PRIMARY LIVER CANCER:

EPIDEMIOLOGICAL AND BIOMARKER DISCOVERY STUDIES

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December 2013

Thesis submitted for Doctor of Philosophy

THESIS ABSTRACT

With previous reports indicating changes in mortality, risk factors and management of primary liver cancer (PLC), evaluation of current trends in the incidence and mortality rates was indicated. Late diagnosis has been implicated to be a major contributor to the high fatality rates of PLC. This work aimed at:

- studying trends of PLC by subcategories globally in general, and in England and Wales, in particular;
- investigating liver-related morbidities of HIV infected patients in an African setting; and
- discovering urinary biomarkers of hepatocellular carcinoma.

The World Health Organisation (WHO) and Small Area Health Statistics Unit (SAHSU) databases were interrogated respectively, in order to achieve the first aim. The second aim was achieved through utilisation of databases of an African-based HIV treatment programme- AIDS Prevention Initiative in Nigeria (APIN), located in Jos, Nigeria. The European Union-funded Prevention of Liver Fibrosis and Cancer in Africa (PROLIFICA) case-control study in three West African countries was the platform through which urinary metabolic profiling was accomplished. Proton nuclear magnetic resonance spectroscopy (NMR) and parallel ultra-performance liquid chromatography mass spectrometry (UPLC-MS) were used for biomarker discovery studies.

Mortality rates of intrahepatic bile duct carcinoma (IHBD) increased in all countries that were studied. Misclassification of hilar cholangiocarcinoma accounted for only a small increase in the rate of IHBD in England and Wales. With over 90% screening rate for viral hepatitides, the rates of hepatitis B (HBV), hepatitis C (HCV) and

HBV/HCV in HIV-infected patients in the APIN programme were 17.8%, 11.3% and 2.5% respectively. There was attenuated immune response as well as significantly lower survival observed in HBV/HIV co-infection, relative to HIV mono-infected patients (p=0.0097). Whereas single urinary metabolites, including acetylcarnitine, N-acetylglutamate, betaine aldehyde, 3'-sialyllactose, methionine among others possessed high discriminatory power to diagnose HCC, a combination of three metabolites: 3'-sialyllactose, methionine and 9-decenoylcarnitine significantly outperformed serum alpha-fetoprotein (AFP) in the diagnosis of HCC in a cirrhosis population (area under the receiver operating characteristic curve; [urinary panel= 0.96] compared to [AFP = 0.64]).

This work informs a critical assessment of current control strategies in the prevention of HCC, and potentially assists in the development of more affordable means of early detection of PLC for most affected regions of the world.

DEDICATION

To: Joyce, Jipon, Julbyen and Jembyen

DECLARATION OF ORIGINALITY

I declare that the work presented in this thesis is my own and all else is appropriately referenced.

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London, UK

December 2013.

ACKNOWLEDGEMENT

I would not have envisaged that my first trip to England in 2006, sponsored by the Royal College of Physicians International office, under the leadership of Professor Roger Williams would lead to significant research collaborations that ensued. That brilliant idea, aimed at exposing young physicians from developing countries to state-of-the-art conferences and interventional endoscopies led to meeting up with Professor Simon D. Taylor-Robinson. Simon is a knowledgeable man, keen to guide and not afraid to develop minds from the scratch. He was able to provide me all the support any PhD student would require in order to achieve research goals.

Together with Dr Andrew Thillainayagam, Simon was able to source for and obtained a grant from The London Clinic that funded my work for the first 3 years of research. This effort will not easily be forgotten, especially during a time when funding for research is difficult to obtain. Both of them helped me to remain focussed and aim high on several occasions.

Drs Shahid Khan and Mireille Toledano, two of my other primary supervisors painstakingly mentored my epidemiological skills. Dr Toledano ensured I attended Epidemiological and Biostatistics taught courses for 6 months, link up with other students in her department and gave me access to the primary liver cancer data being maintained by the Small Area Health Statistics Unit. Dr Khan took time out of his normal working hours to advise, read through and correct my epidemiological work. Through the advice of my supervisors, I was able to access primary liver cancer database from the World Health Organisation that formed the global primary liver cancer mortality chapter of this work.

Professor Phyllis Kanki of Harvard School of Public Health, Boston (MA, USA), the principal sponsor of AIDS Prevention Initiative in Nigeria deserves no less accolade from me. I had collaborated with Professor Kanki since when I worked in Nigeria. Her willingness and support led to my being able to access the HIV database of the programme that formed some chapters of this work. She has a profound mind and mentored two manuscripts that were published in peer reviewed journals. She is devoted to system developments and successfully established state-of-the-art laboratory in developing countries; one of which holds the samples that were used in the laboratory-based aspect of the work presented in this report.

Perhaps, without the grant from the European Union FP-7 bid that ensured the sponsoring of liver cancer work in Gambia, Nigeria and Senegal, my research dream could have ended prematurely. I am grateful to my collaborators from institutions in the UK, France, Nigeria, Gambia and Senegal. Professor Mark Thursz, my Head of Section and principal investigator of PROLIFICA (Prevention of Liver Fibrosis and Carcinoma in Africa) has provided more than anticipated support to me and ensured that I maintained a keen research focus during the process of the current work. Other contributors to PROLIFICA and metabolic profiling include: Professors Tumani Corrah, Elaine Holmes, Edmund Banwat, Edith Okeke, Soulaymane Mboup, Mourtala Ka and Drs Maud Lemoine, Mohamed Shariff, Jeremy Cobbold, Matthew Lewis, Mark McPhail, Anisha Wijeyesekera, Debbie Garside and Ms Mary Crossey. I am a summary of their collective contributions to intellectual development.

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LIST OF ABBREVIATIONS

APC	Annual percent change
AFB	Aflatoxin B
AFP	Alpha fetoprotein
APIN	AIDS Prevention Initiative in Nigeria
ART	Antiretroviral therapy
ASIR	Age-standardised incidence rate
ASMR	Age-standardised mortality rate
ASpIR	Age-specific incidence rate
ASpMR	Age-specific mortality rate
AUC	Area under the receiver operating characteristic curves
BCLC	Barcelona Clinic Liver Cancer
BMI	Body mass index
CA	Carbohydrate antigen
CEA	Carcinoembryonic antigen
CC	Cholangiocarcinoma
Cir	Cirrhosis
CEUS	Contrast-enhanced ultrasound
CT	Computerised tomography
COX	Cytochrome c oxidase
DNA	Deoxyribonucleic acid
ERCP	Endoscopic retrograde cholangiopancreatography
EHBD	Extra-hepatic bile duct
EHCC	Extra-hepatic cholangiocarcinoma
EASL	European Association for the Study of the Liver
FNA	Fine needle aspiration
FBP	Fructose 1,6-biphosphate
GC	Gas chromatography
GLUT 1	Glucose transporter isoform 1
GHIS	Gambia Hepatitis Intervention Study
HBeAg	Hepatitis B e antigen
HBsAg	Hepatitis B surface antigen
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HDV	Hepatitis D virus
HH	Hereditary haemochromatosis
NC	Healthy volunteers
HAART	Highly active antiretroviral therapy

HILIC	hydrophilic interaction chromatography
ICD	International Classification of Diseases, Injuries and causes of Deaths
ICD (-O)	International Classification of Diseases (for Oncology)
IHBD	Intrahepatic bile duct tumours
IHCC	Intrahepatic cholangiocarcinoma
IRB	Institutional review board
IARC	International Agency for Research on Cancer
ICD-O	International Classification of Diseases for Oncology
IHBD	Intrahepatic bile duct carcinoma
IHCC	Intrahepatic cholangiocarcinoma
IL	Interleukin
JUTH	Jos University Teaching Hospital
LFT	Liver function test
LC	Liquid chromatography
NOS	Not otherwise specified
MRA	Magnetic resonance angiography
MRCP	Magnetic resonance cholangiopancreatography
MRI	Magnetic resonance imaging
MRS	Magnetic resonance spectroscopy
MS	Mass spectrometry
MIR	Mortality to incidence ratio
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NK	Natural killer
NCI	National Cancer Institute
DC	Non-cirrhotic liver disease
NMR	Nuclear magnetic resonance spectroscopy
NOS	Unspecified tumours
ONS	Office for National Statistics
OCP	Oral contraceptive pills
OPLS-DA	Orthogonal partial least squares discriminant analysis
PLC	Primary liver cancer
PLT	Primary liver tumours
PSC	Primary sclerosing cholangitis

PLS-DA	Partial least squares discriminant analysis
PTC	Percutaneous transhepatic cholangiography
PET	Positron emission tomography
PEPFAR	President's Emergency Plan for AIDS Relief
PROLIFICA	Prevention of Liver Fibrosis and Cancer in Africa
PCA	Principal component analysis
RNA	Ribonucleic acid
SAHSU	Small Area Health Statistics Unit
STOCSY	Statistical total correlation spectroscopy
SEER	Surveillance, Epidemiology and End Results
TOF	Time of flight
TIGAR	TP53-induced glycolysis and apoptosis regulator
ULN	Upper limit of normal
UK	United Kingdom
US	Ultrasound
USA	United States of America
WHO	World Health Organisation
WGO	World Gastroenterology Organisation

1. INTRODUCTION

1.1 Definition

Primary liver cancers (PLC) are malignant tumours of the liver, the major types of which are: hepatocellular carcinoma (HCC), arising from hepatocytes; and intrahepatic cholangiocarcinoma (IHCC), arising from the biliary epithelium and which is the most common form of intrahepatic bile duct carcinoma (IHBD). Other rare forms include angiosarcoma (arising from liver vessels), hepatoblastoma (from progenitor cells of the liver) and sarcoma (from connective tissue of the hepatic parenchyma). The health importance of these cancers stems from the huge burden of disease they incur causing hundreds of thousand deaths worldwide, and their dismal prognosis (Brown ML *et al*., 2006). Metastases of neoplasms originating from distant organs to the liver are common, most often from the bowel, breast, lung and the kidney (Keighley, 2003).

1.2 Epidemiology

Globally, liver cancer is the third most common cause of cancer-related death (Parkin *et al*., 2005a). At least half of over 600,000 annual worldwide deaths occur in China alone and a majority of the remaining half in sub-Saharan African region. Liver cancer carries a very poor prognosis, with an estimated 5-year survival rate of 3-5% (Shaib and El-Serag, 2004). It is the fifth most common cancer in the world (Parkin, Bray, Ferlay, & Pisani, 2005a), demonstrating a distinct variation in its geographical distribution, with over 80% of the cases occurring in East/Southeast Asia and sub-Saharan Africa (Figure 1). Southern Europe and Japan are the more developed regions of the world with the highest incidence (Parkin, Bray, Ferlay, & Pisani 2005a). HCC is the most common primary liver cancer subtype, accounting for 85%

to 90% of cases (El-Serag and Rudolph, 2007). Intrahepatic bile duct carcinoma, chiefly comprising IHCC is considered to be relatively rare, but is the second commonest primary liver tumour, accounting for 10-25% of PLC (Parkin, 2004).

The major determinant of the distinct global distribution of HCC is variation in the prevalence of risk factors. Whereas hepatitis C virus (HCV) and alcohol is considered to be the most important risk factor for HCC in developed countries, hepatitis B infection (HBV) and aflatoxin exposure are commonly implicated in developing countries (Bosch *et al*., 2005).

HBV and HCV infections account for more than 80% of cases of HCC globally (Bosch, Ribes, Cleries, & Diaz, 2005). The highest incidence of HCC is in the region of Southeast Asia (China, Vietnam, North and South Korea), often attributed to the high endemicity of HBV. In Japan however, HCV is the commonest risk factor (Yoshizawa, 2002). Outside of Southeast Asia, sub-Saharan countries lead, with Cameroon and Mozambique having relatively high HCC rates (Parkin DM *et al*., 2003). Countries in southern Europe generally present with intermediate rates, whereas low incidence areas include countries in North America, South and Central America (Nordenstedt *et al*., 2010) (Figure 1).

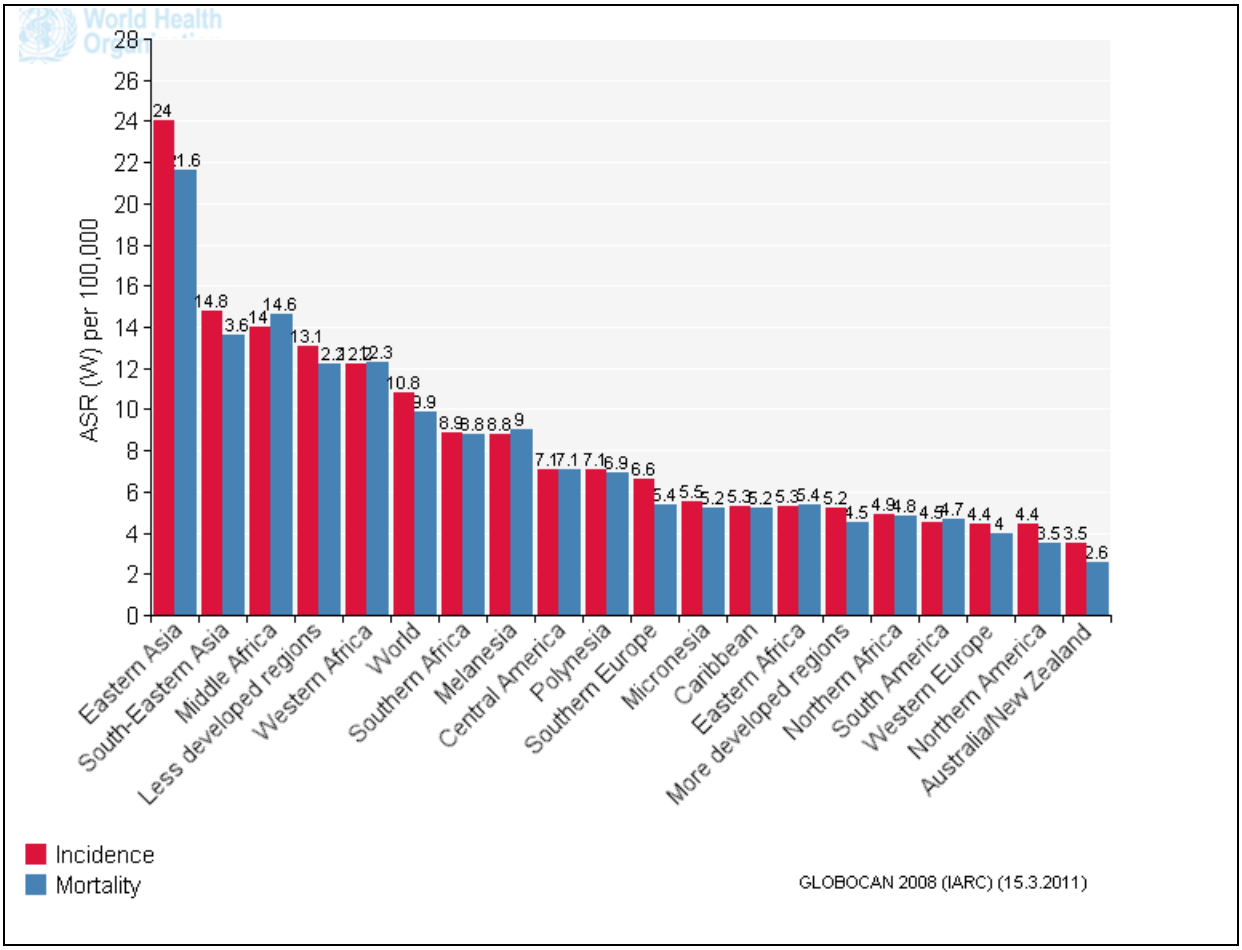

Figure 1. Age-standardised incidence and mortality rates of liver cancer for both males and females from different regions of the world (data from IARC; produced by permission)

The age distribution of HCC depends in most respects, upon the aetiological risk factor, region and gender (Nordenstedt, White, & El-Serag, 2010). While the highest age-specific rates occur among persons aged 75 in industrialised western societies and in regions more predominantly affected by HCV, developing countries such as Gambia and Mali tend to peak between ages 60 and 65 yrs among males (Umoh *et al*., 2011). Age at infection by viral and environmental factors account for the differences observed. While HCV tends to be acquired in adulthood, most carriers of

HBV become infected during childhood. This reflects in the earlier age of peak incidence of HCC in HBV endemic regions compared to regions whose major risk factor is HCV.

Virtually all studies show that males predominate over females in the incidence and mortality of HCC with average ratios of 2:1 to 4:1 (McGlynn *et al*., 2001). Differences in exposure to risk factors might explain this observation. Men are more likely than women to engage in activities that enhance HBV and HCV infection, as well as in the consumption of alcohol and cigarette smoking. The role for sex hormones in hepatocarcinogenesis has been suggested by some researchers (Arakawa *et al*., 1986). Evidence exists to implicate testosterone, as this hormone increases androgen receptor signalling in men, which could promote liver cell proliferation (Yu and Chen, 1993). While an increased susceptibility to liver carcinogenesis was demonstrated in male transgenic mice (Dunsford *et al*., 1990), hypophysectomised males showed an inhibited tendency to develop liver tumour induction by aflatoxin (Goodall and Butler, 1969). In contrast, oestrogen has been suggested to suppress interleukin (IL)-6 mediated inflammation; which could imply a reduction in liver injury and compensatory proliferation in women (Naugler *et al*., 2007).

1.3 Aetiological Risk Factors for Hepatocellular Carcinoma

Several factors have been implicated in the aetiology of primary liver cancer. Cirrhosis of the liver, chronic hepatitis B and C viral infection, dietary aflatoxin and alcohol are the most frequently studied. Risk factors that are still being studied include: obesity, diabetes mellitus, and oral contraceptive drugs, among others.

1.3.1 Cirrhosis

Cirrhosis of the liver is the most important risk factor for HCC as it links most other factors in the pathogenesis of this cancer and is present in approximately 80% of HCC patients (Colombo *et al*., 1991;Tiribelli *et al*., 1989). There is however, variation in the predilection of each underlying cause and stage of cirrhosis in the pathogenesis of liver cancer. For example, the 5-year cumulative risk for HCC ranges between 17-30% in HCV-associated cirrhosis compared to 10-15% among patients with HBV-related cirrhosis. In alcoholic and biliary cirrhosis, it is 8% and 4% respectively (Fattovich *et al*., 2004). A single cohort study found that late stage cirrhosis of the liver; Child-Pugh stage B or C, has a threefold relative risk of HCC compared to stage A (Bolondi *et al*., 2001). This is particularly significant in centres where treatments exist for stage C cirrhosis as this means that the survival of the patients is improved, and thus contributes to the pool of incident cases of HCC.

1.3.2 Non-cirrhotic hepatocellular carcinoma

Most PLC occur in the background of cirrhosis. However, in some patients, HCC arises de novo, without preceding cirrhosis. This is more commonly associated with tumours in patients that have used anabolic steroids, the metabolic syndrome, as well as in patients with type 2 diabetes mellitus. In those patients who have background viral hepatitis, non-cirrhotic HCC occurs much more commonly in less developed regions of the world than in the West. Indeed, metabolic syndrome has been hypothesized to become the most important risk factor for HCC in the future (Siegel and Zhu, 2009;Stickel and Hellerbrand, 2010). Indeed a recent epidemiological study of HCC by the Newcastle group has drawn the attention of the world to a relatively high rate of NAFLD-associated HCC, contributing to 35% of the

aetiological risks identified (Dyson *et al*., 2014). Approximately, 22% of NAFLD-associated HCC patients in that study had background cirrhosis.

1.3.3 Hepatitis C Virus

HCV is an RNA flavivirus, which prior to discovery, was described as Non-A, Non-B hepatitis virus. Its association with HCC has been established from case control, as well as prospective studies. Markers of HCV have been described in about 90% of HCC cases in Japan (Yoshizawa, 2002) and 44-66% of patients in Italy (Fasani *et al*., 1999;Stroffolini *et al*., 1999). Owing to the higher prevalence of HCV, compared to HBV in Western countries, HCV constitutes the most important risk factor for HCC in these countries, as well as Japan. Molecular clock analyses during early 2000 have estimated that HCV began to infect large cohorts of young people in Japan, southern Europe and North America in 1920s, 1940s and 1960s respectively, following the use of contaminated needles and intravenous drug abuse (Armstrong *et al*., 2000). This infection continued to spread in blood product infusions until early 1990s when screening was developed for HCV.

Up to 3% of HCV-infected persons develop HCC after 30 years of infection (Hassan *et al*., 2002), often occurring in the background of advanced fibrosis and cirrhosis. The annual incidence of HCC in HCV-induced cirrhosis is approximately 1-4% (Donato *et al*., 2002a). Environmental and host factors interact to enhance progression to cirrhosis in patients chronically infected by HCV. This has been shown by the fact that the incidence of HCC among Japanese is higher in those living in Japan, compared to those living in Hawaii; who in turn, have a 2.21 higher incidence than native Hawaiians (Maskarinec and Noh, 2004). Old age, as well as older age at the time of infection, heavy alcohol intake of more than 50g per day, male gender, diabetes mellitus, obesity, and co-infection with HBV or HIV have all

been identified as host factors that enhance cirrhosis development in chronically HCV-infected patients (Cramp, 1999). Although, the development of cirrhosis increases HCC occurrence, it is not an absolute requirement; as inflammation continues over time, the many steps required to induce carcinogenesis can occur independent of the evolution of fibrosis.

1.3.4 Hepatitis B Virus

HBV is a DNA virus belonging to the *Hepadnaviridae* family. The World Health Organisation (WHO) has pronounced HBV as the second most important human carcinogen after tobacco (Department of Communicable Diseases Surveillance & Response, 2002). There are estimated 350-400 million people chronically infected with the HBV worldwide. HBV is believed to account for over 50% of cases of HCC globally, having a significant regional variation (Parkin, 2006). Up to 70% of HCC cases in South Korea, 15% in Japan and the majority of cases in sub-Saharan Africa are attributable to HBV (Kim *et al*., 2008). Chronic HBV infection induces chronic inflammation which leads to repeated cellular regeneration, subsequently progressing to HCC, following approximately 25 years of infection. Chronic infection with HBV confers a 10-25% lifetime risk of developing HCC (Seeger and Mason, 2000). The carcinogenic potential of this virus is ensured by its ability to integrate its genetic material into the host genome in or near proto-oncogenes or tumour suppressor genes. However, the vast majority (70 to 90%) of people who develop HCC from HBV infection do so on a background of cirrhosis (El-Serag & Rudolph, 2007). When HBV is acquired in adulthood, there is spontaneous resolution of the acute infection in approximately 90% of cases. Most cases that are acquired vertically, perinatally or by horizontal route of transmission in early childhood often

produce a chronic infection; with less than 10% resolving and majority progressing to various forms of chronic hepatitis B infection sequelae.

