36	[31], [85]	[49]	3	0.33	1	-0.37	-3.33	-1.11	[33], [46], [60], [85], [87]	[101]	6	0.67	4	4.43	28.49	19.00
Malic acid	HMDB0000744		44	21	39	68	118	[46], [45]	2	1.00	2	1.03	4.64	4.64									[46], [45], [101]	[67]	4	0.50	2	1.89	14.93	7.47
L-Valine	HMDB0000883		12	177	50	103	133	[46], [39], [73]	[63]	5	0.40	2	2.56	22.74	9.10	[93]		1	1	1	1.53	7.65	7.65	[46]	[33], [42], [87]	5	0.40	-2	-1.34	-15.40	-6.16
L-Kynurenine	HMDB0000684		53	34	124	77	157	[69], [101]	2	1.00	2	0.37	4.38	4.38	[85]		1	1	1	0.67	5.35	5.35	[87]	[35]	2	0.00	0	0.49	7.45	0.00
L-Aspartic acid	HMDB0000191		19	546	544	265	162	[43], [101]	2	1.00	2	0.91	10.98	10.98			1	0	0	0	0	0	[46], [60]	[35], [24], [24], [85]	6	0.33	-2	-0.70	-3.87	-1.29
Cholesterol	HMDB0000067	LMST01010001	26	34	72	117	163	[23], [45], [73]	4	0.75	3	2.13	12.86	9.65									[23], [24]	[67], [61]	4	0.00	0	0.18	0.17	0.00
C18:1(9Z) carnitine; O-oleoylcarnitine	HMDB0005065	LMFA07070096	26	34	121	470	168		[95], [96]	3	0.67	-2	-3.09	-36.60	-24.40	[105]		1	1	1	0.67	4.68	4.68	[33], [64], [105], [104]		4	1.00	4	4.00	29.02	29.02
D-Glucose	HMDB0000122		12	34	113	543	406		[46], [45]	2	1.00	-2	-1.03	-4.12	-4.12								[24], [24], [42], [61]	[24], [24], [24], [67], [46]	9	0.11	-1	-0.67	2.64	0.29
C2:0 carnitine; Acetyl-L-carnitine	HMDB0000201	LMFA07070050	12	34	95	262	430	[63], [43], [48]	[68]	4	0.50	2	0.72	6.34	3.17								[63], [55], [48], [99]	[67], [105], [68]	7	0.14	1	1.22	1.02	0.15
L-Leucine	HMDB0000687		8	177	78	251	438	[46], [73]	[45]	4	0.25	1	1.60	12.75	3.19								[36], [50], [46], [60]	[35], [33], [42], [87]	8	0.00	0	0.57	-4.93	0.00
Taurine	HMDB0000251		26	78	85	139	496	[69]		1	1.00	1	0.38	4.54	4.54								[35], [64]	[69], [85], [87], [26], [99]	7	0.43	-3	-2.42	-15.39	-6.60
L-Proline	HMDB0000162		19	177	299	490	527	[46]	[101], [49]	3	0.33	-1	-0.10	-2.19	-0.73		[49]	1	1	-1	-1.01	-9.08	-9.08	[24], [67], [46], [85]	[60]	5	0.60	3	1.89	13.80	8.28
Inosine	HMDB0000195		89	177	42	23	533		[101]	1	1.00	-1	-0.12	-1.32	-1.32								[35]	[99]	2	0.00	0	2.95	37.96	0.00
D-Galactose	HMDB0000143		19	177	523	380	539		[46]	1	1.00	-1	-0.51	-3.60	-3.60	[64]		3	0.33	1	0.67	6.02	2.01	[64], [61]	[67], [46], [60]	5	0.20	-1	0.13	1.91	0.38
Glycerol	HMDB0000131		26	177	566	585	571	[63], [45]	[27]	3	0.33	1	0.71	1.94	0.65								[63]	[36], [35], [45]	5	0.40	-2	-0.84	-2.31	-0.92

[a]Reference number in main text of publication reporting significant change
[b]A penalty for contradicting direction of change (See Methods for detail of calculation)
[c]Total vote (+1 given to report of upregulation in HCC, -1 for downregulation)
[d]Reported or estimated log2(fold change)
[e]Log_2(fold change) weighted by score from risk of bias assessment
[f]Weighted log_2(fold change) with discordance penalty applied

Appendix A Table 6. Top 30 metabolites reported to be discriminatory in urine according to the final weighted score and the intermediate steps.