Similar to HCV, several host and environmental factors enhance HCC among those infected with HBV, including: male gender, older age, Asian or African race, cirrhosis, family history of HCC, aflatoxin exposure, alcohol use or HCV and hepatitis D virus (HDV) co-infection. Viral factors that are associated with HCC development include: high levels of HBV DNA and the presence of HBeAg. HBV genotype C conveys a higher risk of HCC than genotype B and genotype D has a higher risk than genotype A (Chan *et al*., 2004;Yang *et al*., 2008). Comparisons between the genotypes commonly encountered in Europe (A and D) and those in Asia (B and C) are yet to be carried out. There remains scanty information on the E genotype, reported to be prevalently restricted to the West African sub-region (Dupinay *et al*., 2010).

1.3.5 Dietary aflatoxin

Aflatoxin B_1 (AFB_1), a mycotoxin produced by the fungus, *Aspergillus* spp*,* was described as a human liver carcinogen by Wogan in 1992 (Wogan, 1992). These fungi grow on several food products including peanuts, cereals, cassava and fermented soy beans; particularly when poorly stored in high moisture conditions of tropical regions of sub-Saharan Africa and eastern Asia. Following ingestion, AFB_1 is metabolised to an active substrate, AFB_1-exo-8,9-epoxide. Transgenic mice studies have suggested that HBV alters the expression of carcinogen metabolising proteins through chronic liver damage and modulates the level of binding of this aflatoxin substrate to DNA (Turner *et al*., 2002). The downstream effect leads to the production of the mutation, P53 249^{ser} with the ultimate result of inhibiting the *p53* tumour suppressor gene.

A synergistic effect exists between HBV and aflatoxin. Most HBV-infected individuals exposed to the toxin have a higher risk of HCC compared to non-exposed persons. A study of a prospective cohort in the mid-1990s from China demonstrated a significant rise in the relative risk of HCC from 7.3 in HBV-infected persons with no aflatoxin exposure to 60 in those who were exposed (Qian *et al*., 1994;Zhang *et al*., 1998). The mutation in the *p53* gene produced by this toxin has been found in 30-60% of all HCC cases from affected regions (Zhang, Wang, Han, & Zhuang, 1998). Additionally, a study from Gambia has shown a strong relationship between cirrhosis of the liver among HBV patients and aflatoxin (Kuniholm *et al*., 2008). This study postulated that aflatoxin enhances hepatocarcinogenesis via promoting cirrhosis of the liver among chronically-infected HBV patients (Kuniholm *et al*., 2008). It remains to be demonstrated whether exposure to this toxin is a risk factor for HCC without the influence of hepatitis B in humans.

1.3.6 Alcohol

Heavy alcohol consumption, defined as the ingestion of more than 50-70g per day for a prolonged period, is an established risk factor for HCC, which can occur independently of the development of cirrhosis. It is known that the consumption of over 80g per day of alcohol for over 10 years increases the risk of HCC five-fold (Morgan *et al*., 2004a). Furthermore, evidence for synergism between heavy alcohol consumption with HCV or HBV to increase HCC risk by promoting cirrhosis exists. A dose-effect relationship study in 2002 in Italy found that HCC incidence doubled in those with HCV infection compared to those who did not have the infection and drank alcohol with a daily intake of more than 60g of alcohol/day (Donato *et al*., 2002a). The mechanisms by which alcohol causes HCC are hypothesised to include

oxidative stress, genetic susceptibility, decreased natural killer (NK) cell function and modulation of DNA methylation (Morgan, Mandayam, & Jamal, 2004a).

1.3.7 Non-alcoholic liver disease and diabetes mellitus

Many of the major risk factors for HCC (HBV, HCV, alcohol) cannot be identified in a proportion of HCC patients. Non-alcoholic fatty liver disease (NAFLD), a condition associated with insulin resistance and which contributes to the metabolic syndrome is reported to be increasing (Leclercq, 2010). About 20% of those who develop NAFLD are complicated by non-alcoholic steatohepatitis (NASH) characterised by hepatocellular damage, chronic inflammation and progressive fibrosis, in addition to steatosis. The progression of NASH ends in cirrhosis, with the development of HCC in some. Recent evidence from a German study suggests that HCC could develop in NAFLD/NASH patients in the absence of cirrhosis (Ertle *et al*., 2011). A significant proportion (41.7%) of individuals with NAFLD/NASH associated HCC in that study had no evidence of cirrhosis. Studies have indicated that up to 90% of all obese persons (BMI >30Kg/m^2) and about 70% of patients with type II diabetes have some form of fatty liver disease (Neuschwander-Tetri and Caldwell, 2003). A 2-3 fold increased risk of HCC in obese men and women, compared to those with normal BMI have been documented in some studies (Moller *et al*., 1994;Wolk *et al*., 2001). HCC was found to be more common among those who had concomitant HCV infection, and conveying only a modest 1.4 fold risk among HBV patients. In the presence of diabetes and obesity, there was a 100-fold excess of HCC in the context of either of the chronic viral hepatitides. With the current global epidemic of obesity (El-Serag & Rudolph, 2007) and diabetes, and with HBV and HCV control programmes in place, it is reasonable to suggest that NASH would likely account for an increasing number of cases of HCC in the future.

1.3.8 Hereditary Haemochromatosis and Iron Overload Syndromes

Hereditary haemochromatosis (HH) is a commonly encountered autosomal recessive disorder, mostly affecting people of North European descent and uncommon in Africa (Powell *et al*., 2000). It is principally caused by mutations in the *HFE* gene (the most important being C282Y, followed by H63D) and is associated with excess iron absorption. Disease manifestation is most common in C282Y homozygotes and C282Y/H63D compound heterozygotes. This mutation leads to excess storage of iron in various organs, including the liver, resulting in liver damage and progressive fibrosis. Some studies have established the association between HH and the development of HCC (Elmberg *et al*., 2003;Fracanzani *et al*., 2001). The risk for the development of liver cancer is particularly enhanced among male diabetics (Yang *et al*., 1998). During the last decade, research has demonstrated that the C282Y mutation in the *HFE* gene is not diagnostic of HH, but may be associated with higher levels of serum ferritin, transferrin saturation and deposition of excess iron in the liver (Fargion *et al*., 2001). A French study has shown that the incidence of HCC was significantly higher among those with this mutation and having alcohol-related cirrhosis compared to those who have HCV-related cirrhosis (Mandishona *et al*., 1998;Nahon *et al*., 2008). Studies in African populations found that patients who ingested iron through drinking locally brewed alcohol in iron pots, have a 10-fold increased risk of developing HCC, after adjusting for other aetiological factors (MacPhail *et al*., 1999;Mandishona *et al*., 1998;Moyo *et al*., 1998).

1.3.9 Oral contraceptive pills

Animal studies have shown that oestrogens and progestogens, contained in oral contraceptive pills (OCP), are inducers and promoters of liver tumours (IARC, 1999). Case-control studies, mostly in the USA and Europe have produced results in

support of the hepatocarcinogenic potential of these pills in relatively young women (Herman *et al*., 1994;Korula *et al*., 1991;Perret *et al*., 1996;Tavani *et al*., 1993;Yu *et al*., 1991). A meta-analysis of these studies found odds ratios of 2.5 and 5.8 in those who had ever used these drugs or had longer duration of use respectively, compared to those who never used them (Yu and Yuan, 2004). This finding is however, not uniform across the world, as some series in Africa and Asia found no difference in the prevalence of HCC between those who took OCPs and those who did not (Kew *et al*., 1990).

1.3.10 Dietary factors

Studies suggesting the role of coffee drinking in modifying the aetiopathogenesis of HCC have been documented. Some epidemiological studies have previously reported that coffee drinking reduced the risk of elevated liver enzymes and cirrhosis (Klatsky *et al*., 2006;Tanaka *et al*., 1998). Indeed, insulin levels have been demonstrated to be low (in contrast to high levels in type 2 diabetes mellitus) in those who drink coffee (El-Serag *et al*., 2006). Animal studies have suggested that coffee reduces carcinogenesis of the liver. Tanaka and colleagues in 1990 found that the incidence of HCC foci in rats given concurrent aminopyrine and sodium nitrite with coffee solution (as drinking water) for 630 days were significantly lower than those rats given only aminopyrine and sodium nitrite (Tanaka *et al*., 1990).

Case-control and cohort studies in Japan (Shimazu *et al*., 2005) and Europe (Bravi *et al*., 2007;Montella *et al*., 2007) evaluating the relationship between increased coffee consumption and HCC, found significant reductions in the risk of HCC. The role of diet in carcinogenesis is not new to HCC as increased dietary fibre has been shown to be associated with lower incidence of colorectal cancers, as well as vitamin C and fruits and vegetables related to lower incidence of gastric and lung cancers

respectively (Gonzalez and Riboli, 2010). If not causal, certain diets may be associated with lower incidence of HCC, or to be synergistic, augmenting the roles of established factors.

Compared to HCC, the aetiology of intrahepatic cholangiocarcinoma (IHCC) is poorly understood and only one out of 10 cases of cholangiocarcinoma are associated with known risk factors (Ben-Menachem, 2007). The important factors are discussed below:

1.4.1 Bile duct factors

In Western industrialised countries, IHCC complicates up to 40% of patients with primary sclerosing cholangitis (PSC) (Shaib & El-Serag, 2004). In contrast, liver fluke infestation (*Opisthorcis viverrini*), known to be endemic in East Asia, is the most implicated risk factor in that region. For this reason, countries in East Asia are known to have the highest rates of IHBD worldwide (Shaib & El-Serag, 2004;Watanapa and Watanapa, 2002). An animal study has suggested that liver fluke infestation promotes carcinogenesis by enhancing the effect of chemical initiators. Following infestation with *O. viverrini*, Syrian hamsters that were fed nitrosamines demonstrated malignant change in their biliary epithelia (Thamavit *et al*., 1978). Approximately, 10% of individuals with intrahepatic bile duct stones develop cholangiocarcinoma (Kubo *et al*., 1995). Congenital fibropolycystic liver disease, bile duct adenomas and biliary papillomatosis are other associated biliary duct factors.

1.4.2 Other factors

Thorotrast, a radiological contrast agent used in the 1930s to 1950s has been strongly linked with IHCC (Sahani *et al*., 2003). Dioxins and nitrosamines, both industrial toxins have demonstrated epidemiological associations with IHBD (Hardell et al. 1984). A recent hospital-based case-control study of risk factors for IHBD in the USA reported that excess alcohol consumption was higher among IHCC patients

(22%) compared to controls (4%) (Shaib *et al*., 2007). A population-based case-control study, also in the USA, involving 535 IHCC patients and 102,782 cancer-free controls found that biliary cirrhosis, alcoholic liver disease, non-specific cirrhosis, diabetes mellitus, thyrotoxicosis and chronic pancreatitis were significantly associated with IHCC (Welzel *et al*., 2007). Other factors that showed a weaker association were obesity, smoking and HCV infection. Additionally, a prospective study involving 11,000 patients with variety of causes of cirrhosis followed up for 6 years in Denmark reported a 10 fold increase in the risk of IHCC compared to the general population (Sorensen *et al*., 1998). Case-control and prospective studies from Korea (Shin *et al*., 1996), Italy (Colombo *et al*.,1991;Donato *et al*., 2001), Japan (Kobayashi *et al*., 2000) and the USA (Shaib *et al*., 2005) have all corroborated the fact that hepatitis B (HBV) and hepatitis C (HCV), have a place in the pathogenesis of intrahepatic bile duct carcinoma. Other putative factors include genetic polymorphisms involving bile salt transporter proteins (Wadsworth *et al*., 2011).

1.4.3 Pathogenesis

Chronic inflammation of the biliary epithelium underlies the pathogenesis of this tumour as recurrent epithelial regeneration is engendered. The resulting increased cholangiocyte turnover may induce mutations of proto-oncogenes and tumour suppressor genes, leading to dysplastic changes and eventually, cholangiocarcinoma (Khan *et al*., 2005b). Alternatively, a putative mechanism involves the initial formation of mutagenic DNA adducts by toxins. Chronic inflammation then provides the “second hit” that exposes the bile duct epithelium to the adducts, resulting in cancer development (Khan *et al*., 2003).

1.5 Clinical Presentation

HCC is asymptomatic in its early stages. A great majority of patients with malignant liver tumours present in advanced stages of the disease with symptoms and signs suggestive of liver decompensation and sometimes, of metastasis (El-Serag *et al*., 2008;Gores, 2000). Nevertheless, increased awareness and the implementation of active surveillance have led to early diagnoses in asymptomatic cases.

Common clinical features of advanced HCC include right upper abdominal pain, weight loss and malaise. In well monitored healthcare systems, the initial observation may be worsening liver enzymes in a patient known to have cirrhosis of the liver. Rare manifestations include acute abdomen from rupture of HCC nodule with intraperitoneal bleeding (Choi *et al*., 2001a). Paraneoplastic presentations have also been documented, but are rare, including: hypercalcaemia, hypoglycaemia, thyrotoxicosis and polycythaemia (extrarenal synthesis of erythropoietin) (Eastman *et al*., 1992;Sakisaka *et al*., 1993).

Clinical signs, such as cachexia in advanced cases, jaundice, palmar erythema, gynaecomastia and signs of portal hypertension (due to associated cirrhosis of the liver) may be noticed. A hard, nodular liver on abdominal palpation has been described to be typical in many African populations, owing to the very late stage at which the patients present (Umoh *et al*., 2011). Hepatic bruits, due to hyper-vascularisation of the tumour can be detected in up to 20% of patients (Kew M., 1996).

1.6.1 Hepatocellular carcinoma

HCC is one of very few cancers where a diagnosis can be made in the absence of histology as it can be diagnosed on radiological criteria alone. Triple-phase helical computerised tomography (CT), and dynamic contrast enhanced magnetic resonance imaging (MRI) (Choi et al. 2001b) is usually embarked upon as soon as a suspicious lesion is detected during routine surveilance. The hallmark of diagnosis using these techniques is based on arterial enhancement, followed by delayed hypointensity of the tumour in the portal venous and delayed phases (described as "washout") respectively. HCC derives its blood supply from the hepatic artery, in contrast to the remainder of the non-cancerous liver tissue (which receives blood supply from both artery and portal venous system). Decreased or absence of contrast in the tumour tissue during the venous and delayed phases is the hallmark of "washout". The performance of CT and MRI is affected by the size of the lesions. The accuracy of MRI to detect lesions less than 2 cm falls to 33%, but >90% for lesions larger than 2 cm (Ebara *et al*., 1986). Although, "washout" has a high sensitivity of 90% and specificity of 95%, about a third of HCC cases do not have this feature. Liver biopsy for histological diagnosis is usually unavoidable for this group. Indeed, in many centres in developing countries, biopsy remains the only option for confirming diagnosis of HCC (Ladep and Taylor-Robinson, 2007). For some less developed countries, the World Gastroenterology Organisation (WGO) guidelines recommend the use of AFP for HCC diagnosis (Ferenci *et al*., 2010). Serum levels of AFP above 200 ng/L are highly specific for HCC diagnosis in patients with cirrhosis (Bruix and Sherman, 2005). However, reports have indicated that the sensitivity of AFP falls drastically when a cut-off value of 200 ng/L is used since only a third of

patients with HCC have levels above that threshold (Ebara *et al*., 1989;Torzilli *et al*., 1999).

The diagnosis of HCC can be established if:

a focal liver mass >2 cm is identified on one imaging technique, demonstrating characteristic contrast enhancement features on the arterial phase with venous washout on MRI, CT or CEUS;

(i) a focal hepatic mass with atypical imaging findings, or a focal mass detected in a non-cirrhotic liver, should undergo a biopsy.

(ii) Nodules smaller than 1 cm should be followed up for 2 years at 3-4 monthly intervals, based on the updated algorithm of the American Association for the Study of the Liver (AASLD) (Figure 2).

(iii) If no further growth is demonstrated, a return to routine surveillance is recommended.

Percutaneous liver biopsy performed under CT guidance yields higher sensitivity and specificity than when US alone is utilised (Durand et al. 2001).

1.6.2 Intrahepatic cholangiocarcinoma

Intrahepatic cholangiocarcinoma is difficult to diagnose, as there are no definite imaging findings that describe the lesions accurately, no serum markers of proven high sensitivity and specificity, and the histological pattern is non-specific. In some instances, diagnosis is achieved at autopsy. Biochemical investigations offer limited utility in the diagnosis of cholangiocarcinoma. Liver function tests may show an obstructive pattern of raised alkaline phosphatase, bilirubin and gamma-glutamyl transpeptidase. Tumour markers for cholangiocarcinoma; carbohydrate antigen 19-9

(CA 19-9), carcinoembryonic antigen (CEA) and carbohydrate antigen 125 (CA-125), all are associated with low sensitivity and specificity and cannot be relied upon for diagnostic purposes (Hultcrantz *et al*., 1999;Khan *et al*., 2002a). Owing to its low cost, widespread availability and non-invasive nature, abdominal US is the initial imaging modality employed. A pulsed colour Doppler sonography may demonstrate a tumour-induced compression (or thrombosis of the portal vein or artery). A combination of MRI and magnetic resonance cholangiopancreatography (MRCP) is used (where available) as a second line, otherwise high resolution contrast-enhanced spiral CT is used (Figure 3). Potential resectability is considered before confirming IHCC histologically, for example by endoscopic retrograde cholangiopancreatography (ERCP) and percutaneous transhepatic cholangiography (PTC). Although, these latter techniques are often required to obtain washings, brushings, intraductal biopsies and/or therapeutic interventions, MRCP has been more commonly utilised in recent years for the diagnosis of IHBD (Khan *et al*., 2002a;Mazen *et al*., 2007;Simmons and Baron, 2007).

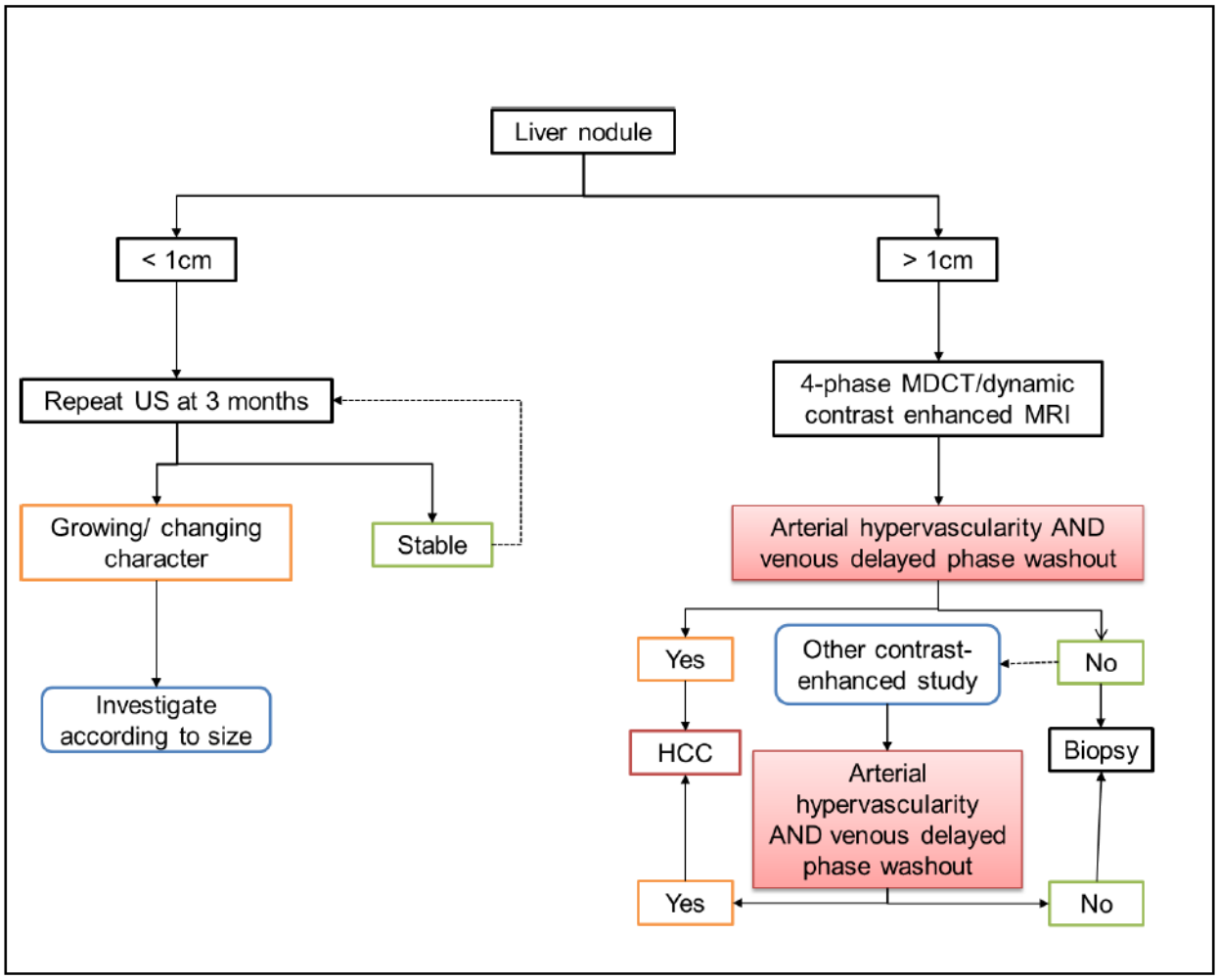

Figure 2. Diagnostic algorithm for suspected HCC (CT, computed tomography; MDCT, multidetector CT; MRI, magnetic resonance imaging; US, Ultrasound) (Bruix and Sherman, 2011)

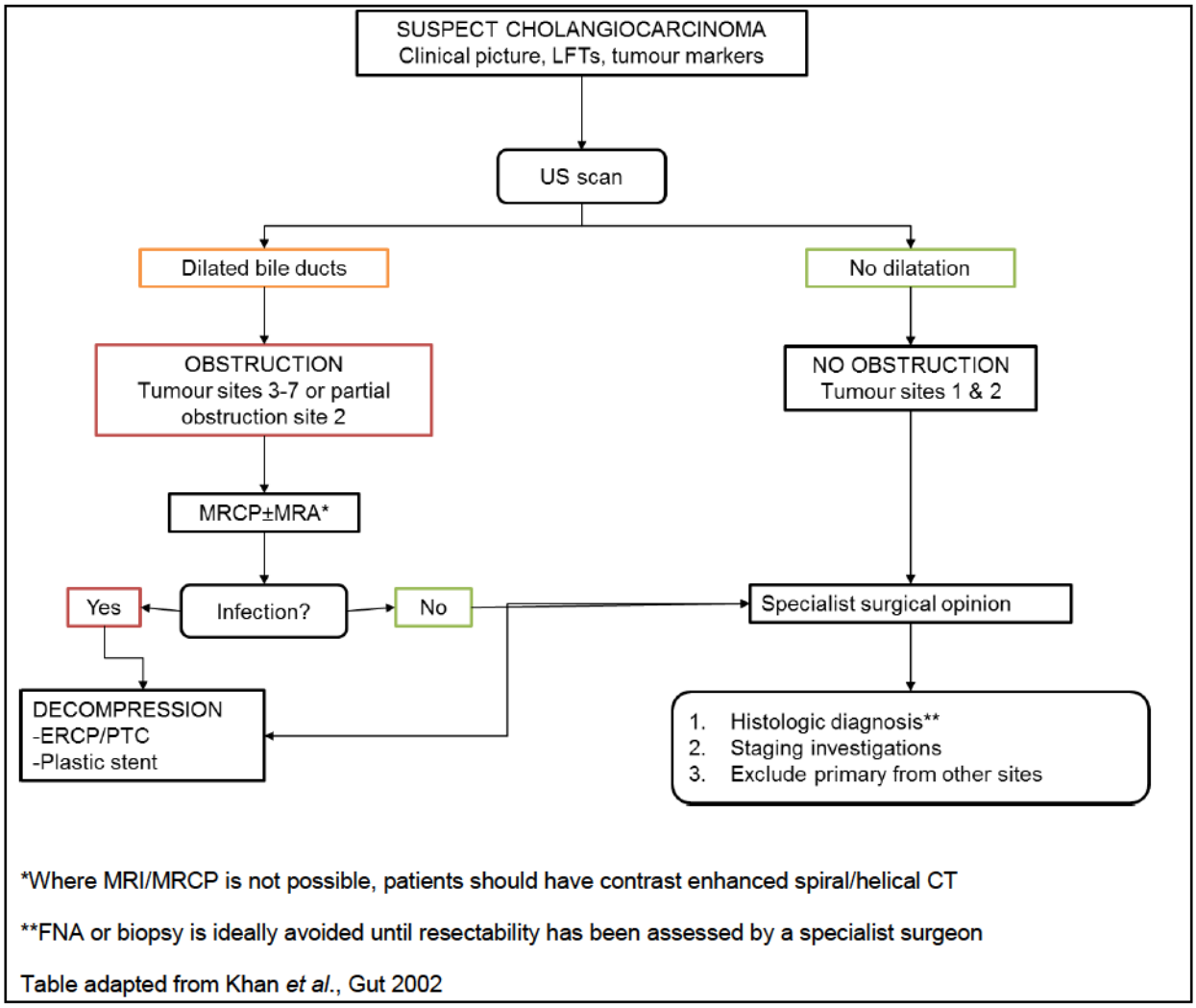

Figure 3. Diagnostic algorithm for cholangiocarcinoma (adapted from Khan *et al.*, 2002)

1.7 Surveillance and Early Detection

Surveillance of at-risk populations improves the treatment and outcome of HCC, since it leads to detection of tumours at early stages, when they are amenable for curative therapies such as resection, ablation and/or transplantation. High risk populations include virtually all those with cirrhosis and HBV-infected men (>40 years) and women (> 50 years) (El-Serag, Marrero, Rudolph, & Reddy, 2008). A

breakdown of groups of high risk people for whom screening for liver cancer has been recommended is shown in table 1.

Table 1. Groups in whom HCC screening and surveillance are recommended (El-Serag, Marrero, Rudolph, & Reddy, 2008)

Hepatitis B carriers
Asian males >40 yrs
Asian females >50 yrs
All cirrhotic hepatitis B carriers
Family history of HCC
Africans over age 20 yrs
Non-hepatitis B cirrhosis
Hepatitis C
Alcoholic cirrhosis
Genetic haemochromatosis
Primary biliary cirrhosis
Possibly; non-alcoholic steatohepatitis, autoimmune hepatitis, α1 anti-trypsin deficiency

Liver US and AFP are the most commonly used modalities for HCC surveillance. The performance of US is however, operator-dependent, the technology employed, presence of cirrhosis and size of the tumour (Bolondi *et al*., 2001). Most studies have identified a sensitivity of above 60% and specificity, over 90% for US. The sensitivity is particularly low if detecting a tumour nodule in a cirrhotic liver (Kim *et al*., 2001). The upper limit of normal (ULN) serum AFP, 20ng/mL has a low sensitivity of 25% to 65% for detection of HCC and thus fails to serve as a sole screening modality (Trevisani *et al*., 2001). Furthermore, elevated values (in the absence of malignancy) of AFP occur in conditions associated with high degree of liver regeneration, such as chronic HCV (Bayati *et al*., 1998). Other serum-based tests, such as des-γ-carboxyprothrombin and lectin bound AFP, present variable sensitivities and require more robust data to validate their usefulness (Ishida *et al*., 2010;Marrero *et al*., 2009). The current guidelines recommend the use of standard, non-contrast US at 6-12 months frequency to screen for HCC in high risk individuals (Bruix & Sherman, 2005;Trevisani *et al*., 2002).

1.7.1 Advantages of screening

If PLC is discovered at an early stage, effective treatments are available, possessing the advantage of improving survival. Owing to ethical issues, results of survival benefits from surveillance programmes have been scanty. Zhang and colleagues, working in China reported a survival benefit in a surveyed at-risk population compared to a non-surveyed population, in the only available randomised trial performed to date. Although, the adherence was noted to be lower than 60%, this study of nearly 19,000 HBV infected patients showed that testing of serum α-fetoprotein (AFP) and abdominal ultrasound (US) at 6 monthly intervals improved survival (37% reduction in HCC-related mortality) (Zhang et al. 2004). This benefit

could however, be annulled if curative managements, are lacking as observed by a similar study in another province of China (Chen et al. 2003).

1.8 International Classification of Diseases

Historically, the classification of diseases started in the mid-1800s, then known as International List of Causes of Death. The World Health Organisation (WHO) took over from the International Statistical Institute in 1948 about the time the 6th revision was published, which included causes of morbidity for the first time (World Health Organisation, 1949). The International Classification of Diseases, Injuries, and Causes of Death (ICD) has since become adopted as the International standard diagnostic categorisation for all general epidemiological and clinical use, among which are: general health situation of population groups and monitoring of incidence and mortality of diseases in relation to personal and resource variables. It provides the basis for the compilation of national mortality and morbidity statistics by WHO member states. The 6th version and indeed all the other revisions were necessitated by the need to keep abreast with advances in medical knowledge and nomenclature.

The ICD assigns a three character alphanumeric code to every major condition, a fourth character is often added for more exact specification. For cancer classification, each tumour is assigned a topographical code, correlating with the anatomic sites of involvement. These topography codes are applicable to all tumours, regardless of their growth behaviour; whether benign, malignant, *in situ* or uncertain. Hepatocellular carcinoma (HCC) and Intrahepatic bile duct tumours (IHBD) were recognised as distinct entities by cancer registries for the first time in 1968 (eighth revision of ICD) (World Health Organisation, 1967). The ninth revision (ICD-9) (World Health Organisation, 1975) was adopted for the coding of incidence and mortality

data in England and Wales between 1979 and 1994, after which ICD-10 came into use for incidence data (World Health Organisation, 1992). Changes in ICD classification can impact upon direct comparability of disease rates over time. Aggregation of categories of diseases is often necessary before any valid comparison is possible. From 2001 however, both cancer registration and mortality data in England and Wales are recorded using the ICD-10 (Table 2). It is important to note that different countries adopt ICD changes at different time points; the impact on epidemiological trends of which remains to be extensively studied.

Table 2. Subsets of liver tumours under by ICD codes

SUBSET NAME	ICD-10 CODE
Malignant liver tumours (PLC) **Primary liver tumour (mainly HCC)**	C22 (malignant neoplasm of the liver and intrahepatic bile ducts) C22.0 (hepatocellular carcinoma)
Other malignant liver tumours	C22.2 (hepatoblastoma)
	C22.3 (angiosarcoma of the liver)
	C22.4 (other sarcomas of the liver)
	C22.7 (other specified carcinomas of the liver)
Intrahepatic bile duct	C22.1 (intrahepatic bile duct carcinoma)
Unspecified liver tumours	C22.9 (liver, unspecified)

1.9.1 Current Knowledge

Epidemiological studies of primary liver cancer (PLC) towards the end of the last century revealed upward trends in the incidence and mortality rates of PLC in some developed industrialised countries of the world, including England and Wales. However, despite progress made in the management of PLC, the prognosis of this cancer remains abysmal. The high case-fatality associated with HCC is often due to late presentation by most patients, absence of effective screening tools and personnel, besides poor health delivery systems in most developing countries. The current screening tools for HCC remain the use of ultrasound (US) and serum alpha fetoprotein (AFP). While US is operator-dependent and presents increased risk of missed diagnoses, in addition to being an impractical tool for use in many developing countries, AFP has a low sensitivity for detecting HCC (Farinati *et al*., 2006). Also, adherence to 6 monthly HCC surveillance is poor, even in resource rich countries. An ideal screening tool with higher sensitivity and specificity, easy accessibility, and which is cost-effective and culturally acceptable is a necessity in curbing the growing problem of HCC.

1.9.2 Gaps in knowledge

The impact of recent management advances and emerging risk factors on the epidemiology of PLC in England and Wales, as well as globally, is yet to be adequately studied. Although improved case ascertainment, diagnostic transfer, and evolution in aetiologic factors have all been postulated as explanations for earlier increasing international trends, widespread substantive evidence is lacking to prove

any of these hypotheses. The utility of the current screening tools (AFP and US) for primary liver cancer are limited by logistical, technical and personnel issues. Simple, effective and cheaper alternatives for screening are required, particularly in resource-limited countries where HCC is most common. The performance of pilot screening tools determined by metabonomic studies is promising, but limited by small sample sizes.

1.9.3 Contribution of current work

This work comprised an analysis of recent trends in mortality rates of PLC worldwide; including a comprehensive study of the incidence and mortality rates of PLC by subgroups in England and Wales. This was enabled by the fact that there is a robust cancer registration in the UK compared to many other countries. The impact of viral hepatitis co-infection of HIV patients on liver related morbidities in the African setting was examined as well. Importantly, urinary metabolic profiling for biomarkers of HCC was undertaken in a large sample study of subjects from West Africa, with view to establish a robust, easily accessible and applicable tool (dip-stick) for the screening of HCC.

1.10 Overall aims

The main aims of my study were to a) characterise the mortality trends of PLC subtypes globally, b) determine the most recent trends in the incidence and mortality rates of primary liver cancer in England and Wales, c) study the impact of viral hepatitis on HIV-infected persons, and d) metabolically profile HCC using urine, with an ultimate view of enabling a novel urinary diagnostic screening test for HCC.

1.10.1 Specific objectives

1. To determine the age-standardised mortality rates by ICD subcategories of primary liver cancer in countries across different regions of the world.
2. To determine the age-standardised incidence and mortality rates of primary liver cancer by ICD subcategories in England and Wales.
3. To determine the rates and impact of liver-related morbidities on HIV in an African setting.
4. To determine the impact of HBV on survival of HIV-infected patients on antiretroviral therapy.
5. To analyse the metabolic profile in urine of HCC patients from Gambia, Senegal and Nigeria using proton magnetic resonance spectroscopy (NMR) and ultra-performance liquid chromatographic mass spectrometry (UPLC-MS).

2. INTERNATIONAL TRENDS IN MORTALITY RATES OF PRIMARY LIVER CANCERS

2.0 ABSTRACT

Background: As the risk factors for primary liver cancer (PLC) are likely to be specific for the different sub-groups, studies of mortality rates by specific PLC subtypes and gender of several countries may provide useful information regarding prevention and treatment. Global trends in the mortality rate of major subcategories of PLC from 1996 to 2009 were determined.

Method: Recent primary liver cancer mortality data from the National Cancer Registries of 8 countries: representative of Europe, Asia-Pacific and North America were obtained from the World Health Organisation (WHO) database. The age-standardised mortality rates (ASMR) of different subcategories of PLC were calculated, and the rates for hepatocellular carcinoma (HCC), intrahepatic bile duct tumours (IHBD) and unspecified liver tumours (Liver NOS) were reported in the present study. Trends in the ASMR of HCC and IHCC were evaluated using a regression method in which a least squares regression line was fitted to the natural logarithm of the rates.

Results: Over the study period, high ASMR of HCC were noted in males from Japan (17.4/100,000), Hong Kong (15.3/100,000) and Spain (4.8/100,000). Relatively lower ASMR of HCC were observed in Norway (1.0/100,000), England and Wales (1.4 /100,000), Romania (1.6/100,000), Australia (2.2 /100,000) and USA (2.3/100,000). The trends of ASMR of HCC has been declining in Japan, Hong Kong and Spain (APC; -4%, -2% and -1% respectively). ASMR of HCC increased in men in England & Wales (+7%), Romania (+5%), USA (+3%) and Australia (+1%). Rising trends in

mortality from IHCC for all evaluated countries were observed. Hong Kong registered the highest mean ASMR of IHBD of 2.4/100,000. Although the burden of IHBD was least in Romania (0.2/100,000), the greatest increase (APC: +18%) was noted in that Eastern European country.

Conclusion: While a global increase in ASMR of IHBD was observed over the study period, mortality from HCC is increasing only in countries with relatively lower ASMR while decreasing in countries with high rates, suggesting differences in the control of risk factors for HCC such as HBV vaccination and or surveillance and management of incident HCC. Factors that may be contributing to the global widespread increase in mortality from IHBD warrant further studies.

Recent data from the USA (El-Serag and Mason, 1999), France (La *et al*., 2000), Italy (La *et al*., 2000) and Japan (Kato *et al*. 1990) showed rising mortality rates of primary liver tumours. Epidemiological reviews indicate that mortality rates are falling in many developing countries (McGlynn *et al*., 2001). In England and Wales, excess mortality from PLC during 1968-1998 was attributable to IHBD *(Taylor-Robinson et al*., 2001). Also, looking at international trends in the mortality rates of hepatobiliary tumours, Khan and colleagues in 2002 (Khan *et al*., 2002b) trawled WHO mortality databases and observed widespread increase in the mortality rate for IHBD. Technological advances in the management of liver diseases, including PLC have been realised within the last decade and are assumed to have impacted on the mortality rate for PLC. Several countries have published guidelines, aimed at improving the management of PLC. Despite availability ultrasound for screening at-risk populations, most recent data from England and Wales have shown that mortality rate for PLC has maintained its relentless upward pattern, particularly with regards to both IHBD and HCC in men and women.

2.1.1 Rationale for the study

Given their dynamic nature, further studies on trends in the mortality of PLC by sub-types are required for the evaluation of intervention systems and proper health planning. The extent to which control programmes for HBV and HCV would achieve any significant impact can be determined by continuous monitoring of incidence and mortality rates of PLC. There has been increasing consumption of alcohol globally (Donato *et al*., 2002b;Hasumura and Takeuchi, 1991;Pincock, 2003), as well as an epidemic of obesity (James *et al*., 2001a;James. 2008). The global epidemic of obesity, as well as rise in the consumption of alcohol may act against any gains

reminiscent upon improved preventive methods that would have been in place in the last decade towards reducing the burden of PLC. Nevertheless, active mobilisation for surveillance of HCC to achieve early detection and meet criteria for curative therapies is being pursued in resource-rich countries (Khan *et al*., 2002a;Ryder, 2003;Song *et al*., 2010).

Trends in mortality rates may reflect progress in the management of PLC and mortality data are readily available (as death registration are mandatory in many countries) (Sharp *et al*., 2001). Also, as the prognosis of PLC remains poor, mortality data for PLC can and has been used as proxy for incidence. The study of ASMR of PLC of the recruited countries can provide a fair idea of the incidence of this emerging epidemic. Moreover, population-weighted correlation analyses had demonstrated that liver cancer mortality mirrors its incidence (Neuberger *et al*., 1985). The data for PLC incidence held by the International Agency for Research on Cancer (IARC) could be used but are limited to 3 digit ICD-10 codes and hence fail to meet the criteria for site specific study of PLC incidence. In this current study, 4-digit ICD-10 codes have been used in order to facilitate investigation of specific PLC sub-types, as the need to study tumours by subcategories was been highlighted by Percy and co-workers (Percy *et al*., 1990); whose report pointed out the enhanced accuracy of studying diseases by specific ICD codes. This study aimed to examine temporal changes of mortality from PLC by subtypes (four digit ICD codes) in select countries, representative of different regions of the world.

2.1.2 Hypothesis

Mortality rates of PLC are increasing in several countries around the world.

2.1.3 AIMS

The aims of the present study were to primarily calculate ASMR of PLC in countries from different regions of the world and to determine trends in the mortality rates of IHBD and HCC in particular.

2.2 METHODS

Liver cancer mortality statistics were obtained from the Department of Health Statistics and Informatics of the World Health Organisation (WHO). Population numbers, given by 5-year bands, were obtained from the official WHO database. Data were acquired from representative countries of all the health regions of the world. Included were countries that had optimal cancer registry data of mortality in the most recent past, as well as 5 year bands population counts for the period of mortality data. Countries where data were unavailable or incomplete were excluded. On one or both of these bases, countries from Africa, South America and East Asia were excluded. Included in the study were: Australia (Australasia), United States of America (North America), Japan, Romania (Eastern Europe), Spain (Southern Europe), Hong Kong (Asia), Norway and both England and Wales (Northern Europe). ASMR of PLC from 1996 and 2009 were calculated by direct standardisation, using 1960 world Standard population to control for age and sex structure differences in the population of the countries (WHO, 2011).

Specific time periods for which data were utilised for this study are outlined below:

- 1998 – 2006 for Australia
- 1996 – 2008 for Japan
- 1999 – 2009 for Romania
- 1999 – 2008 for Spain

- 1998 – 2008 for Norway
- 2001 – 2008 for Hong Kong
- 1999 – 2005 for USA
- 2001 – 2007 for England and Wales

The percentage change in ASMR was calculated. The annual percent change (APC) for HCC, IHBD and Liver NOS in both genders, from the beginning and the end of the periods studied were calculated. These were determined using a regression method in which a least squares regression line was fitted to the natural logarithm of the mortality rates.

2.3 RESULTS

2.3.1 Hepatocellular carcinoma: General pattern

A non-uniform pattern of trend in the mortality from HCC was found when latest ASMR were compared with the earlier data of the respective countries that were studied (Tables 3 and 4). In both sexes, increases in ASMR of HCC were observed in Australia, England and Wales, USA and Romania (Figure 4). In men, the highest increase in the mortality rate of HCC occurred in England and Wales (APC: +7%), followed by Romania (APC: +5%), and USA (APC: +3%), in that order. The least increase of 1% was observed in Australia. In women, rising trends in mortality rate of HCC were noted to be similar in England and Wales (APC: +4%) and Romania (APC: +5%). In contrast, mortality rates of HCC declined in Japan (APC: -4%), Hong Kong (APC: -2%) and Spain (APC: -1%).

2.3.2 Intrahepatic bile duct tumours: General pattern

In all studied countries there were increased ASMR of IHBD in both sexes with the exception of Hong Kong, where a 1% year on year decline was noted amongst

females (Tables 3 and 4). The highest increase was observed in men in Romania (APC: +18%). Consistent with the declining mortality rate of IHBD amongst females from Hong Kong, the smallest increase was recorded amongst men in this country (APC: +1%). Australia, England and Wales, Spain and USA in that order had intermediate rates of increase (5% to 6%) in ASMR of IHBD (Figure 5).

2.3.3 Unspecified liver tumours: General pattern

Falling ASMR of unspecified liver tumours were found in both men and women in most of the studied countries; including England and Wales, Japan, Hong Kong and Spain; as well as, females in the USA and Romania. During 1998 and in both sexes, no deaths were registered to unspecified liver tumour in Norway (Figure 6). However, inconsistent fluctuations were noted throughout the rest of the study period in Norway (Tables 3 and 4).

2.3.4 Australia

In Australia, there were marginal increases in the ASMR of HCC in both sexes between 1998 and 2006. Data were unavailable for 2005 and for which reason; the ASMR for that year was linearly extrapolated. A total of 244 (ASMR: 1.89) males and 64 (ASMR: 0.41) females died due to HCC in 1998 in Australia, compared to higher numbers in 2006 [males: 346 (2.21) and females: 83 (0.45)]. This rise was inconsistent, producing an overall increase of 1% in men and no significant change in mortality among women during the study period.

A smooth and more sustained increase in the ASMR of IHBD in both sexes was documented in Australia. One hundred and eleven (111) men (ASMR of 0.83) and 93 females (ASMR: 0.52) died due to IHBD in 1998, rising to 214 (ASMR: 1.22) in men and 184 (ASMR: 0.91) in women, corresponding to a higher average annual

percent (APC) increased death rates in females of 7% than males (5%). While the ASMR of liver tumours of unspecified sub-site experienced a similar rise in females (5%) and males (5%).

2.3.5 England and Wales

In England and Wales, there was a sustained increase in the total number of deaths due to HCC. Six hundred and forty seven (647) people died in 2001, rising progressively to 1065 in 2007. The increased ASMR was more consistent amongst men (from 1.14 in 2001 to 1.71 in 2007) than women, resulting in APC of 7% and 4% respectively.

There was a sustained rise in the mortality trend of IHBD in both sexes between 2001 and 2007. The absolute number of deaths (ASMR) in 2001 for males and females were 454 (0.92) and 537 (0.79) respectively. The mortality rate rose to peak in 2006 for males at 1.21, while that for females rose to 1.12/100,000 in 2007. There was a slight decline in the ASMR of this tumour amongst men in 2007. The mortality rates of IHBD increased by an annual average of 5% and 6% in men and women respectively.

In both males and females, deaths from unspecified liver tumours declined between 2001 and 2007 (APC decreases of 6% in men and 9% in women).