			Overall ranking					HCC vs Cirrhosis								HCC vs non-cirrhotic liver disease								HCC vs Healthy							
Compound name	HMDB identifier	LMSD identifier	Rank: Number of reports	Rank: Vote count	Rank: log2(Fold change)	Rank: log2(Fold change), RoB weighted	Rank: Final weighted score	Reports of upregulation*	Reports of downregulation*	Number of reports	Discordance penalty[a]	Vote count[b]	log2(Fold change)[c]	log2(Fold change), RoB weighted[d]	Final weighted score[e]	Reports of upregulation*	Reports of downregulation*	Number of reports	Discordance penalty[a]	Vote count[b]	log2(Fold change)[c]	log2(Fold change), RoB weighted[d]	Final weighted score[e]	Reports of upregulation*	Reports of downregulation*	Number of reports	Discordance penalty[a]	Vote count[b]	log2(Fold change)[c]	log2(Fold change), RoB weighted[d]	Final weighted score[e]
Creatinine	HMDB0000562		2	2	2	1	1	[37], [54], [81]		3	1	-3	-3.10	-19.61	-19.61	[37], [54]		2	1	-2	-2.06	-13.41	-13.41		[37], [54]	2	1.00	-2	-2.06	-13.41	-13.41
Hippuric acid	HMDB0000714		1	1	1	2	2	[54], [74]		2	1	-2	-2.06	-8.25	-8.25	[37], [54]		2	1	-2	-2.06	-13.41	-13.41		[37], [54], [80], [74]	5	0.80	-4	-4.13	-22.70	-18.16
Trimethylamine N-oxide	HMDB0000925		3	3	3	4	3	[54]		1	1	-1	-1.03	-4.13	-4.13	[37], [54]		2	1	-2	-2.06	-13.41	-13.41		[54], [80], [35]	3	1.00	-3	-2.95	-16.40	-16.40
Aminocaproic acid	HMDB0001901		45	37	5	5	4																		[35]	1	1.00	-1	-3.47	-27.79	-27.79
Glycocholic acid	HMDB0000138	LMST05030001	28	37	4	3	5																	[35]		2	0.50	1	5.49	43.93	21.97
L-Carnitine	HMDB0000062		6	4	11	6	6	[37], [54]		2	1	2	1.23	7.98	7.98	[37], [54]		2	1	2	1.23	7.98	7.98	[54]		1	1.00	1	0.61	2.45	2.45
Nicotinic acid	HMDB0001488		45	37	19	7	7																		[35]	1	1.00	-1	-2.06	-16.47	-16.47
L-Threonine	HMDB0000167		7	5	12	8	8	[74]		1	1	1	0.61	2.45	2.45									[94], [74], [35]		3	1.00	3	2.05	12.73	12.73
L-Xylote	HMDB0060256		11	7	6	9	9		[74]	1	1	-1	-1.03	-4.13	-4.13										[94], [74]	2	1.00	-2	-2.06	-10.32	-10.32
Phosphate	HMDB0001429		11	7	6	9	9		[74]	1	1	-1	-1.03	-4.13	-4.13										[94], [74]	2	1.00	-2	-2.06	-10.32	-10.32
Urea	HMDB0000294		11	7	6	9	9		[74]	1	1	-1	-1.03	-4.13	-4.13										[94], [74]	2	1.00	-2	-2.06	-10.32	-10.32
D-Xylitol	HMDB0002917		11	7	6	12	12		[74]	1	1	-1	-1.03	-4.13	-4.13										[94], [74]	2	1.00	-2	-2.06	-9.29	-9.29
Serotonin	HMDB0000259		11	7	6	13	13		[54]	1	1	-1	-1.03	-4.13	-4.13		[54]	1	1	-1	-1.03	-4.13	-4.13		[54]	1	1.00	-1	-1.03	-4.13	-4.13
L-Cysteine	HMDB0000574		45	37	33	14	14																		[35]	1	1.00	-1	-1.51	-12.12	-12.12
Cysteic acid	HMDB0002757		45	37	36	15	15																		[35]	1	1.00	-1	-1.32	-10.58	-10.58
N-Acetylneuraminic acid	HMDB0000230		45	37	38	16	16																	[35]		1	1.00	1	1.28	10.25	10.25
Succinic acid	HMDB0000254		28	22	31	17	17																		[94], [35]	2	1.00	-2	-1.70	-9.83	-9.83
Adenine	HMDB0000034		45	37	47	18	18																		[35]	1	1.00	-1	-1.18	-9.48	-9.48
Normetanephrine	HMDB0000819		45	37	47	18	18																		[35]	1	1.00	-1	-1.18	-9.48	-9.48
Glycerol	HMDB0000131		28	22	13	20	20		[74]	1	1	-1	-1.03	-4.13	-4.13										[74]	1	1.00	-1	-1.03	-5.16	-5.16
L-Arabinose	HMDB0000646		28	22	13	20	20		[74]	1	1	-1	-1.03	-4.13	-4.13										[74]	1	1.00	-1	-1.03	-5.16	-5.16
Adenosine	HMDB0000050		28	22	37	22	22	[79]		1	1	1	0.61	3.07	3.07									[35]		1	1.00	1	0.68	5.42	5.42
5-Hydroxyindoleacetic acid	HMDB0000763		28	22	13	23	23									[54]		1	1	-1	-1.03	-4.13	-4.13		[54]	1	1.00	-1	-1.03	-4.13	-4.13
Alpha-Hydroxyhippuric acid	HMDB0002404		28	22	13	23	23									[54]		1	1	-1	-1.03	-4.13	-4.13		[54]	1	1.00	-1	-1.03	-4.13	-4.13