2.3.6 Norway

ASMR of HCC in both males and females from Norway undulated but showed a general pattern of decline during 1998 to 2008. In 2008, these were 1.07 and 0.28 compared to 1.23 and 0.40 in 1998 in males and females with APCs of -4% and -10% respectively. The ASMR of IHBD in men and women increased more than 2 and 4 folds respectively during 1999-2008. In 1998, no case of death due to liver

NOS was documented. There were inconsistent increases in the ASMR of unspecified liver tumour in both men and women in Norway until 2004, when it became smoother (APC increases of +11% and +17% in males and females respectively).

2.3.7 Spain

ASMR of HCC in Spain showed an overall decline; from 5.09 (men) and 1.37 (women) in 1999 to 4.59 (men) and 0.95 (women) in 2008, corresponding to APC decreases of 1% and 4% in men and women respectively. ASMR of IHBD in both sexes in Spain showed a rising, but inconsistent trend (APC increased by 6% in men and 5% in women). A small but fluctuating decline in the ASMR of liver NOS was recorded in both genders between 1999 and 2008 (APC: +4% and +5% in men and women respectively).

2.3.8 Romania

There was a more than 2-fold increase in the absolute number of deaths and ASMR of HCC amongst men in Romania between 1999 and 2009. This was 199 (ASMR: 1.21) in 1999 and 412 (ASMR: 2.54) in 2009 (APC: +5%). A less consistent, but rising ASMR of HCC was also noted amongst Romanian women (APC: +5%).

The most dramatic increase in the mortality rate of IHBD, in both sexes, was found in Romania. Whereas in 1999 both males and females had 9 deaths (0.06 and 0.05 ASMR respectively) the numbers rose to 62 (ASMR: 0.37) amongst males and 45 (ASMR: 0.19) amongst females in 2009. APC of +18% and +15% of ASMR of IHBD in males and females respectively were observed during the study period.

Liver NOS was noted to be the commonest cause of PLC subtype death in Romania. 1019 males (6.53) and 622 (2.97) females died due to this tumour in 1999. An

inconsistent increase in men (APC: +1%) is seen while the rate was stable amongst women in Romania.

2.3.9 Japan

With the exception of IHBD, HCC and liver NOS realised a falling ASMR in Japan between 1996 and 2008. There were recorded 21,871 deaths among men in Japan and 8443 among women in Japan due to HCC in 1996, corresponding to ASMR of 21.52 and 5.75 /100,000 respectively. These rates have steadily fallen from 1996 to recent values in 2008; 13.02 in males and 3.83 in females (APC: -4% and -3% respectively). A similar, but less rapid decline in the ASMR of liver NOS was also noted.

There was an approximately 35% increase in the total number of deaths due to IHBD in both men and women in Japan between 1996 and 2008. This corresponds to a marginal rise in the ASMR from initial values of 0.79 (males) and 0.46 (females) in 1996 to 1.07 (males) and 0.63 (females) in 2008 (APC of +2% in both men and women).

2.3.10 Hong Kong

In Hong Kong, data from 2001 to 2008 showed falling ASMR in all tumour subtypes in both sexes except for IHBD in males. Deaths due to IHBD affected 120 males (ASMR: 2.53) in 2001, rising to 164 (ASMR: 2.66) in 2008 (APC: +1%). Mortality rates of HCC and liver NOS in both sexes, as well as IHBD in females, declined during the study period.

2.3.11 United States of America

In general, there was a progressive rise in the number of deaths due to PLC subtypes in the US population between 1999 and 2005. The ASMR correspondingly

demonstrated a mild increase with the exception of a static rate in females, exclusive to HCC. Significant and steady increases in the ASMR of HCC and IHBD in men were noticed. The ASMR of HCC in men in America increased from 2.08 in 1999 to 2.45 in 2006 (APC: +3%) and that for IHBD rose from 0.63 to 0.81 correspondingly (APC: +5%). The mortality rate of IHBD increased in women in USA by 4% (Figure **7**).

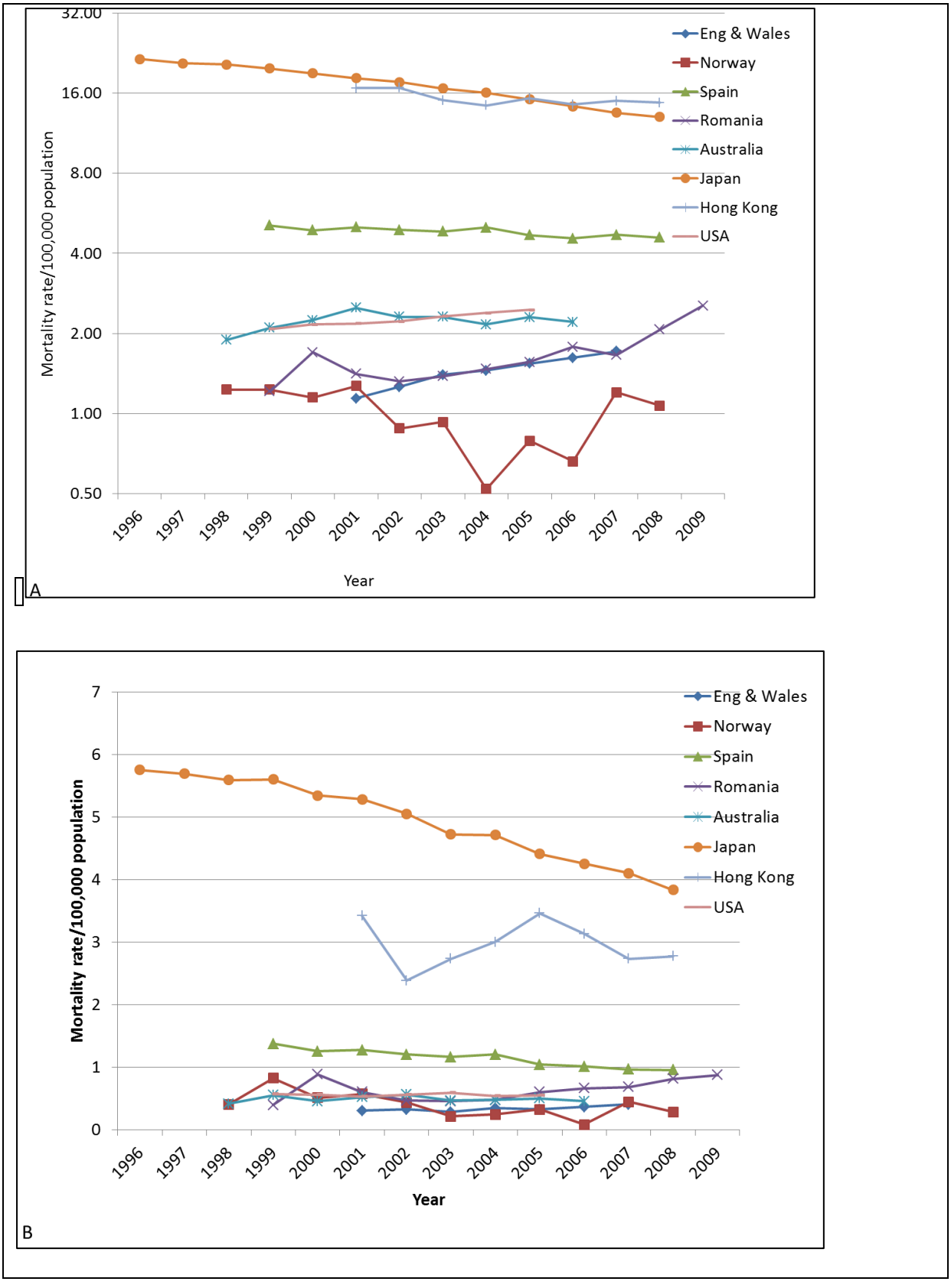

Figure 4. Age-standardised mortality rates of HCC in (A) men and (B) women of selected countries of the world

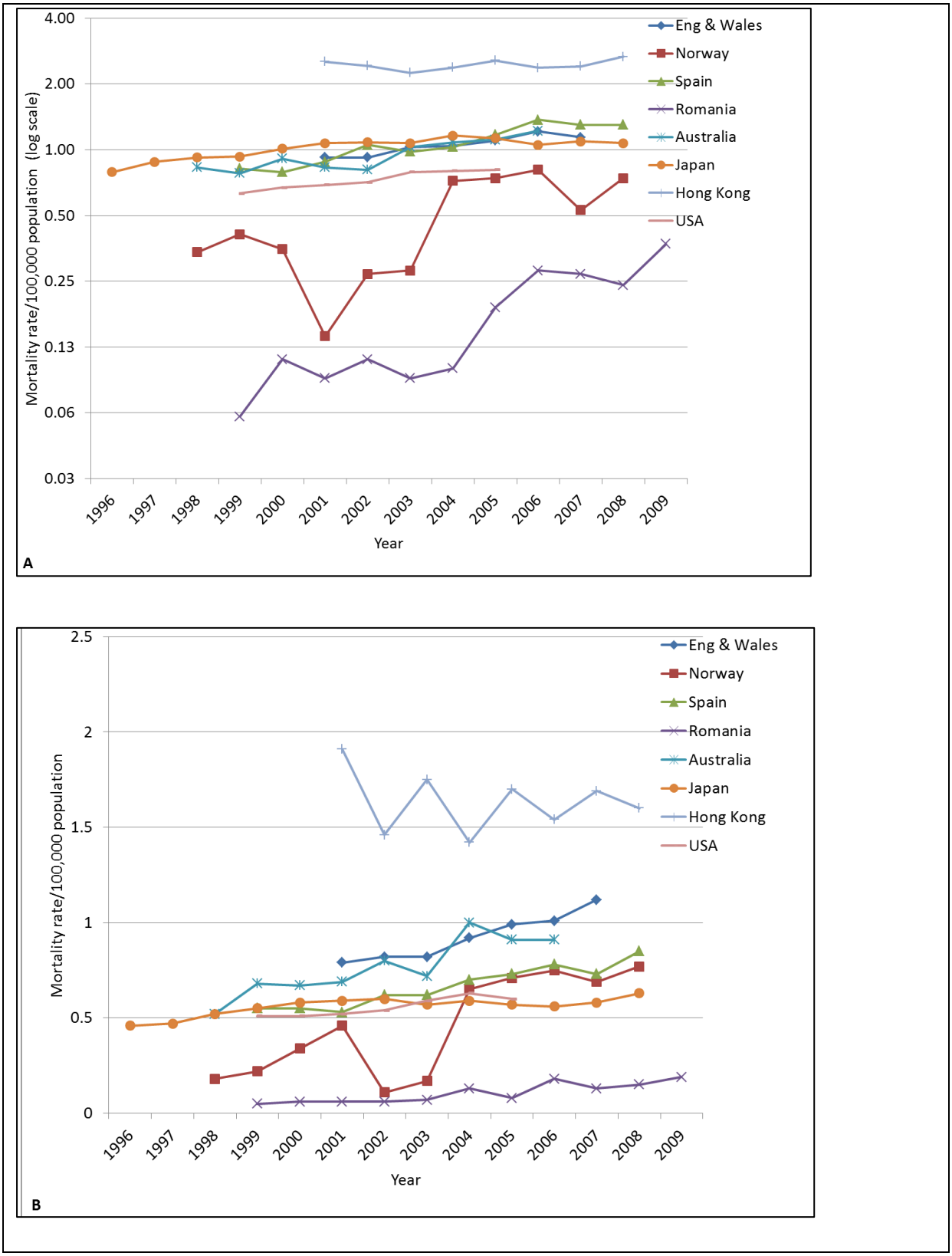

Figure 5. Age-standardised mortality rate of intrahepatic bile duct tumours in (A) men (log scale) and (B) women (numerical scale) in selected countries of the world

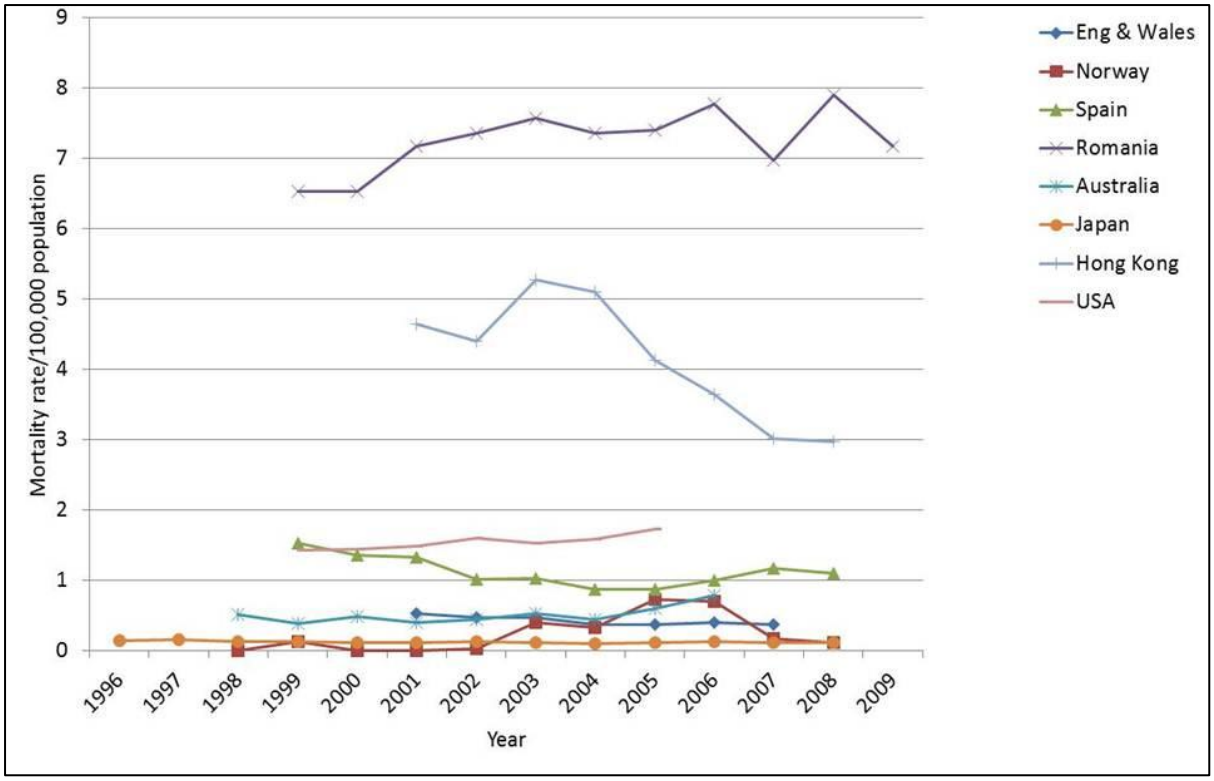

Figure 6. Age-standardised mortality rate of Liver tumours, not otherwise specified (Liver NOS) in men for selected countries of the world

Table 3. ASMR and number of deaths with percentage change for selected primary liver cancer subtypes in men by countries

Tumour	Australia			England and Wales			USA			Japan		
	1998	2006	APC	2001	2007	APC	1999	2005	APC	1996	2008	APC
HCC	1.89(244)	2.21(346)	+1%	1.14(476)	1.71(820)	+7%	2.08(3708)	2.45(4992)	+3%	21.52(21871)	13.02 (20397)	-4%
IHBD	0.83(111)	1.22(214)	+5%	0.92(454)	1.14(608)	+5%	0.63(1208)	0.81(1780)	+5%	0.79(830)	1.07(1684)	+2%
Liver NOS	0.52(67)	0.79(126)	+5%	0.53(237)	0.37(195)	-6%	1.44(2721)	1.73(3722)	+3%	0.15(162)	0.12(195)	-2%

Table 3 continued

Tumour	Hong Kong			Norway			Spain			Romania		
	2001	2008	APC	1998	2008	APC	1999	2008	APC	1999	2009	APC
HCC	16.69(736)	14.75(774)	-2%	1.23(48)	1.07(45)	-4%	5.09(1703)	4.59(1889)	-1%	1.21(191)	2.54(412)	+5%
IHBD	2.53(120)	2.66(164)	+1%	0.34(9)	0.74(30)	+11%	0.82(273)	1.30(569)	+6%	0.06(9)	0.37(62)	+18%
Liver NOS	4.65(211)	2.98(176)	-7%	0.00(0)	0.12(6)	-8%	1.54(565)	1.11(498)	-4%	6.53(1019)	7.17(1188)	+1%

Table 4. ASMR and number of deaths with percentage change for selected primary liver cancer subtypes in women by countries

Tumour	Australia			England and Wales			USA			Japan		
	1998	2006	APC	2001	2007	APC	1999	2005	APC	1996	2008	APC
HCC	0.41(64)	0.45(83)	0%	0.30(171)	0.40(245)	+4%	0.57(1382)	0.52(1525)	0%	5.75(8443)	3.83(9783)	-3%
IHBD	0.52(93)	0.91(184)	+7%	0.79(537)	1.12(763)	+6%	0.51(1344)	0.60(1750)	+4%	0.46(688)	0.63(1382)	+2%
Liver NOS	0.30(48)	0.27(57)	+5%	0.25(167)	0.13(103)	-9%	0.67(1945)	0.71(2225)	+1%	0.07(115)	0.06(145)	-2%

Table 4. continued

Tumour	Hong Kong			Norway			Spain			Romania		
	2001	2008	APC	1998	2008	APC	1999	2008	APC	1999	2009	APC
HCC	3.42(166)	2.77(194)	0%	0.40(26)	0.28(19)	-10%	1.37(696)	0.95(652)	-4%	0.39(77)	0.87(205)	+5%
IHBD	1.91(110)	1.60(123)	-1%	0.18(6)	0.77(45)	+17%	0.55(283)	0.85(558)	+5%	0.05(9)	0.19(45)	+15%
Liver NOS	1.37(75)	0.91(65)	-8%	0.00(0)	0.13(13)	-1%	0.62(358)	0.41(300)	-5%	2.97(622)	2.88(680)	0%

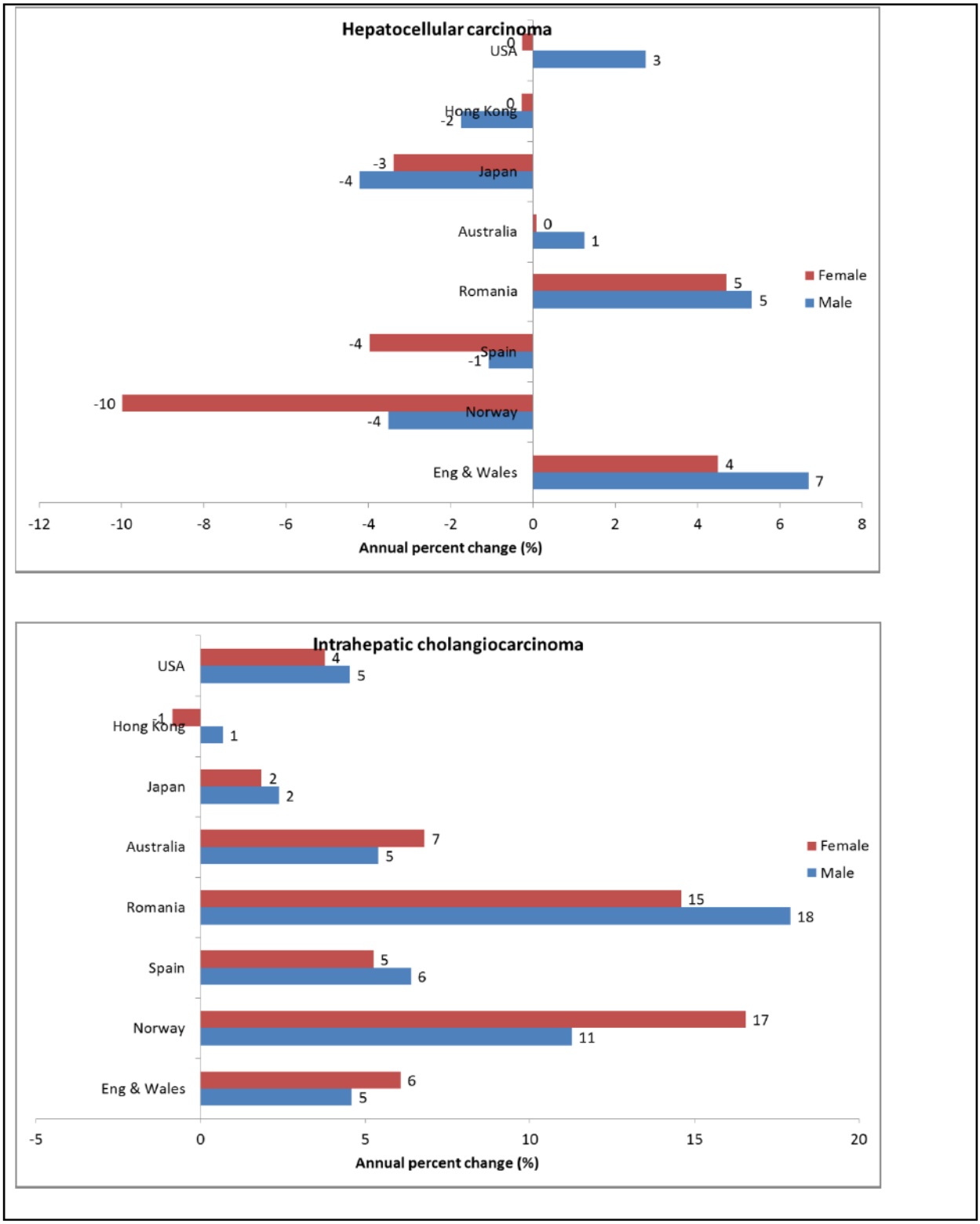

Figure 7. Annual percent changes of mortality rates of hepatocellular carcinoma and intrahepatic cholangiocarcinoma for by countries

2.4 DISCUSSION

2.4.1 Hepatocellular carcinoma

This study has found a distinct pattern of mortality from HCC for countries that were included in the present study, demonstrating salient changes from earlier findings by Khan and co-workers who had reported increases in ASMR of HCC in France, Japan, Australia and the USA between 1979-1997 (Khan *et al*., 2002). In contrast to their data, declining ASMR of HCC in both sexes in countries including Japan, Spain and Hong Kong were observed. Conventionally, Japan and Hong Kong have been described as high incidence countries due to the high prevalence of aetiological risk factors for HCC in these countries, while Spain falls within the southern European axis, considered to have intermediate risk. The rest of the countries: namely, England and Wales, Norway, Romania, USA and Australia with rising mortality rates of HCC are classified as low risk countries. I thus report rising trends in ASMR of HCC in low risk and decreasing trends in high risk countries.

Differences in the prevalence and control of HBV and HCV, alcohol consumption, immigration and obesity could be playing significant roles in explaining the distinctive mortality trends observed. HCV has been implicated as a major contributor to the rising incidence of HCC in Western industrialised countries (Di Bisceglie, 1997). While HCV is the most incriminated risk factor in Japan, United States and several European countries, the period of initial spread of infection and implementation of control measures could be responsible for the varied mortality trend of HCC observed across some of the countries studied. For example, molecular clock analyses have estimated that HCV began to infect large cohorts of young people in Japan, southern Europe and North America in 1920s, 1940s and 1960s respectively consequent upon the use of contaminated needles and intravenous drugs

(Armstrong, Alter, McQuillan, & Margolis, 2000). Population-based HCV prevalence rates of Spain, UK and Norway were estimated at approximately 1% and for Romania, above 2%. HBV prevalence of the earlier aforementioned countries is about 2%, while the value is more than 2% for Romania. With the exception of Norway that has ≤ 11L per capita alcohol consumption in adults above 15 years of age, during 2002, all the other 3 European countries had values above 11L per capita (Ribes *et al*., 2008). The findings of this study corroborate the importance of these risk factors, as there were higher mortality rates of HCC in Spain, England and Wales and Norway, in that order. Factors such as alcohol and obesity complicate and accelerate pre-existing liver diseases. Notably, the HCC mortality trend for Romania reflects the enormous additive effects of these risk factors in that the percentage change of mortality rate was highest in that country.

In my study, I could not determine the reason for the low mortality relative to prevalent risk factors for HCC for Romania, but it could potentially be due to a defective coding practice. In Romania, the mortality rate for unspecified liver tumours (Liver NOS) was higher than that for the major subcategories of PLC, namely, HCC and IHBD. Nevertheless, overall the mortality rate for PLC was highest in this Eastern European country compared to the other European countries selected in my study. It is not completely apparent what accounts for this excess mortality rate for Liver NOS in Romania. The impact of coding practices on epidemiological studies of PLC has been well recognised as alluded to by Taylor-Robinson and colleagues (Taylor-Robinson *et al*., 2001). In order to mitigate this, follow-up enquiries by cancer registries could be embarked upon to ensure appropriate assignment of correct International Classification of Diseases (ICD) category to tumours (that is, whenever there were unclear diagnoses on death certificates). Abandonment of this procedure

in 1993 was associated with an increase in the number of deaths assigned to Liver NOS in England and Wales (Office for National Statistics, (ONS)). It is likely that if this rigorous process of checking unclear diagnoses on death certification were embarked upon in Romania, the rates obtained for Romania might well have been different. Studies of ICD coding practice and the basis of diagnosis of PLC (only recorded in cancer incidence data in some countries) may be helpful to determine if some misclassification of HCC to Liver NOS is responsible for the unexpectedly low ASMR of HCC in Romania.

A recent population-based incidence study of HCC in Japan showed that by 2000, the incidence of HCV-related HCC started to decline in both sexes (Tanaka *et al*., 2008). Additionally, HBV control efforts in Japan could be contributory to the success being observed in the declining mortality rate for HCC in that country. Tajiri and colleagues recently reported decreasing trends in the incidence of childhood HCC following the nationwide introduction of passive-active HBV immunisation for high-risk babies in Japan from 1986 (Tajiri *et al*., 2011). The trend in the mortality rate of HCC for Japan in the current study confirms falling mortality rates. Since the spread of HCV in Europe and North America came after Japan, and with widespread practice of primary prevention of HCV, it is anticipated that mortality rates may start to decline within the next two decades in these regions if HCV control measures are optimised. Already, declines in mortality rates have been documented among female populations in Norway, Hong Kong, Spain and the USA; and in men within the first three aforementioned countries. However, recent evidence suggests that emerging risk factors such as obesity, diabetes mellitus and factors promoting alcohol consumption might offset any declines in HCC death rates. Moreover, a global "epidemic" of obesity has been reported by some researchers (Angulo, 2002;James,

Leach, Kalamara, & Shayeghi, 2001a). Also, driven by cultural and marketing factors, alcohol consumption has increased in many industrialised nations (Donato *et al*., 2002b;El-Serag and Mason, 2000a;Hasumura & Takeuchi, 1991;Jewell and Sheron, 2010;Morgan *et al*., 2004b;Pincock, 2003).

Variation in the implementation of guidelines for screening at-risk populations and of management of HCC could contribute to the differences in the ASMR observed between the countries studied. Surveillance programmes for HCC could potentially improve early detection and hence survival of patients with HCC as they are amenable to curative therapy. Improvement in survival from HCC as a result of surveillance has been shown in reports from China (Wong *et al*., 2008), Japan (Tanaka *et al*., 2006) and Spain (Zapata *et al*., 2010). This benefit however, is nullified if no facilities exist for the treatment of patients detected during such screening programmes. The utilisation of screening and surveillance programmes for HCC is unlikely to be pursued with similar rigour in all countries studied. For example, in the USA, although, 84% of hepatologists reported to have routinely screened their patients for HCC by 1998 (Chalasani *et al*., 1999), a recent study found that routine surveillance in 13,002 cirrhosis patients was adequately carried out in only 12% of the patients (Davila *et al*., 2011). Although, that study did not determine if missing screening was due to failures of the physician to recommend tests or patients to adhere to investigations, it demonstrates a low adherence to guidelines. This lack of adherence to surveillance guidelines may be mitigating the control efforts in many countries of the world. The management of HCC in Japan has achieved remarkable success, owing to widespread adherence to their national guidelines that led to the detection of early stage HCC nodules in more than 60% of surveyed patients, reported in one study (Izumi, 2010). Indeed, following the

publishing of the Japanese HCC guidelines in February 2005, it has become a widely adopted HCC treatment strategy in that country (Song *et al*., 2010). This is likely to have contributed to the recent declining trend in the mortality rate for HCC in Japan in this present study. While it is unlikely to be the sole reason for the variation in the death rates due to HCC in the studied countries, studies of utilisation and/or implementation of surveillance for HCC in these countries are warranted.

Immigration factors could be contributory to the observed trends of mortality rate for HCC. Countries that have sustained increases in the ASMR of HCC in this study included England and Wales, USA and Australia. The New York Times reported in 2010 that Europe was the most favoured immigration destination, followed by North America (Anon, 2010). People from less developed countries, with high prevalence of risk factors for HCC, carry with them their original propensity to contribute to the indigenous pool of HCC cases. This has been documented by studies in the USA, Australia and England and Wales. In the USA for example, the incidence of liver cancer was found to be higher among Japanese living in Hawaii than among native Hawaiians (Maskarinec & Noh, 2004). Similar findings have been documented by studies in England and Wales (Haworth *et al*., 1999a) and Australia (Khlat *et al*., 1993).

Furthermore, improved management of end-stage liver disease could paradoxically add to the sustained increase in the mortality rate for HCC in some of the developed countries. Improvement in the management of cirrhosis of the liver may lead paradoxically, to an increase the mortality from HCC, especially when there is sub-optimal concurrent surveillance and management of incident cases. For example, reviewing the risk for HCC in decompensated alcoholic cirrhosis, Morgan and co-workers found that significant alcohol consumers who abstained had higher

incidence of HCC than those who continued to drink (Morgan, Mandayam, & Jamal, 2004a). Thus with increasing accessibility of advanced techniques for the management of end stage liver disease, there should exist resilient screening and treatment programmes for incident cases of HCC.

2.4.2 Intrahepatic bile duct carcinoma

Similar to the findings of earlier reported data, mortality from IHBD has been rising in both sexes in all the countries studied. It is notable that this tumour continues to be rising as a cause of death in Japanese men and women despite declining rates of HCC from 2000 in this population. While marked increases were observed for Norwegian, Romanian, Australian and Spanish populations, the trends indicated smaller increases in Japan, England and Wales and the USA. Only females from Hong Kong failed to demonstrate a rise in IHBD mortality.

This widespread global increase in the mortality rate for IHBD was first reported by Khan *et al.* in 2002. An increase in the incidence of aetiological risk factors might be responsible for this global trend. Of all the risk factors, primary sclerosing cholangitis (PSC) is the commonest predisposing in western industrialised world. It is however, known that PSC-related IHBD commonly occurs in young people, peaking in the fifth decade (Shaib *et al*., 2004). One would expect a change in the trend towards younger age of diagnosis for this tumour if PSC were the reason for the increase. PSC incidence has not been shown to be rising and the increase in mortality in this study was higher in patients above 75 years of age. It would be expected that a marked rise in middle age group would be recorded if PSC were the reason for the increasing trend in mortality from IHBD. Environmental toxins might have some aetiologic role in the current trend. Spatio-temporal analysis of IHBD mortality between 1981 and 2004 in England and Wales has shown strong evidence for spatial

clustering in rural areas, suggesting a role for environmental carcinogens in these agricultural land use areas (Khan SA *et al*., 2008). Liver flukes in Southeast Asia are established risk factors for IHBD and for which reason, this tumour is one of the commonest malignancies in that region (Nakanuma Y *et al*., 1997). However, these flukes are not common in western industrialised countries.

The progressive increase in the ASMR of IHBD may also represent an artefactual pattern, due to improved diagnostic yield (case ascertainment) and/or misclassification from ICD coding (diagnostic transfer). Improved case ascertainment from increased availability of better imaging modalities (CT, MRI and ERCP) could potentially be responsible for the increase in the ASMR of IHBD. These could have enhanced better characterisation of hepatobiliary tumours that would have previously been described as unspecified or incorrectly as extra-hepatic cholangiocarcinoma. This does not seem to be the case as an expected increase in the detection of early stage disease, consistent with improved diagnostic yield and survival is yet to be documented (Shaib, Davila, McGlynn, & El-Serag, 2004). Also, the expected plateau in the epidemiological trend characteristic of increases due to improved case finding has not occurred in any of the countries. Additionally, since the definitive diagnosis of IHBD is based not only on imaging, but also on clinical and histological parameters, better designed epidemiological studies may help to determine the contribution of case ascertainment to the observed trends.

Errors in death certification may impact the mortality trend of IHBD (Sharp *et al.,* 2001). Diagnostic transfer between hilar cholangiocarcinoma and IHBD has been documented to be responsible for 13% overestimation of IHBD and underestimation by 15% of extrahepatic cholangiocarcinoma by Welzel and colleagues (Welzel *et al*., 2006) in 2006 in the USA. Allowing for this, the authors still found an overall increase

in IHBD in the US during the period of the study (1979-2001). In England and Wales, there was a 94% incorrect coding of hilar cholangiocarcinoma as IHBD, during the period 1990-2004 (Khan SA *et al*., 2010). Diagnostic transfer is common especially due to cross referencing to the topographic code C22.1 of Klatskin tumours (histology coded, M8162/3) in the second version of the ICD-oncology of 1994. It is pertinent to note that coding practices differ between countries and might present with the pattern of differences in the ASMR observed. Transfers can also occur between Liver NOS and IHBD. This does not appear to be significant as shown by the results obtained in this study, especially as the percentage increase in the mortality rate for IHBD is not commensurate with the percentage decrease in ASMR of Liver NOS in the respective countries. Moreover, there were observed simultaneous increases of ASMR of Liver NOS and IHBD in some of the countries studied. Thus diagnostic transfer alone does not explain the rising pattern observed in the mortality trend of IHBD in the countries studied. This widespread rise in IHBD mortality is therefore likely secondary to a genuine global increase in the incidence of this tumour.

2.4.3 Summary and conclusions

Mortality rates of IHBD have been increasing in all countries studied. In contrast, there was an inconsistent pattern in the mortality trend for HCC. While countries that were previously known to have high prevalence of risk factors for HCC are recording declining mortality rates, there were substantial increases in mortality rates of HCC in Romania, England and Wales, Australia and the USA. Liver NOS was the most common PLC subtype in Romania, where a marked increase in the mortality rate of HCC in both genders was found. Differences in risk factors and ICD coding practices, and immigration from regions highly endemic for risk factors to some of the industrialised nations could all play roles in the observed mortality trends. Moreover,

variations in the prevention of risk factors, such as HBV vaccination, surveillance of at-risk populations and management of HCC may also be contributory.

If diagnostic transfer could be ruled out, predominantly rising trend in the mortality rate for IHBD could reflect a true increase in the risk factors of this tumour. It could also mean certain co-factors (possibly, genetic), acting on the established mutual risk factors for HCC and IHBD to determine the eventual outcome to either, to account for the differential trends. Additionally, the rising mortality rate for IHBD, despite falling rates for HCC in Japan confirms the poorer prognosis of IHBD and which may in part explain the relentless increases observed in the ASMR of IHBD in studied countries.

Future research directions

To fully understand the reasons for the changing trends in the mortality rate for PLC subcategories, further epidemiological studies will be required. A concurrent study of the incidence and mortality trends of HCC, IHBD and Liver NOS, would suggest whether there has been improvements in the survival from PLC during this period. Furthermore, with the continued high mortality rates of HCC in some of the countries, there is an urgent requirement for more sensitive screening tools and early detection of this lethal tumour. More accurate, reliable and readily available screening tools in resource-limited countries that are universally applicable would also contribute immensely towards curbing the menace of these tumours, particularly in developing nations where much less is known about the most current population based mortality rates.

3. INCIDENCE AND MORTALITY OF PRIMARY LIVER CANCER IN ENGLAND AND WALES

3.0 ABSTRACT

Background: Mortality rate of PLC in England and Wales have been rising. Studies of changes in the modes of diagnosis and ethnic distribution of this tumour in England and Wales are yet to be embarked upon. This study aimed to explore recent trends, modes of diagnosis, ethnic distribution and the mortality to incidence ratio of primary liver cancer by subtypes in England and Wales.

Methods: Incidence (1979-2008) and mortality (1968-2008) data for primary liver cancer for England and Wales were obtained from the Office for National Statistics (ONS), being maintained by the Small Area Health Statistics Unit of Imperial College London. The age-standardised incidence and mortality rates of HCC, IHBD and Liver NOS were calculated. Trends in rates and basis of diagnosis of PLC and subcategories: hepatocellular carcinoma, intrahepatic bile duct and unspecified liver tumours, were analysed over the study period. Also, changes in the mode of diagnosis and distribution of these tumours by ethnic groups were explored. Partial least squares regression analyses were used to calculate the degree of change. I further extrapolated the estimated 5 and 10 year survival patterns of these tumours using complement of mortality to incidence ratio (1-MIR).

Results: Age-standardised mortality rate of PLC overall increased in both sexes: from 2.56 and 1.29/100,000 in 1968 to 5.10 and 2.63/100,000 in 2008 for men and women respectively. For men, the annual percent changes in the mortality rate of hepatocellular carcinoma and intrahepatic bile duct carcinoma were +2% and +9% respectively. The use of histology for confirmation of primary liver cancer diagnosis

increased from 35.7% of registered cases in 1993 to plateau at about 50% during 2005 to 2008. Afro-Caribbeans comprised 3% and 5% of hepatocellular carcinoma and intrahepatic bile duct carcinoma registrations, respectively. Survival from PLC is estimated to get poorer in 10 years (2018) relative to 2008, particularly as a result of IHBD.

Conclusions: Incidence and mortality of PLC, and particularly IHBD, have continued to rise in England and Wales. Changes in the modes of diagnosis may be contributing. Differing patterns in immigrant populations need to be monitored in the future.

3.1 INTRODUCTION

The incidence of primary liver cancer (PLC), otherwise coded as malignant neoplasms of the liver and intrahepatic bile duct (MNL) in the International Classification of Diseases (ICD) version 10, in many industrialised countries is increasing (Nordenstedt, White, & El-Serag, 2010). Major PLC subcategories include: hepatocellular carcinoma [HCC], intrahepatic bile duct carcinoma [IHBD] and also tumours where the precise histological diagnosis is not specified by the reporting physicians [Liver NOS]. Previous epidemiological studies of PLC in England and Wales have documented increases in mortality and incidence rates, but these data have yet to be updated covering the past decade (Haworth, Soni, V, & Balarajan, 1999a;Taylor-Robinson *et al*., 2001). Taylor-Robinson and co-workers reported that the mortality rate of IHBD in England and Wales almost doubled between 1979 and 1999, while rates of HCC remained stable (Taylor-Robinson *et al*., 2001). A study of the incidence of liver cell cancer by West and colleagues during 1971-2001, reported a rising trend of HCC among men in England and Wales (West *et al*., 2006). The rate of PLC is indeed likely to be higher in migrant populations (especially from regions known to be endemic to risk factors associated with PLC) than in the indigenous white population.

3.1.1 Rationale for study

A further study of the incidence and mortality of PLC will update information on rising trends in order to ascertain if these have continued or not. The need to update these trends is also borne out of the alarming increasing chronic liver disease death rates in the UK while deaths secondary to cardiovascular, blood, respiratory diseases have been falling at the same time (Figure 8). The current global epidemic of obesity (James *et al*., 2001b;James, 2008) and increased alcohol consumption (Pincock,

2003); both factors associated with HCC may be contributing to the burden of PLC. Additionally, the need to evaluate intervention endeavours, such as provision of guidelines (Khan *et al*., 2002a;Ryder, 2003) and advances in the management of PLC engendered this study. Moreover, a combined study of mortality and incidence can better provide information on the control of risk factors and survival of cases than would be available from either alone. Furthermore, the modes of diagnosis of the different tumour subtypes of PLC in the England and Wales will be studied concomitantly. This aspect of the study will lend support, in part, to the validity of the diagnosis of PLC in England and Wales.

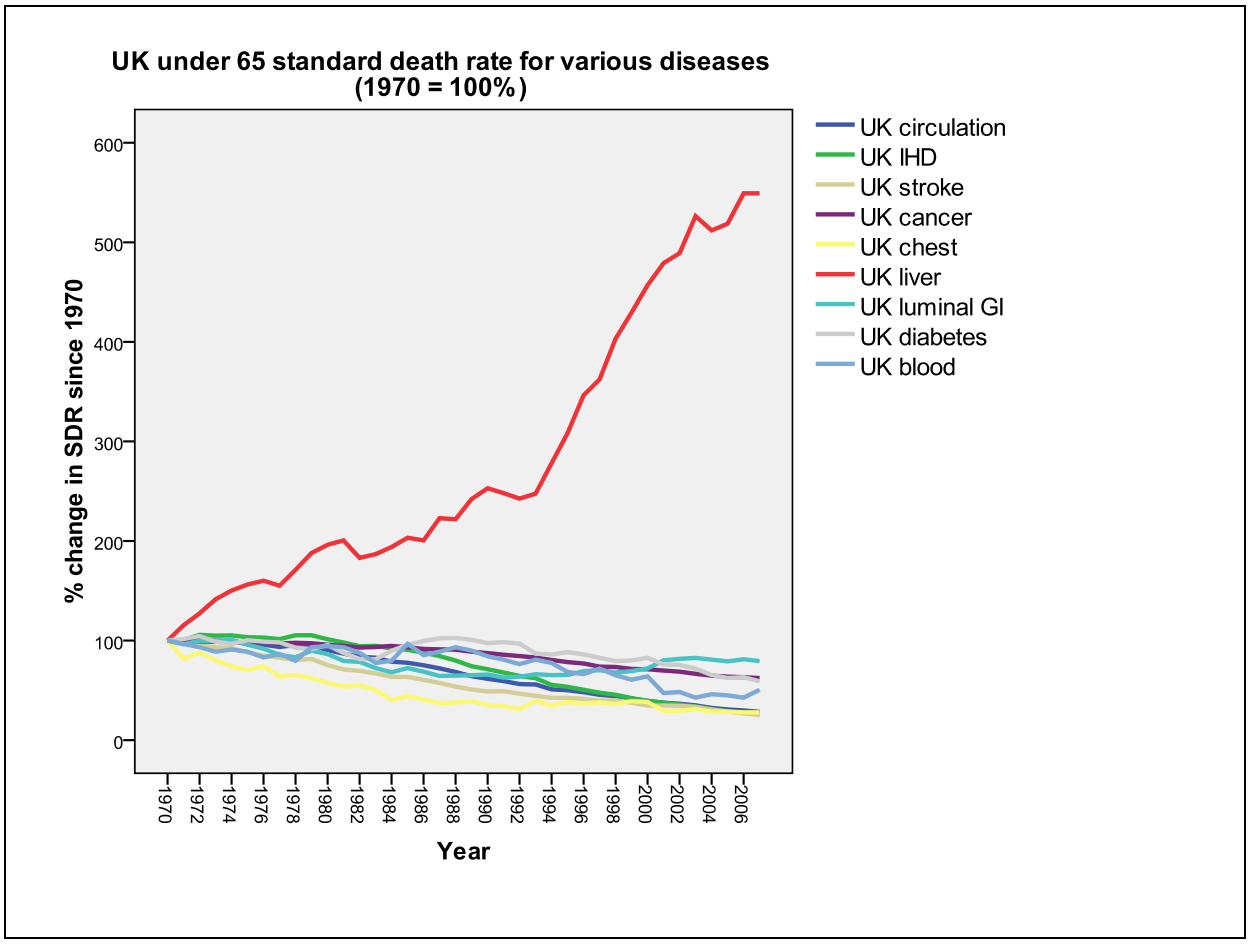

Figure 8. Standard death rates percentage changes of common diseases in the UK from 1970-2006 (acknowledgement from: British Liver Trust)

This study of the incidence and mortality of PLC will not only update previously documented trends, but may garner insights into the impact of diagnostic and management algorithms on PLC trends during the last decade. With rising rates in deaths from chronic liver disease during 1970 through to 2006, and also reported increases in alcohol consumption (Pincock, 2003), it is hypothesised that mortality from PLC in England and Wales is increasing. I analysed trends in the mortality and incidence rates of PLC overall and PLC subcategories in England and Wales, and examined trends in the basis of diagnosis of PLC. Whereas earlier data during a 5 year period (1988-1992) have suggested that the incidence of cirrhosis and liver cancer were higher among ethnic minorities, this study was limited by the fact that it did not tease out the different subcategories of primary liver cancer, included a group of people in whom PLC is not highly prevalent (20-69 years) and was not based on data from the population (Balarajan and Raleigh, 1997;Haworth *et al*., 1999b). The present study analysed ethnic variations in the incidence of HCC and IHBD using data for the whole population of England and Wales during 1979 to 2008. This work is relevant because previous observed increases in the incidence of PLC in England and Wales is likely related to environmental factors (recent immigration). Defining high-risk populations by ethnicity can provide invaluable information for screening and surveillance programmes. Important also is the fact that population-based relative ethnic distribution of PLC in England and Wales has not previously been reported. The finding of this study could yield information that will be relevant to individualised surveillance and early detection of PLC; as well as lend support to coordinated preventative strategies.