Phenyl acetate	HMDB0040733		28	22	13	23	23									[54]		1	1	-1	-1.03	-4.13	-4.13		[54]	1	1.00	-1	-1.03	-4.13	-4.13
Pyrimidine	HMDB0003361		28	22	13	23	23		[74]	1	1	-1	-1.03	-4.13	-4.13										[74]	1	1.00	-1	-1.03	-4.13	-4.13
1, 3-Dimethyluric acid	HMDB0001857		11	7	21	27	27	[54], [79]		2	1	2	1.23	5.52	5.52	[54]		1	1	1	0.61	2.45	2.45								
Pyridoxal	HMDB0001545		45	37	62	28	28																	[35]		1	1.00	1	0.99	7.94	7.94
Pyroglutamic acid	HMDB0000267		45	37	63	29	29																		[35]	1	1.00	-1	-0.94	-7.55	-7.55
C2:0 carnitine; Acetyl-L-carnitine	HMDB0000201	LMFA07070050	11	7	21	30	30	[54]		1	1	1	0.61	2.45	2.45	[54]		1	1	1	0.61	2.45	2.45	[54]		1	1.00	1	0.61	2.45	2.45
Choline	HMDB0000097		11	7	21	30	30	[54]		1	1	1	0.61	2.45	2.45	[54]		1	1	1	0.61	2.45	2.45	[54]		1	1.00	1	0.61	2.45	2.45
D-Galactose	HMDB0000143		11	7	21	30	30	[54]		1	1	1	0.61	2.45	2.45	[54]		1	1	1	0.61	2.45	2.45	[54]		1	1.00	1	0.61	2.45	2.45
Dimethylglycine	HMDB0000092		11	7	21	30	30	[54]		1	1	1	0.61	2.45	2.45	[54]		1	1	1	0.61	2.45	2.45	[54]		1	1.00	1	0.61	2.45	2.45
Guanosine triphosphate	HMDB0001273		11	7	21	30	30	[54]		1	1	1	0.61	2.45	2.45	[54]		1	1	1	0.61	2.45	2.45	[54]		1	1.00	1	0.61	2.45	2.45
Indoleacetic acid	HMDB0000197		11	7	21	30	30	[54]		1	1	1	0.61	2.45	2.45	[54]		1	1	1	0.61	2.45	2.45	[54]		1	1.00	1	0.61	2.45	2.45
N-acetylated amino acid			11	7	21	30	30	[54]		1	1	1	0.61	2.45	2.45	[54]		1	1	1	0.61	2.45	2.45	[54]		1	1.00	1	0.61	2.45	2.45
N6, N6, N6-Trimethyl-L-lysine	HMDB0001325		11	7	21	30	30	[54]		1	1	1	0.61	2.45	2.45	[54]		1	1	1	0.61	2.45	2.45	[54]		1	1.00	1	0.61	2.45	2.45
Creatine	HMDB0000064		3	5	20	70	38	[54], [80]		2	1	2	1.23	5.52	5.52	[54]		1	1	1	0.61	2.45	2.45	[54], [80]	[35]	3	0.33	1	0.11	-4.05	-1.35
Oxoglutaric acid	HMDB0000208		7	7	21	30	40	[54]		1	1	1	0.61	2.45	2.45	[54]		1	1	1	0.61	2.45	2.45	[54]		2	0.50	1	0.61	2.45	1.23
Pyruvic acid	HMDB0000243		11	37	34	41	41	[54]		1	1	1	0.61	2.45	2.45		[54]	1	1	-1	-1.03	-4.13	-4.13		[54]	1	1.00	-1	-1.03	-4.13	-4.13
Dopamine	HMDB0000073		45	37	65	43	42																	[35]		1	1.00	1	0.71	5.71	5.71
L-Serine	HMDB0000187		28	22	40	44	43	[74]		1	1	1	0.61	2.45	2.45									[74]		1	1.00	1	0.61	3.07	3.07
Uric acid	HMDB0000289		45	37	66	45	44																	[35]		1	1.00	1	0.69	5.50	5.50
Hypoxanthine	HMDB0000157		11	37	32	39	45		[79]	1	1	-1	-1.03	-5.16	-5.16									[35]	[94]	2	0.00	0	-0.53	-1.11	0.00
1-Methylnicotimide	HMDB0000699		28	22	40	54	54								[54]			1	1	1	0.61	2.45	2.45	[54]		1	1.00	1	0.61	2.45	2.45
3-Hydroxyphenylacetic acid	HMDB0000440		28	22	40	54	54								[54]			1	1	1	0.61	2.45	2.45	[54]		1	1.00	1	0.61	2.45	2.45
Acetic acid	HMDB0000042		28	22	40	54	54	[54]		1	1	1	0.61	2.45	2.45	[54]		1	1	1	0.61	2.45	2.45								
D-Glucose	HMDB0000122		28	22	40	54	54								[54]			1	1	1	0.61	2.45	2.45	[54]		1	1.00	1	0.61	2.45	2.45
Dimethylamine	HMDB0000087		28	22	40	54	54								[54]			1	1	1	0.61	2.45	2.45	[54]		1	1.00	1	0.61	2.45	2.45
L-Methionine	HMDB0000696		28	22	40	54	54	[54]		1	1	1	0.61	2.45	2.45	[54]		1	1	1	0.61	2.45	2.45								
Agmatine	HMDB0001432		45	37	105	61	60																	[35]		1	1.00	1	0.58	4.68	4.68
Taurine	HMDB0000251		45	37	107	64	63																	[35]		1	1.00	1	0.52	4.13	4.13
Dihydrouracil	HMDB0000076		45	37	108	68	67																	[35]		1	1.00	1	0.50	3.97	3.97
L-Alanine	HMDB0000161		45	37	109	69	68																		[35]	1	1.00	-1	-0.49	-3.95	-3.95
Glycine	HMDB0000123		3	114	39	60	69	[74]	[54]	2	0	0	-0.42	-1.67	0.00		[54]	1	1	-1	-1.03	-4.13	-4.13	[94], [74]	[80]	3	0.33	1	0.20	0.98	0.33