3.1.2 Hypothesis

Incidence and mortality rates of primary liver cancer are continuing to increase in England and Wales.

3.1.3 Aims

- To study the mortality (1968-2008) and incidence (1979-2008) trends with respect to PLC sub-site categories, particularly, HCC, IHBD and Liver NOS in order to determine any change(s) attributable to any specific tumour subtype.
- To study trends in the mode of diagnosis of PLC subtypes during 1993-2008.
- To determine the ethnic distribution of PLC subtypes.

3.2 MATERIALS AND METHODS

Incidence (1979-2008) and mortality (1968-2008) data for PLC [C22 as coded by the 10th version of International Classification of Diseases] and PLC subcategories [C22.0 hepatocellular carcinoma (HCC), C22.1 intrahepatic bile ducts (IHBD), C22.9 liver tumours not otherwise specified (NOS)] were extracted from the National Cancer Registry of England and Wales held at the Small Area Health Statistics Unit (SAHSU) of Imperial College London and maintained by the Office for National Statistics (ONS). Mid-year population estimates by 5-year bands for England and Wales for the period from 1968 to 2008 were obtained from SAHSU and ONS databases. Data were analysed using Epi Info^{TM} statistical software (version 3.5.1, 2008, Atlanta GA, USA). Age-standardised incidence rates (ASIR) and mortality rates (ASMR) per 100,000 were calculated. The European standard population was used as the reference for direct age standardisation. To define patterns of change over time, we evaluated trends in ASMR and ASIR of PLC and IHBD using a least

squares regression line fitted to the natural logarithm of the mortality and incidence rates.

Diagnoses of specific PLC sub-types are usually based on several modalities of investigation. The connotation, 'Basis of Diagnosis', as practiced by coding officers during registration of incident cases of cancer, describes the most advanced method used in making a diagnostic decision for each case. The basis of diagnosis of PLC has been recorded by all Cancer Registries in England and Wales since 1993 based on the following codes: 1 (imaging/radiology); 2 (other special tests such as clinical opinions and tumour markers); 3 (cytology); and 4 (histology). In clinical practice, histology of the liver is hierarchically ranked the gold standard of diagnosis, followed by imaging, cytology and serum tumour markers ("others"), in that order. The proportion of cases diagnosed using these modalities was determined for PLC overall, and specifically for HCC, IHBD and Liver NOS.

Information on ethnicity, recorded at the time of registration (incidence data), was obtained for PLC and subcategories for every year. To reflect regional ethnic affiliation, rather than countries of origin, we categorised Indians, Pakistanis and Bangladeshis together as 'South Asians'. Other ethnic categories were as follows: white, Afro-Caribbeans, black Africans, Chinese, and other. We then calculated the proportion of PLC and subcategories registered in the incidence data, among each ethnic group annually. These proportions were compared with the proportion of each ethnic group in the national population for each year. Projected mid-2008 population estimates by ethnic group released by the Office for National Statistics were utilised to calculate the proportion of each ethnic group for each year (Office for National Statistics (ONS), 2011).

Mortality to incidence ratios (MIR) has been utilized to not only interrogate the completeness of data in cancer registries (Parkin and Bray, 2009), but additionally as a proxy to estimate 5 year survival of cancer patients(Pisani *et al*., 1993). The MIR for PLC overall, as well as for HCC and IHBD by sex were calculated. These were derived from the crude mortality and incidence rates of the various tumours for the last 10 years of the study (1999-2008). The rates were then projected linearly to 2018 and MIR were calculated. The projected 5 and 10 year survival patterns by gender were than calculated using the complement of MIR (1-MIR). This method of survival estimated has recently been validated for site specific tumours (Asadzadeh *et al*., 2011).

3.3 RESULTS

3.3.1 Age-standardised mortality rates (ASMR)

Rising trends in mortality rate of PLC in England and Wales during 1968-1996 has been previously reported (Taylor-Robinson *et al*., 2001). Overall, the absolute mortality and ASMR of PLC continued to increase during 1996-2008 in both males and females. In men, this rose from 1052 cases (ASMR: 3.70 per 100,000) in 1996 to 1731 cases (ASMR: 5.10 per 100,000) in 2008 and in women; from 770 cases (1.93 per 100,000) to 1178 cases (ASMR: 2.63 per 100,000) in the same period (Figure **9**). The trend was more consistent in men than women. For HCC, men experienced a rise in the mortality rate (from 1.49 to 2.60 per 100,000 in 1996 to 2008 respectively) whereas women had a relatively stable rate.

There was a steady increase in the mortality rates of IHBD in both men and women, which persisted with a similar annual percent change of 9% and 10% in men and women respectively during 1968-2008. The numbers of those registered without a specific histological category (Liver NOS) fluctuated during 1968-1990, from which time it started to fall. This was 287 and 219 in 1996 and has fallen to 200 and 130 in 2008 for men and women respectively.

3.3.2 Age-standardised incidence rates (ASIR)

Over the last decade, there was an upward trend in the incidence of PLC among men in England and Wales, contrasted to a relatively static rate among women. There was also a marginal rise in the ASIR of HCC among men contrasted to a non-significant decline in the rate in women (774-1083 and 371-306 cases in men and women respectively) (**Figure 10**).

IHBD markedly increased in both genders (annual percent change [APC]: 10% in both men and women) during 2001 to 2008 (437-639 and 473-672 in men and women respectively). The ASIR of unspecified liver tumours in both sexes has stabilised during 1995 to 2008.

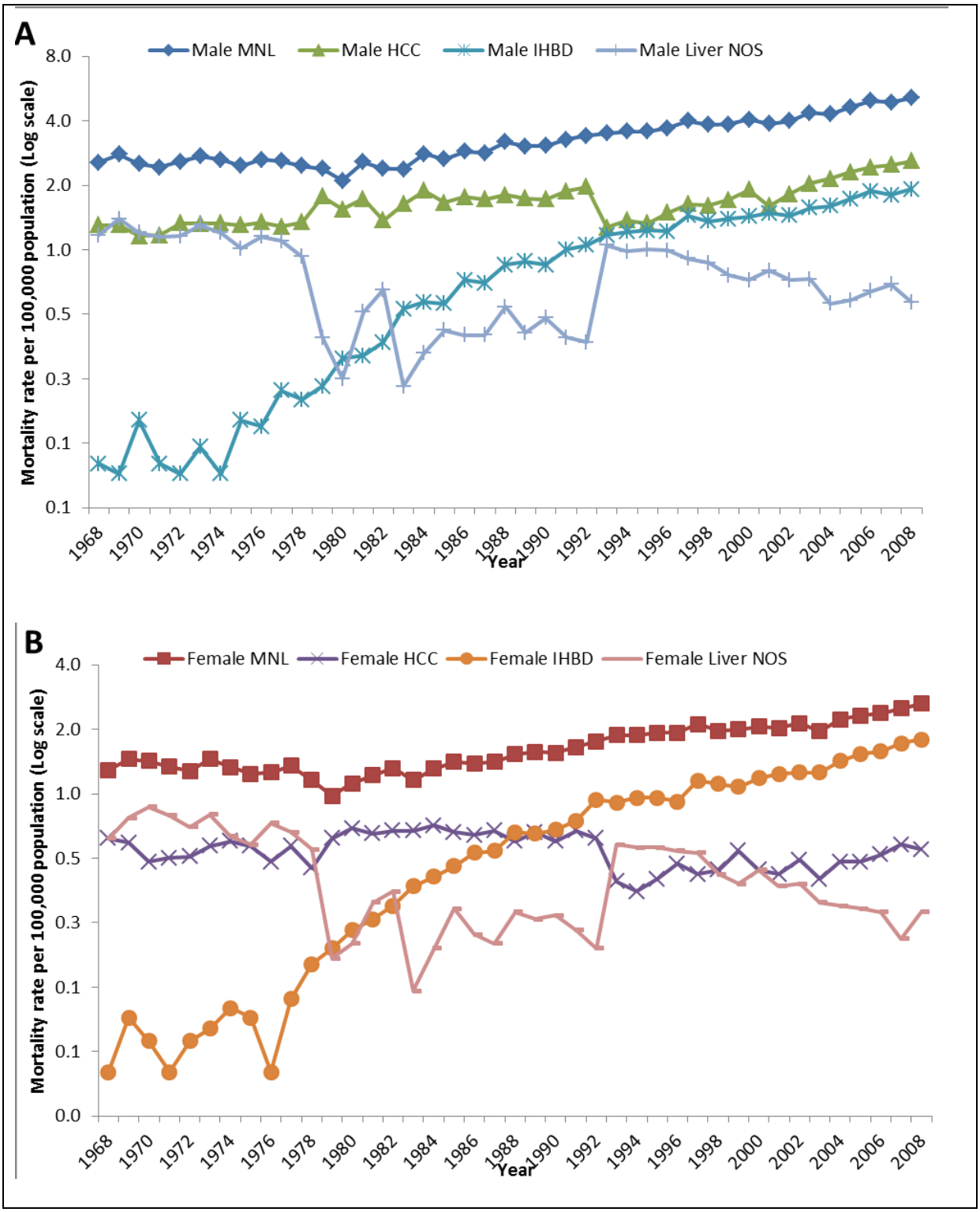

Figure 9. Age-standardised mortality rates of primary liver cancer (MNL), hepatocellular carcinoma (HCC), intrahepatic bile duct carcinoma (IHBD) in (A) menand (B) women in England and Wales, 1968-2008

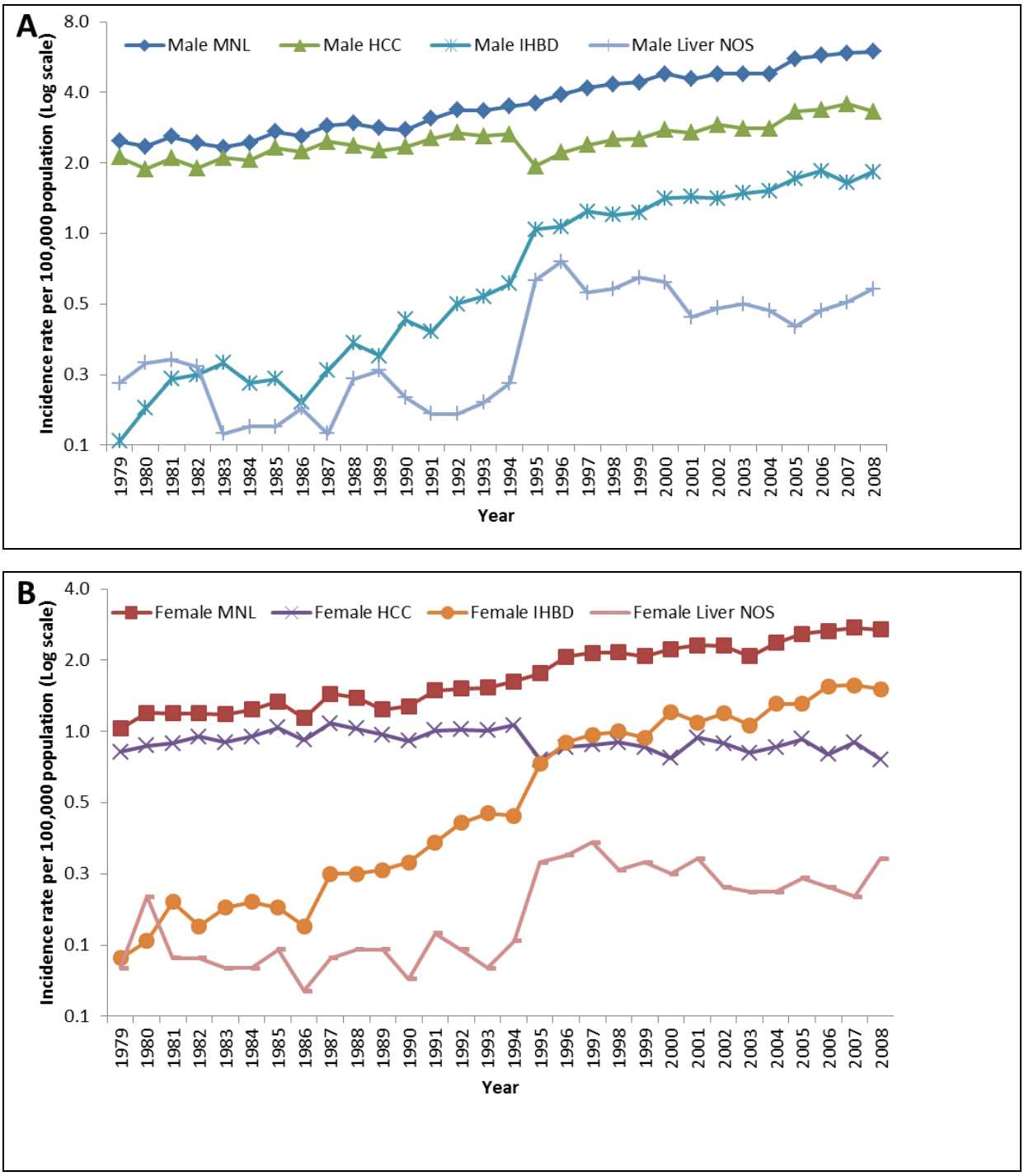

Figure 10 Age-standardised incidence rates of primary liver cancer (MNL), hepatocellular carcinoma (HCC), intrahepatic bile duct carcinoma (IHBD) in (A) menand (B) women in England and Wales, 1979-2008

3.3.3 Basis of Diagnosis

There were increases in the proportion of patients registered to have required histology to establish specific diagnosis of PLC during the last 4 years of the study (2005-2008). Imaging was the basis of diagnosis of HCC from 1993 up until 2005, when it was overtaken by histology (Figure 11). For IHBD, imaging and histology were relied upon with approximately equal proportion during 1993-1997. However, from 1998, histological confirmation was the most common modality on which the diagnosis of IHBD was based. The proportion of PLC registrations having unsophisticated means of diagnosis remained low during 1993-2008. Cytology is increasingly less relied upon in the diagnosis of liver tumours. However, for IHBD, cytology has slightly been increasing and “others” (including: those diagnosed by unsophisticated means, tumour markers and clinical opinion) have dropped dramatically from 31% in 1994 to 5% of cases in 2008.

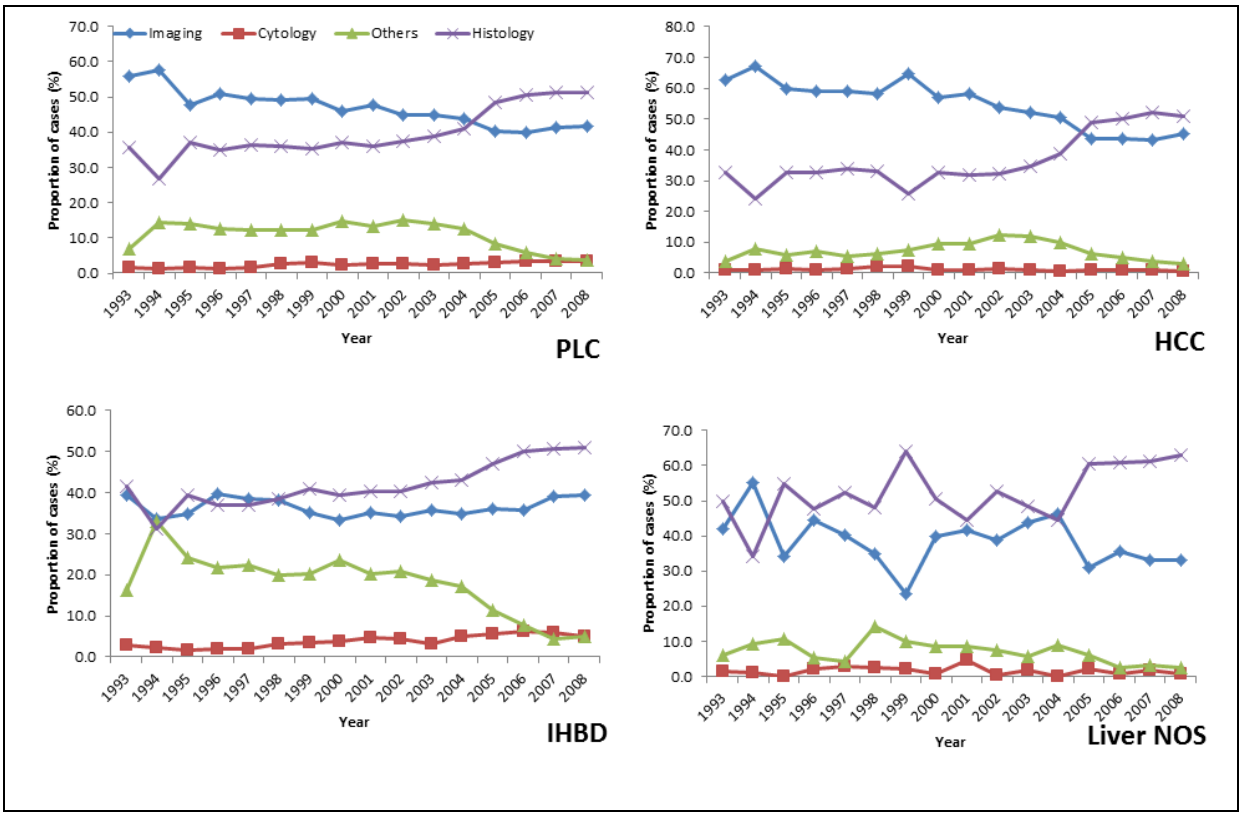

Figure 11. Proportion of registrations (incidence) of PLC, HCC, IHBD and Liver NOS by the basis of diagnosis, England and Wales, 1993-2008 (PLC; Primary liver cancer, HCC; Hepatocellular carcinoma, IHBD; Intrahepatic bile duct carcinoma, Liver NOS; Unspecified liver tumours)

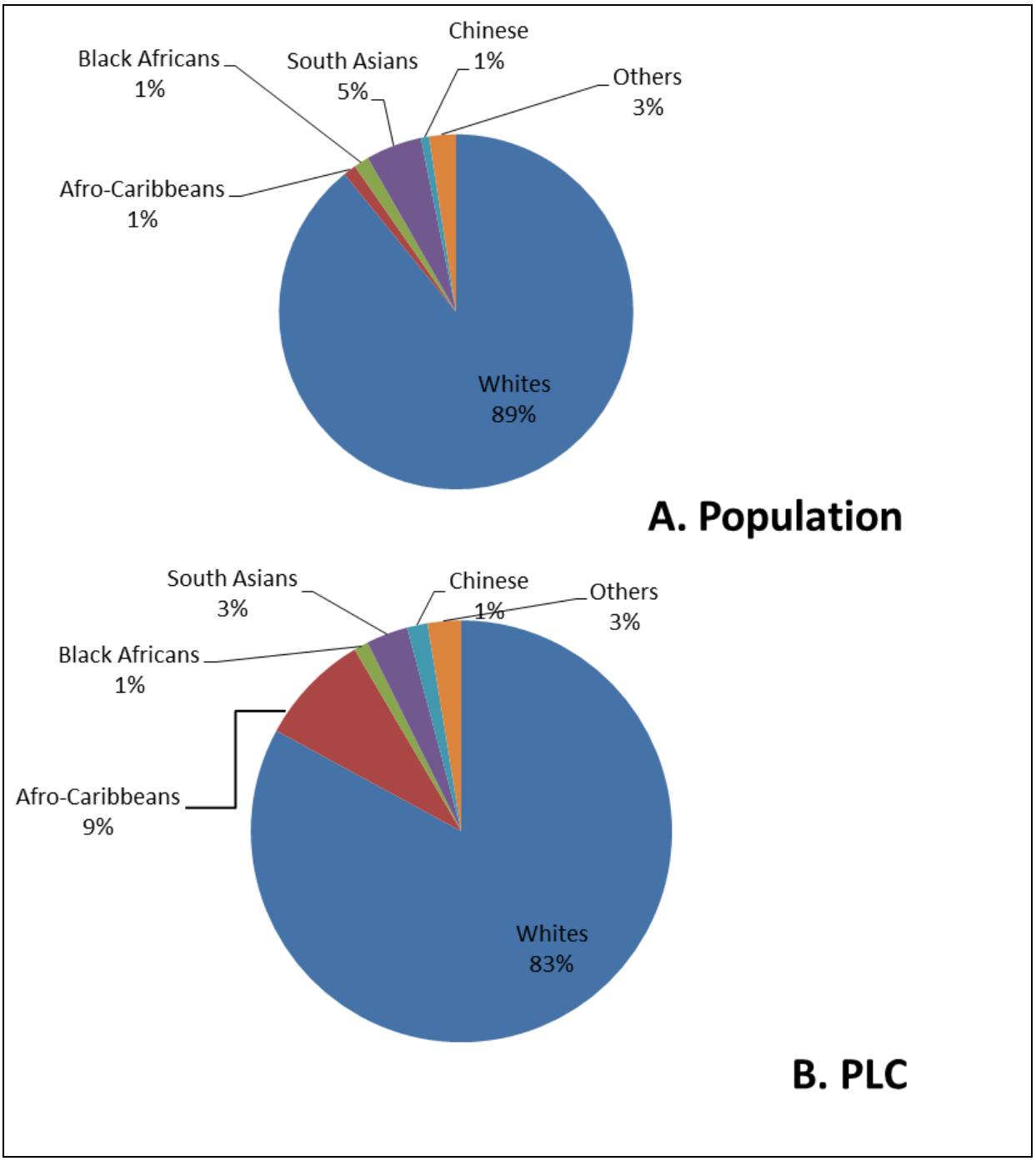

Figure 12. Comparison of ethnic groups in England and Wales (2008), as recorded in the (A) population of England and Wales, and the registration of (B) PLC by ethnic groups; in 2008; PLC: primary liver cancer