Phenylacetylglutamine	HMDB0006344	7	37	34	41	71	[54]		1	1	1	0.61	2.45	2.45	[54]	1	1	-1	-1.03	-4.13	-4.13	[54]	2	0.50	-1	-1.03	-4.13	-2.06
N-Acetyl-L-aspartic acid	HMDB0000812	45	37	110	73	72															[35]		1	1.00	1	0.45	3.63	3.63
DL-O-Phosphoserine	HMDB0001721	45	37	111	74	73															[35]		1	1.00	1	0.44	3.55	3.55
L-Cystine	HMDB0000192	45	37	112	75	74															[35]		1	1.00	1	0.40	3.20	3.20
L-Proline	HMDB0000162	11	37	116	115	76	[74]	[79]	2	0	0	-0.42	-2.71	0.00							[74]		1	1.00	1	0.61	3.07	3.07
L-Phenylalanine	HMDB0000159	45	37	114	93	93															[35]		1	1.00	1	0.33	2.67	2.67
Citric acid	HMDB0000094	7	114	64	71	115	[74]		1	1	1	0.61	2.45	2.45	[54]	1	1	-1	-1.03	-4.13	-4.13	[74]	[80]	2	0.00	0	-0.42	-2.09	0.00
Xanthine	HMDB0000292	28	114	113	116	116		[79]	1	1	-1	-1.03	-5.16	-5.16							[35]		1	1.00	1	0.69	5.50	5.50