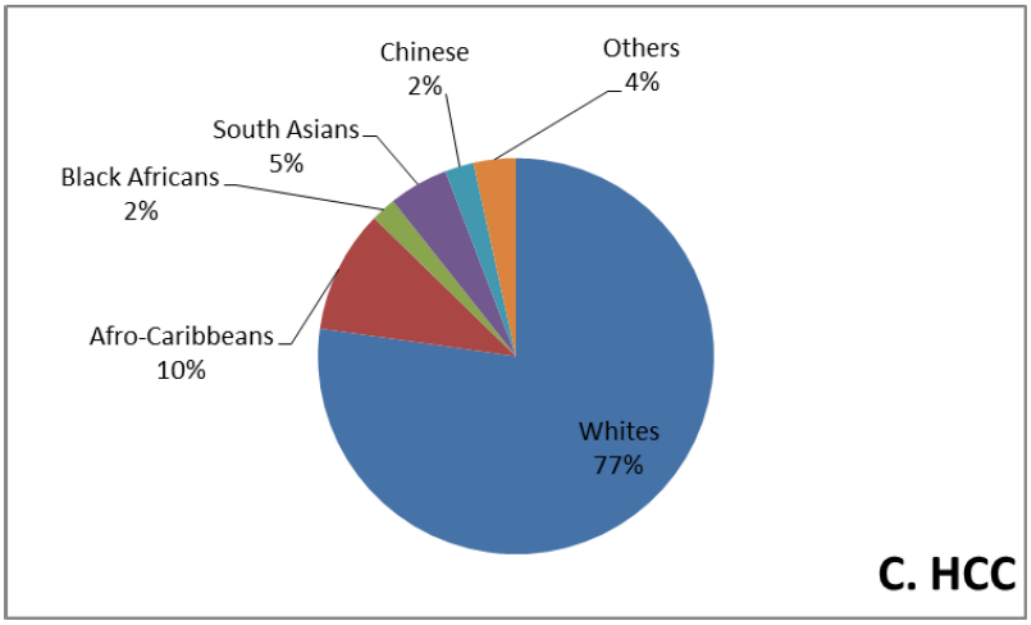

Figure 13. Proportion of ethnic groups registered to have hepatocellular carcinoma (HCC) in England in Wales in 2008

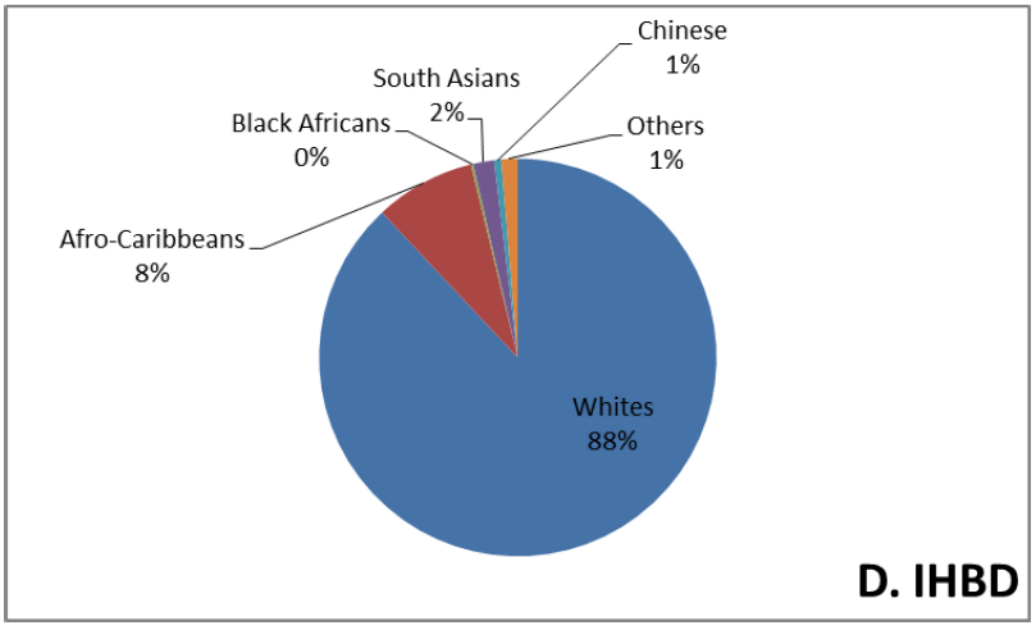

Figure 14. Proportion of ethnic groups registered to have intrahepatic cholangiocarcinoma (IHBD) in England in Wales in 2008

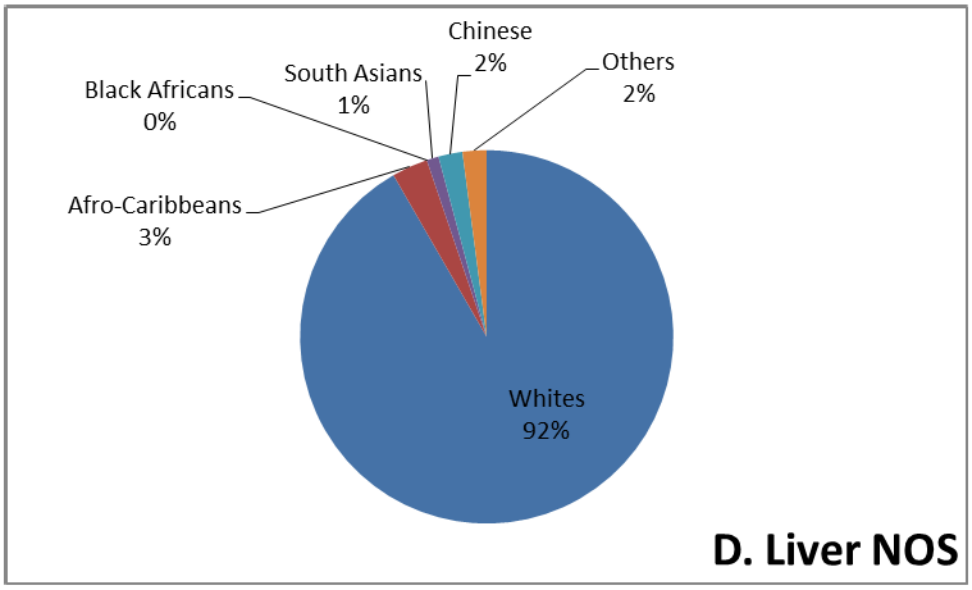

Figure 15 Proportion of ethnic groups registered to have unspecified liver tumours (Liver NOS) in England in Wales in 2008

3.3.4 Distribution of Primary Liver Cancer by Ethnicity

The completeness of ethnic information in the incidence data, recorded during the study period, ranged from 19% to 51% of cases that were registered to have had PLC (Table 5). For HCC and IHBD, there was an initial increase (50.3% and 57.7% respectively) in the proportion of those registered with ethnicity from 1993 to 1998, after which it remained static for two years. Following that, there was a sharp drop in 2001 to 19% for both HCC and IHBD, rising steadily to 42% and 41% respectively in 2007.

Table 5. Cases of primary liver cancer (PLC) in whom ethnicity was registered in the incidence data in England and Wales, 1993-2008 (percentages in parentheses)

Year	Primary liver cancer (PLC)						
	All groups	**Whites**	**Afro-Caribbeans**	**Black Africans**	**South Asians**	**Chinese**	**Others**
1993	563(38.3)	554(98.4)	2(0.4)	0(0.0)	7(1.2)	0(0.0)	0(0.0)
1994	570(36.4)	561(98.4)	1(0.2)	1(0.2)	4(0.7)	0(0.0)	3(0.5)
1995	605(35.9)	587(97.0)	2(0.3)	2(0.3)	7(1.2)	4(0.7)	3(0.5)
1996	888(46.5)	863(97.2)	3(0.3)	3(0.3)	7(0.8)	4(0.5)	8(0.9)
1997	990(48.7)	964(97.4)	1(0.1)	4(0.4)	11(1.1)	7(0.7)	3(0.3)
1998	1075(51.4)	1034(96.2)	5(0.5)	8(0.7)	15(1.4)	5(0.5)	8(0.7)
1999	1003(47.7)	953(95.0)	11(1.1)	3(0.3)	15(1.5)	8(0.8)	13(1.3)
2000	977(41.9)	913(93.4)	24(2.5)	8(0.8)	17(1.7)	5(0.5)	10(1.0)
2001	443(19.1)	365(82.4)	35(7.9)	8(1.8)	11(2.5)	7(1.6)	17(3.8)
2002	529(21.3)	461(87.1)	23(3.9)	8(1.5)	18(3.4)	9(1.7)	10(1.9)
2003	508(20.7)	436(85.8)	20(4.3)	11(2.2)	25(4.9)	3(0.6)	13(2.6)
2004	741(28.4)	647(87.3)	30(4.0)	18(2.4)	20(2.7)	6(0.8)	20(2.7)
2005	908(31.8)	784(86.3)	57(6.3)	17(1.9)	26(2.9)	8(0.9)	16(1.8)
2006	1156(39.0)	962(83.2)	96(8.3)	13(1.1)	49(4.2)	10(0.9)	26(2.2)

2007	1276(41.3)	1058(82.9)	110(8.6)	14(1.1)	41(3.2)	20(1.6)	33(2.6)
2008	1273(40.3)	1135(89.2)	43(3.4)	12(0.9)	34(2.7)	11(0.9)	38(3.0)

The proportion of ethnic groups in the population of England and Wales in 2008 with the corresponding distribution of ethnicity amongst cancer registrations for HCC, IHBD and Liver NOS was compared. Year 2008 was chosen as it is the most recent year for which data were available for the study. White people comprised 89% of the population and the corresponding proportions of cases of IHBD and HCC were 85% and 92% respectively (Figure **12**). In contrast, Afro-Caribbeans constituted about 1% of the population of England and Wales in 2008, yet 3%, 5% and 1% of HCC (Figure 13), IHBD (Figure 14) and Liver NOS (Figure 15), respectively were registered to people whose ethnic background was Afro-Caribbean in that year. Similarly, the proportions of PLC registration in black Africans and Chinese were slightly higher than the proportion of these ethnic groups in the population as a whole (1% versus 2% respectively) (Figure **12**).

3.3.5 Mortality to incidence ratio

Women are projected to experience lower survival than men in general. For HCC, both men and women will experience rising MIR during the period of 10 years from 2008 (i.e. 2018), although this is projected to plateau for men by 2014 (Figure **16**). These data suggest that a higher proportion of women than men with IHBD will survive less.

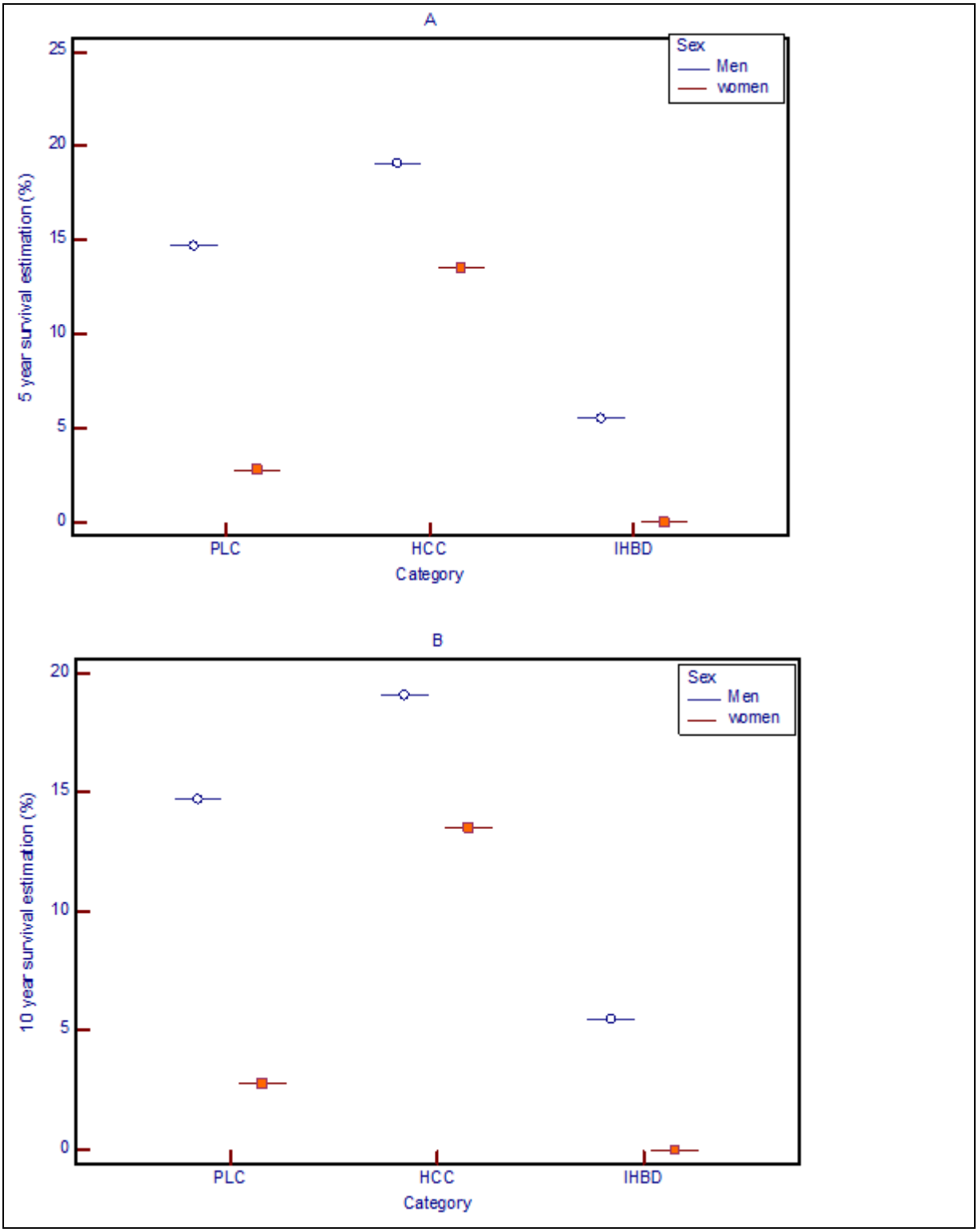

Figure 16. Estimated relative survival (A) 5 year and (10) year projections; obtained from complement of mortality to incidence ratios (1-MIR)*100 that was extrapolated from 2008 to 2018.

3.4 DISCUSSION

This study has presented for the first time both mortality and incidence rates simultaneously for primary liver cancer and shown that they have continued to rise in England and Wales throughout the last decade. The greatest rise was in IHBD in both men and women (44% increase: from 910 cases in 2001 to 1311 cases in 2008). Reassuringly, histological confirmation of PLC subcategories has become more widespread in diagnostic practice and less accurate modes of diagnosis have been declining as the sole mode of diagnostic confirmation. Generally, the registration of IHBD and HCC was proportionately highest among people whose ethnicity was registered as Afro-Caribbean.

Using data gathered towards the end of the last century, Taylor-Robinson and colleagues, as well as West *et al.* in separate studies, reported increases in mortality and incidence of PLC, respectively (Taylor-Robinson *et al*., 2001;West *et al*., 2006). Global studies of PLC was reported by Khan and colleagues which revealed widespread increase in mortality from IHBD in all western countries that were studied (Khan *et al*., 2002b). Whereas a variable mortality trend from HCC was found in studies across countries, the present study noted that both IHBD and HCC were rising in incidence and mortality in England and Wales. In contrast to the other countries, however, the rate of increase in the mortality from PLC in England and Wales was greatest for IHBD, compared to HCC (Khan *et al*., 2002b).

The observed increase in the incidence and mortality rates of PLC in England and Wales is either a result of an epidemiological artefact or real. For instance, misclassification of hilar cholangiocarcinoma to IHBD in increasing the incidence of IHBD has been reported in England and Wales (Khan *et al*., 2011). Another study in

the USA has also shown that although coding error resulted in 15% overestimation of IHBD, there was a significant proportion of increase that was not explained by misclassification (Welzel *et al*., 2006). A true increase may thus underlie the trend in the mortality and incidence being reported in the present study. Furthermore, the fact that both incidence and mortality increased supports a non-artefactual effect and suggests there has not been any significant improvement in survival from PLC, despite changes in the diagnosis and treatment of PLC during the last decade.

While it has been a common cause of liver tumour-related death in England and Wales, IHBD is a less common PLC than HCC worldwide. Aetiological associations of IHBD are ill-understood, and are still being studied. Chronic infestation by liver fluke is associated with IHBD in South-East and East Asia (Shaib & El-Serag, 2004;Watanapa & Watanapa, 2002). Primary sclerosing cholangitis (PSC) is the commonest known predisposing factor for IHBD in the Western world, often associated with up to a tenth of cases of cholangiocarcinoma (Cullen and Chapman, 2003;Levy and Lindor, 2003). However, the incidence of PSC has not been shown to be increasing and thus cannot explain the current rise in the incidence of IHBD (Kingham *et al*., 2004). Environmental toxins and genetic dispositions (Wadsworth *et al*., 2011) have also been noted to be responsible for sporadic cases of IHBD and may underlie some of the observed differences in the present study.

Changes in the basis of diagnosis may have resulted in increased case ascertainment and thus contributed to the rising trends in the incidence and mortality rates observed. These data show that the proportion of cases reported to cancer registries that had histology underpinning the diagnosis of PLC has been increasing. What this suggests is that suspicious “nodules” identified during routine screening or investigations incompletely characterised by dynamic imaging are being subjected to

liver biopsy in many centres across England and Wales and hence diagnosed. Recent technological advances in the management of PLC (Bruix & Sherman, 2005;Bruix & Sherman, 2011;Ryder, 2003), including the setting up of tertiary centres, modern hepatobiliary imaging, image-guided biopsies and widespread availability of magnetic resonance cholangiopancreatography (MRCP) may have contributed to the recent trends. The role of case ascertainment is further buttressed by the rising trend in the histological confirmation of PLC shown in this study, suggesting a more aggressive surveillance for PLC.

It would be expected that an outcome of improved diagnostic yield should lead to detection of early stage PLC and improvement in survival. There is yet a study to be undertaken that describes the impact of changes in screening guidelines and management of PLC in England and Wales. As MIR has been recently validated to be a proxy indicator for cancer survival estimations (Asadzadeh *et al*., 2011), the data in the present work confirm that PLC will continue to be associated with high case fatality, mostly contributed to by IHBD. However, Shaib and colleagues (Shaib, Davila, McGlynn, & El-Serag, 2004), has recently shown that although an increase in the incidence of IHBD was noted during 1975 to 1999, there was neither a significant increase in the proportion of those found with localised disease, nor in the survival of those diagnosed. Lead-time bias could be responsible for the observed mutual increase in the incidence and mortality rates of PLC in the face of improvements in diagnosis.

The exploration of ethnic information confirms that the proportion of sub-Saharan Africans and South Asians living in England and Wales that have PLC is higher than the proportion of indigenous white people (Balarajan & Raleigh, 1997;Grulich *et al*., 1992). My population-based analysis confirms that the registration of HCC among

Afro-Caribbeans, sub-Saharan black Africans and Chinese is higher than among indigenous white people in England and Wales. This finding is similar to a population-based study in the USA in 2008 that reported a higher incidence of HCC among immigrant populations, compared to Caucasians (Wong and Corley, 2008). Higher prevalence of HBsAg carriage status among immigrant populations may in part have predisposed ethnic minorities to HCC. The rates of HCC in people from Asian and African backgrounds were however, not as alarming as expected, perhaps reflecting the positive impact of HBV vaccination in the control of this cancer. A landmark study in Taiwan confirmed that HBV immunisation in children, introduced in 1984 significantly halved the annual incidence of HCC from 0.70/100,000 in 1981-1986 to 0.36/100,000 in 1990-1994 (Chang *et al*., 1997).

The underlying reasons for the higher proportional registration of IHBD in particular among Afro-Caribbean populations are not clear and require further investigation. It suggests either a high prevalence of aetiological factors and/or more willingness to register ethnic information by Afro-Caribbean people (the latter being unlikely). Ulcerative colitis is associated with PSC, which in turn is responsible for about 10% of IHBD (Boberg and Lind, 2011). Reports of PSC and indeed ulcerative colitis among ethnic minorities are scanty. A 15-year study, published 3 decades ago intriguingly observed a significant rarity of ulcerative colitis among Afro-Caribbeans (Benfield and Asquith, 1986). Recent case control and cohort studies have implicated HCV, HBV, cigarette smoking, obesity, gallstones and alcohol consumption, among others to be contributory to IHBD, although some of these associations are weak (Nordenstedt *et al*., 2012;Palmer and Patel, 2012). A descriptive study during the latter part of 2000 found a higher prevalence of obesity,

cigarette smoking, alcohol drinking and cirrhosis of the liver from HCV in Afro-Caribbeans, compared to sub-Saharan black Africans (Mann *et al*., 2008).

HCV-related HCC has been identified as one of many reasons for the rise in HCC incidence in some developed countries, including England and Wales (El-Serag and Mason, 2000b). The incidence of HCV in England and Wales rose significantly between 1960 and 1980 (Sweeting *et al*., 2007), owing to the practice of intravenous drug use (IDU) and the prevalence of HCV in the blood donor system prior to donor screening procedures in 1992. HCC would have only recently started to emerge in patients that were infected during this period as progression to end-stage liver disease (cirrhosis and HCC) can take 25-30 years (Higuchi *et al*., 2002). Alcoholic liver disease has also been reported to have more than doubled between 1990 and 2003 in England and Wales (Thomson *et al*., 2008). With economic and social factors that have promoted consumption of alcohol, the influence of alcohol on the current rising incidence of HCC is likely to be exponential and has contributed to the current observation. A population-based study of the impact of alcohol consumption, obesity and other factors has recently lent support to the contribution of these lifestyle factors to rising incidence of PLC. A recently published case-control study of the European Prospective Investigation into Nutrition and Cancer (EPIC) cohort reported higher odds of HCC in people who smoked cigarettes, drank alcohol and/or were obese, compared to controls (Trichopoulos *et al*., 2011). *Per capita* alcohol consumption in the UK has increased by 150% in the past 50 years (Pincock, 2003)], a factor that could well have added to the rising incidence of HCC.

Diabetes mellitus (DM), a recognised risk factor for HCC, is higher among migrant populations than among indigenous whites of England and Wales (Oldroyd *et al*., 2005). Since the mid-1990s, Gray and colleagues found that DM coexisting with

HCV infection was significantly higher among Afro-Caribbeans than other ethnicities (Gray *et al*., 1995). That study also reported that persistent moderate elevations in serum transaminases of studied patients was higher among Afro-Caribbeans than in other studied ethnic minorities, a factor associated with poor outcome. This may therefore contribute to the higher and rising rate of HCC in Afro-Caribbeans, compared to the rest of studied ethnic minorities. Indeed, a study of obesity among ethnic minorities found that the highest rate of obesity was among Afro-Caribbeans (30%) relative to 19% in the reference white population.

I note that the registration of ethnicity information was lacking in more than 60% of PLC of the ONS database. Although data during the most recent year with 40% registration of ethnic information were utilised in the analysis, it is possible that a disproportionate registration of ethnic information would have skewed the findings. With progressive increases in ethnic registration during liver cancer registration, follow-up analyses should provide a better distribution of PLC by ethnic groups in England and Wales.