[a]Reference number in main text of publication reporting significant change

[b]A penalty for contradicting direction of change (See Methods for detail of calculation)

[c]Total vote (+1 given to report of upregulation in HCC, -1 for downregulation)

[d]Reported or estimated log2(fold change)

[e]Log_2(fold change) weighted by score from risk of bias assessment

[f]Weighted log_2(fold change) with discordance penalty applied

Appendix B – Publications and presentations

Publications

1. **U MRA** and Taylor-Robinson SD. Comments on Gabbani, et al. Metabolomic analysis with H-1 NMR for non-invasive diagnosis of hepatic fibrosis degree in patients with chronic hepatitis C. *Digestive and Liver Disease.* 2018;50(2): 209-210.
2. **U MRA**, Shen Y-LE, Alkhatib A, Cartlidge C, Holmes E and Taylor-Robinson SD. *Candidate biomarkers for the diagnosis of hepatocellular carcinoma from metabonomic studies: a systematic review.* Available from: http://www.crd.york.ac.uk/PROSPERO/display_record.php?ID=CRD42018095412. [Accessed 06/08/2019].
3. Vorkas PA, **U MRA** and Li JV. Tissue multiplatform-based metabolomics/metabonomics for enhanced metabolome coverage. In: Theodoridis G., Gika H., Wilson I. (eds) *Metabolic Profiling. Methods in Molecular* Biology, vol 1738. Humana Press, New York, NY; 2018. p. 239-260.
4. Oleribe OO, Salako BL, Akpalu A, Anteyi E, Ka MM, Deen G, et al. (including **U MRA**) Public private partnership in in-service training of physicians: the millennium development goal 6-partnership for African clinical training (M-PACT) approach. *Pan African Medical Journal.* 2018;29:77.
5. Cartlidge CR, **U MRA**, Alkhatib A and Taylor-Robinson, SD. The utility of biomarkers in hepatocellular carcinoma: review of urine-based ^{1}H-NMR studies–what the clinician needs to know. *International Journal of General Medicine*. 2017;10:431-442.

Conference presentations

1. **The Liver Meeting, AASLD, San Francisco, CA, USA, 11/2018**
   [Oral presentation] Elucidating serum and urinary hepatocellular carcinoma diagnostic biomarker panels: insight from the United Kingdom and West Africa
2. **Annual Meeting of West African College of Physicians, Freetown, Sierra Leone, 11/2018**
   [Oral presentation] Large-scale discovery and validation of blood and urinary discriminant metabolites as potential biomarkers for the diagnosis of hepatocellular carcinoma in West Africa
3. **Global Health Forum: Cancer Technologies, Institute of Global Health Innovation, London, UK, 10/2018**
   [Invited speech] Novel diagnostic biomarkers for hepatocellular carcinoma using metabolic profiling: challenges and breakthroughs
4. **Annual Meeting of British Association for the Study of the Liver York, UK, 09/2018**
   [Poster presentation] Elucidating serum and urinary hepatocellular carcinoma diagnostic biomarker panels: insight from the United Kingdom and West Africa
5. **Conference on Liver Disease in Africa Nairobi, Kenya, 09/2018**
   [Oral & poster presentation] Large-scale discovery and validation of blood and urinary discriminant metabolites as potential biomarkers for the diagnosis of hepatocellular carcinoma in West Africa

6. **Personalized Medicine, Gordon Research Conference Hong Kong SAR, China, 07/2018**
   [Poster presentation] Elucidating serum and urinary hepatocellular carcinoma diagnostic biomarker panels: insight from the United Kingdom and West Africa
7. **The International Liver Congress, EASL Paris, France, 04/2018**
   [Poster presentation] Towards elucidating a universal panel of diagnostic biomarkers for early hepatocellular carcinoma

Appendix C – Research as art: A dream of metabonomics

Artwork made as part of Graduate School's Summer Showcase 2018

Our body operates an extremely intricate network of metabolites – a diverse array of molecules that perform functions ranging from the storage and release of energy, the construction of components of our cells, to the communication between cells. To maintain a healthy functioning body, components of the network need to be in fine balance. Subtle disruption may signify the beginning of disease processes.

This is what we study in the field of metabonomics – how this network of metabolites changes in health and disease. Through this artwork, I want to take the viewer on a journey of our research, with a hint of my specific project.

Starting from the human figure, it may represent our study participants who are willing to offer us a biological sample of theirs for a glimpse into their metabolism. The samples we collect, typically blood or urine, contain a complex mixture of metabolites, a snapshot of the metabolism.

Going clockwise from the figure, we see delicate metabolic profiles – fingerprints of the metabolite mixtures obtained from analysing the samples by sensitive chemical techniques.

With these fingerprints, we can deduce the levels of different metabolites present in the samples for us to construct a picture of the metabolic network, represented in the two-sided painting. On one side we have the full network – each blob is a metabolite and each line is the conversion from one to another. The question we ask is: is there abnormality in levels of certain metabolites in the disease group compared to the healthy group?

Using statistics, the answer is revealed on the other side, certain metabolites are raised, some are reduced. This is extremely useful as it gives us insight into the disturbance of metabolism relating to the disease state. More importantly, these metabolites could be used as indicators for us to detect a disease or to monitor its progression. For example, for my project, I am trying to find any such disruptions in patients with hepatocellular carcinoma (HCC), the main primary liver cancer.

This leads us to the model, the final stage of the cycle. Using information from data analysis, we can build a model that uses the levels of the indicator metabolites to predict the disease state of new samples. By repeating the cycle again and again, we can validate our model, which ultimately allows us to develop useful tools. For my case, it would be a new test that is better than existing ones for us to detect HCC tumours earlier, increasing the chance of a cure.

This takes us back to the human figure. This is what all this effort is about. At the end of the day, we want to bring the results of the work back to the study participants – a new diagnostic tool, an insightful biological understanding that can inform treatment strategies, etc.

Or perhaps the human figure is the researcher, like me, who is the driving force of this cycle? Anyhow, it is a cycle of metabonomics, more than that, a dream of humanity.