The major strength of the present study is the fact that the population cancer registry data of England and Wales were used. I have been able, for the first time, to demonstrate trends in both mortality and incidence together of PLC, the basis of diagnosis and ethnic distribution for the whole population of England and Wales. The information on the basis of diagnosis was objective evidence of the modality of investigation for PLC during the period when data on mode of diagnosis were collected routinely across the country. The ethnic trends in PLC registrations presented here provide important insight on high risk groups and possible focus for prevention strategies. However, due to the limited number of individual registrations recorded with ethnicity, the estimations of ethnic distribution are subject to error,

based on the ethnic proportions in the population as a whole. I advocate further studies to corroborate and expand these findings. To determine the impact of guidelines and of improved management of PLC, information on adherence to surveillance schedules, tumour stage at diagnosis, ethnicity, and survival of patients will need to be gathered. With such a low fatality rate, the search for better performing screening and diagnostic tools should be a priority as current tools have low sensitivities and specificities. Newer techniques, including the use of urinary metabonomics hold some promise but wait on validation experiments. The Imperial group has recently reported the impressive diagnostic performance of panels of urinary metabolites in discriminating HCC in some African populations (Shariff *et al*., 2010;Shariff *et al*., 2011). Until the latter is achievable, follow-up studies of these changing trends in the mortality and incidence of PLC in England and Wales need to be closely monitored.

3.4.1 Conclusion

Mortality from PLC has continued to increase in both sexes in England and Wales. The incidence of IHBD increased in both genders, while that of HCC increased only among males. Increasing use of histological confirmation of PLC and subcategories lends support to better characterisation of liver tumours. While the proportional registration of all major categories of PLC was greatest in Afro-Caribbeans, there were modestly higher proportions of sub-Saharan black Africans and Chinese with HCC, compared to indigenous white populations. Better characterisation of PLC is being achieved in England and Wales, providing opportunities for targeted preventive programmes.

3.4.2 Future research directions

The pattern of change in mortality trends for PLC warrants a close examination of data to study the epidemiological factors underlying the current rapid increase in incidence and mortality of PLC in general and IHBD in particular. Further studies of the pathogenesis of HCC and IHBD are engendered.

Additional data on the impact of changing patterns of risk factors, role of increased alcohol consumption and contribution of obesity will be helpful to facilitate successful preventative measures. There is also need to evaluate adherence to screening and surveillance guidelines by physicians and patients for the detection of potentially treatable tumours to determine how this impacts the current incidence and mortality trends.

4. ROLE OF TUMOUR MISCLASSIFICATION IN THE RISING TRENDS IN INTRAHEPATIC BILE DUCT CARCINOMA IN ENGLAND AND WALES

4.0 ABSTRACT

Background: Cholangiocarcinomas (CC) can be sub-divided into intrahepatic (IHBD) or extrahepatic (EHCC). Hilar, or 'Klatskin', tumours are anatomically extrahepatic. Most international studies, including England and Wales, report increasing IHBD and decreasing EHCC incidence. The second edition of the International Classification of Diseases for Oncology (ICD-O-2) assigned 'Klatskin' tumours a unique histology code (8162/3), but this was cross-referenced to the topography code for intrahepatic (IHBD), rather than extrahepatic bile duct carcinomas (EHCC). Under the third ICD-O edition, Klatskin tumours are cross-referenced to either IHBD or EHCC. The impact of changing ICD-O classifications and the potential misclassification of hilar/Klatskin tumours on IHBD incidence rates in England and Wales was studied.

Methods: Age-standardised incidence rates (ASIR) of IHCC and EHCC in England and Wales between 1990 and 2008 were calculated. I then transferred all 'Klatskin' tumours from IHBD to EHCC and reanalyzed rates from 1995, when ICD-O-2 was introduced in England and Wales.

Results: In England and Wales, during 1990-2008, ASIR of IHBD rose from 0.43 to 1.84/100,000 population in males and from 0.27 to 1.51/100,000 in females; but fell for EHCC (0.78-0.51; 0.62-0.39). After transferring all 'Klatskin' tumours from IHBD to EHCC, there remained a marked increase in ASIR of IHBD and a decrease in ASIR for EHCC, as only 1% of CC were reportedly Klatskin.

Conclusions: Changes in ICD-classification may be influencing observed changes in IHBD and EHCC incidence rates. Coding misclassification is likely to have been skewing CC registration to an intrahepatic site, thereby contributing to the rise in intrahepatic bile duct tumours.

4.1 INTRODUCTION

Cholangiocarcinoma (CC) is a lethal tumour, arising in the epithelium of bile ducts. CC is divided into intrahepatic cholangiocarcinoma (IHBD) and extrahepatic (EHCC). IHBD is the second most common PLC worldwide, after HCC (Khan *et al*., 2008). CC arising at the liver hilum (hilar CC) is anatomically defined as a subset of EHCC, since the bifurcation of the hepatic ducts lies outside the liver parenchyma. IHCC are conventionally documented to account for 5-10% of all CC cases; hilar CC for 60–70%; and EHCC for 15–20% (Khan *et al*., 2002a;Klatskin, 1965a;Nakeeb *et al*. 1996). The eponym 'Klatskin' tumour has been adopted for hilar CC, particularly in the USA, after the American hepatologist who first described the unique features of these tumours in 1965 (Klatskin, 1965b). The terms 'hilar' and 'Klatskin' are used interchangeably.

IHBD and EHCC have distinct clinical and morphological features (Khan *et al*., 2002a;Klatskin, 1965b;Nakeeb *et al*., 1996). Previous epidemiological studies from England and Wales showed that ASMR of IHBD increased markedly over a 30-year period after 1968, from 0.10 to 1.49 in men and 0.05 to 1.24 in women (Taylor-Robinson *et al*., 2001). There was a 15-fold increase in age-specific mortality rates in those aged 45 years and above; and since 1993, IHBD are the commonest recorded cause of liver tumour-related death in England and Wales (Taylor-Robinson *et al*., 2001). Age-standardized incidence rates (ASIR) for IHBD increased concomitantly, approximately 12-fold (West *et al*., 2006). These studies showed an accompanying fall in mortality and incidence rates for EHCC (Taylor-Robinson *et al*., 2001;West *et al*., 2006). Recently, a number of international studies have reported increasing mortality and incidence rates for IHBD and decreasing rates for EHCC, over the last

few decades (Khan *et al*., 2002;McGlynn *et al*., 2006;Patel, 2001;Patel, 2002;Shaib & El-Serag, 2004;Shaib *et al*., 2004).

The reasons for these dynamic trends in different sub-groups of CC are unclear. The trends may reflect genuine changes in the incidence of these tumours. However, given the complexity over how CC are classified and several revisions of the International Classification of Diseases (ICD) coding system for liver and biliary tract tumours over the past three decades, trends in CC rates could theoretically be influenced by coding misclassification. This is particularly likely if hilar/Klatskin tumours, which account for the majority of CC and are in fact extrahepatic, are misclassified as intrahepatic tumours. To date, only one published study has examined this issue (Welzel *et al*., 2006). This investigation, performed in the USA examined the impact of classification of Klatskin CC on IHBD and EHCC incidence rates using data from the Surveillance, Epidemiology and End Results (SEER) cancer registry program of the United States National Cancer Institute (NCI) (Welzel *et al*., 2006). Studying data from 1992 to 2000, before ICD-O-3 was introduced, the investigators found that 91% of the Klatskin CC reported between 1992 and 2000 were incorrectly coded as IHCC, rather than EHCC, resulting in an overestimation of IHBD incidence by 13% and a similar underestimation of EHCC incidence**.** No similar studies have been done elsewhere.

The aim of this study was to:

- analyse incidence trends in IHBD and EHCC in relation to changes in ICD-O classification, and to investigate the impact of potential misclassification of hilar/Klatskin tumours on site-specific incidence rates for bile duct tumours in England and Wales

4.2 METHODS

The bi-axial International Classification of Diseases for Oncology (ICD-O) of the WHO classified CC as intra- or extrahepatic. The ICD-O was introduced in 1979 and assigns two codes dependent upon the anatomical topography and morphology (based on histology) of the tumour (Fritz A *et al*., 2000). Topography codes are defined in the neoplasm section of the ICD, and are applicable to all tumours, regardless of whether their growth behaviour is malignant, benign, *in situ* or uncertain. A number of different revisions of the ICD and ICD-O have been introduced over the past 40 years. In the 10th revision of the ICD, currently in use for cancer registration statistics in England and Wales, HCC is coded as C22.0, IHBD as C22.1 and of the EHCC as C24.0 (World Health Organisation, 1992). These codes include several morphological/histological sub-codes for more specific delineation of tumours, as outlined in ICD-O, including: 8180/3 for combined hepatocellular carcinoma and CC, 8160/3 for CC, 8010/3 for carcinoma 'not otherwise specified (NOS)', 8140/3 for adenocarcinoma NOS, 8000/3 for 'malignant neoplasm' and 8162/3 for Klatskin tumours. IHBD (coded to C22.1) are considered a PLC (C22), whereas EHCC (C24.0) are recognised as a subset of biliary tract cancers (C24).

In the first edition of the ICD-O, hilar/Klatskin tumours were not assigned a specific morphology/histology code and could therefore be classified as either intrahepatic (C22.1) or extrahepatic (C24.0). In the second edition (ICD-O-2), 'Klatskin' tumours were given a unique histology code, 8162/3, but this was cross referenced to the topography code for *intra*- rather than extrahepatic bile duct tumours (Percy C *et al*., 1990;Welzel, McGlynn *et al*., 2006). ICD-O-2 came into use in the US in 1992 and in England and Wales in 1995. In ICD-O-3, which came into use in the US in 2001 but

later in the UK in 2008, the histology code 8162/3 was cross-referenced to either intra *or* extrahepatic bile duct tumours. Thus, hilar/Klatskin tumours may have been misclassified in all versions of the ICD-O.

4.2.1 England and Wales cancer registration data

Registration data, including full details of histological classification, for all cancers coded as IHBD (C22.1) and EHCC (C24.0) in the whole of England and Wales, 1990 to 2008, were extracted from the National Cancer Registry held at the Small Area Health Statistics Unit (SAHSU) at Imperial College London. Annual population estimates were obtained from the ONS.

4.2.2 Data analysis

The total number of cases and the ASIR of IHBD, EHBD and Klatskin tumours were analysed by year and sex. ASIR in England and Wales were standardised using the 2001 European standard population as the reference population. Age-specific incidence rates (ASpIR) were calculated using the following age groups: 20-44, 45-64, 65-74 and over-75 years. Age-standardised incidence rate for IHBD and EHCC specifically between 1990 and 2008 was calculated. IHBD was defined by topography code C22.1 (intrahepatic bile duct) and histology codes 8140, 8160, 8161, 8020, and 8010. EHCC was defined by topography code C24.0 and histology codes 8010, 8020, 8041, 8070, 8140, 8144, 8160, 8161, 8260, 8310, 8480, 8490, and 8560. All ‘Klatskin’ tumours (8162/3) were removed from the IHBD dataset and included in the EHCC dataset and ASIR trends reanalyzed.

4.3 RESULTS

4.3.1 Total numbers of cases, 1990 to 2008

Between 1990 and 2008, the total number of cases reported to have IHBD (C22.1) rose from 226 to 1311. Male cases increased from 116 to 639, and females from 110 to 672. In the same period, the number of cases reported to have EHCC (C24.0) declined from 465 to 329. The decline in males was from 211 to 170 and in females from 254 to 159 (Table 7).

4.3.2 Incidence rates

In England and Wales, ASIR of IHBD (C22.1) increased from 0.34 to 1.67 per 100,000 population/year between 1990 and 2008 (Figure **17**). An increase from 0.43 to 1.84 was seen in males and from 0.27 to 1.51 in females. In the same time period, ASIR of EHCC (C24.0) declined from 0.70 to 0.45 (Figure 18): a reduction from 0.78 to 0.51 in males and from 0.62 to 0.39 in females. All 82 cases with histology code 8162/3 (i.e. 'Klatskin' tumours specifically) were then removed from the IHCC (C22.1) dataset and included with the EHCC (C24.0) dataset, prior to reanalysis of the incidence rates. There was still a marked increase in the ASIR of IHBD (C22.1), even when 8162/3-coded tumours were excluded, from 0.87 to 1.39 per 100 000 population, between 1995 (when ICD-O-2 was introduced) and 2008 (Figure 18). The rise was from 1.04 to 1.51 in males and from 0.73 to 1.29 in females. Concurrently, even after all the Klatskin tumours were included in the EHCC (C24.0) data, a marked decrease in ASIR remained from 0.55 in 1995 to 0.47 in 2008.

Table 6. Number of cases per year of IHBD (C22.1) and EHCC (C24.0), between 1990 and 2008 in England and Wales.

	No. of cases with IHBD			No. of cases with EHCC		
Year	F	M	Total	F	M	Total
1990	110	116	226	254	211	465
1991	127	99	226	226	215	441
1992	167	142	309	256	233	489
1993	176	151	327	235	200	435
1994	194	168	362	216	178	394
1995	297	291	588	210	168	378
1996	362	305	667	189	172	361
1997	413	361	774	157	139	296
1998	417	359	776	194	151	345
1999	385	367	752	189	144	333
2000	504	420	924	138	127	265
2001	473	437	910	138	110	248
2002	506	441	947	127	109	236
2003	474	464	938	126	110	236
2004	573	489	1062	100	110	210
2005	592	559	1151	135	139	274
2006	676	608	1284	155	162	317
2007	691	557	1248	138	186	324
2008	672	639	1311	159	170	329
Total	7709	6973	14682	3342	3034	6376

Table 7. Total number and relative percentages of Klatskin tumours (histology code 8162/3) classified as C22.1 (IHBD) and C24.0 (EHCC) per year in England and Wales, between 1995 and 2008. ICD-O-2 was in use throughout this period.

Year	C22.1 (IHBD) Number (%)	C24.0 (EHCC) Number (%)	Total
1995	3 (100%)	0 (0%)	3
1996	6 (100%)	0 (0%)	6
1997	6 (100%)	0 (0%)	6
1998	11 (92%)	1 (8%)	12
1999	9 (100%)	0 (0%)	9
2000	6 (86%)	1 (14%)	7
2001	9 (100%)	0 (0%)	9
2002	10 (91%)	1 (9%)	11
2003	10 (100%)	0 (0%)	10
2004	12 (86%)	2 (14%)	14
2005	10 (100%)	0 (0%)	10
2006	18 (95%)	1 (5%)	19
2007	17 (100%)	0 (0%)	17
2008	15 (94%)	2 (6%)	17
Total	**142**	**8**	**150**

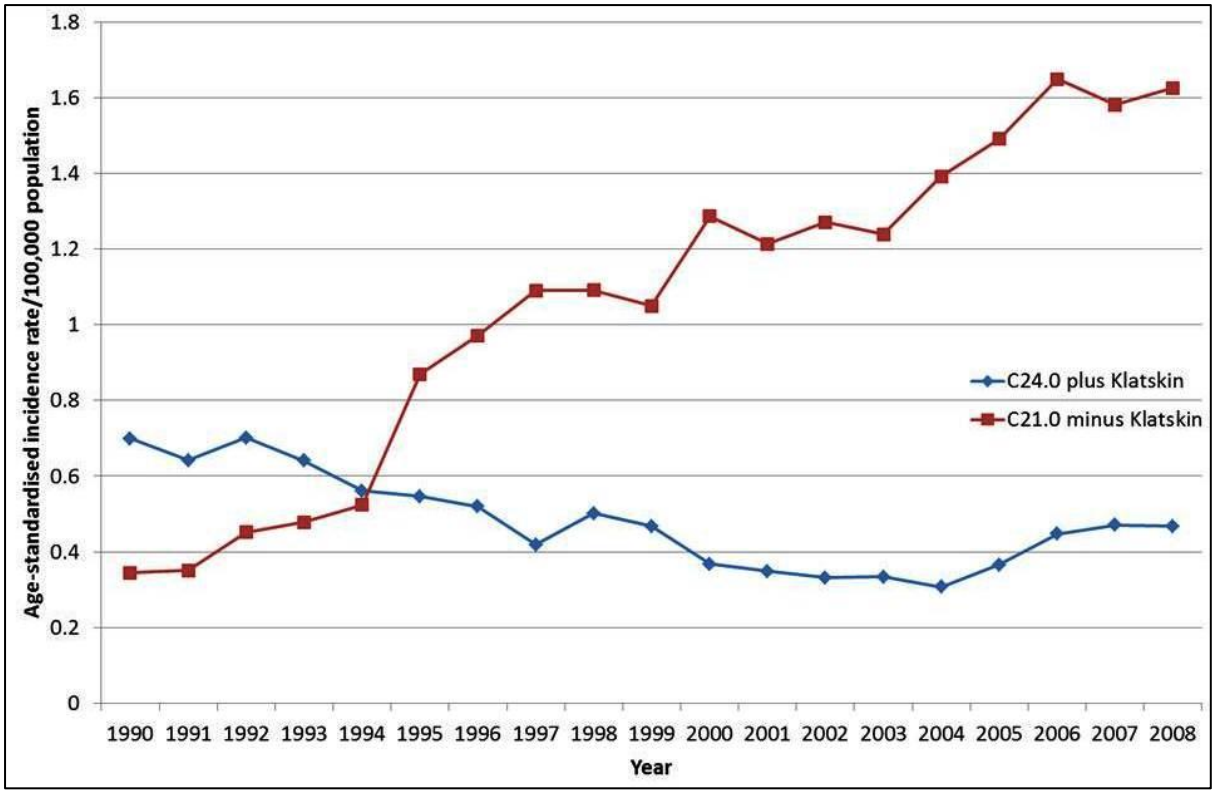

Figure 17. Comparison of age-standardised incidence rates per 100,000 population for tumours coded to C22.1 (excluding M8162/3) and C24.0 (including M8162/3), between 1990 and 2008 in England and Wales. Males and females combined.

4.3.3 Age-specific incidence rates (ASpIR)

In England and Wales, ASpIR analysis showed that the greatest increase in incidence for IHBD (C22.1), excluding 8162/3 (Klatskin tumours), occurred in the age group of 75+ years (Figure 15A). This was the case for both sexes. The decline in incidence rates of EHCC (C24.0), including 8162/3 (Klatskin tumours), was most marked in those over 75 years (Figure **19**). Again, this pattern was seen in both sexes.

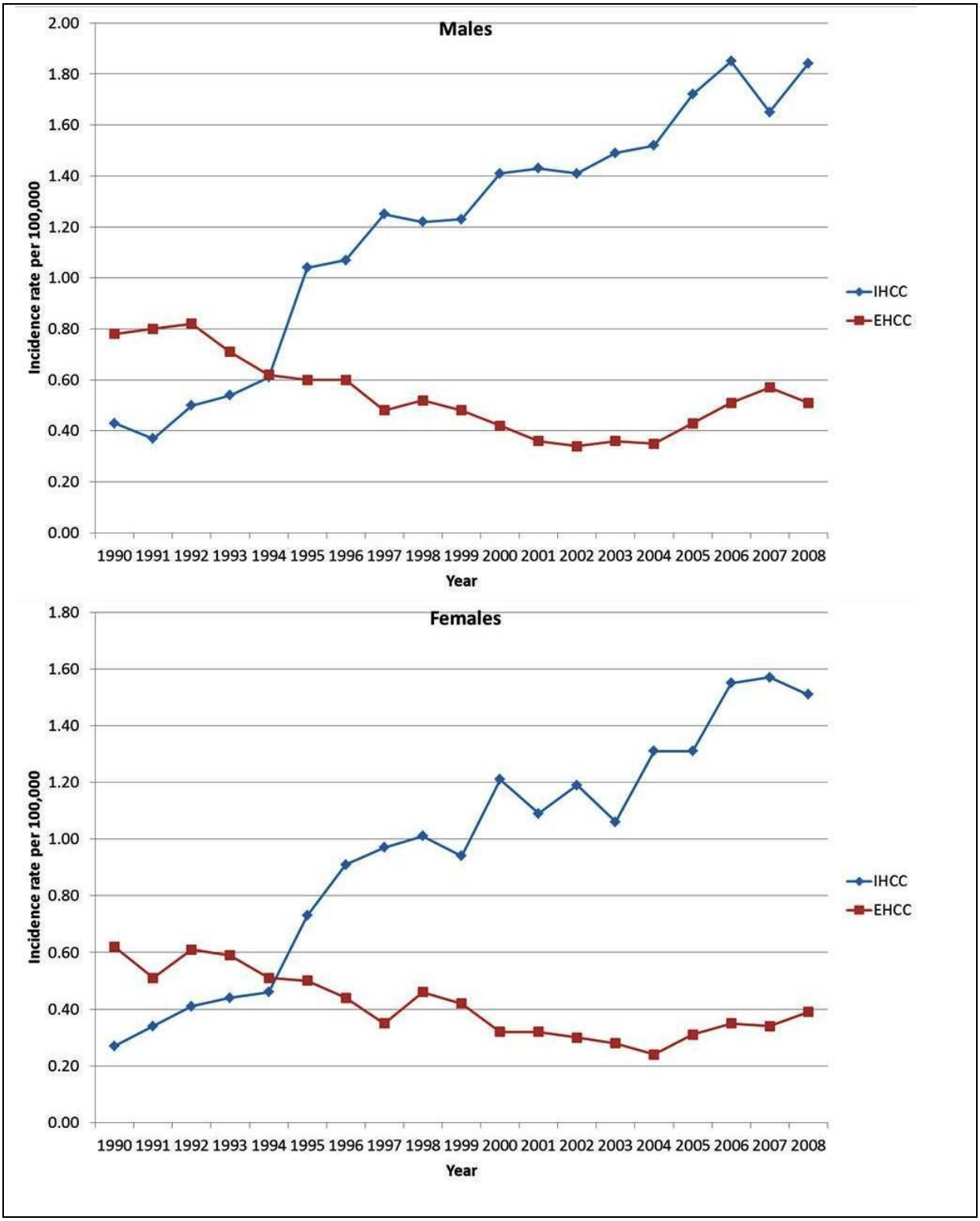

Figure 18. Comparison of age-standardised incidence rates (ASIR) per 100,000 population for tumours coded to C22.1 (IHCC, intrahepatic bile duct carcinoma) and C24.1 (EHCC, extra-hepatic bile duct) between 1990 and 2008 in England and Wales in males and females.

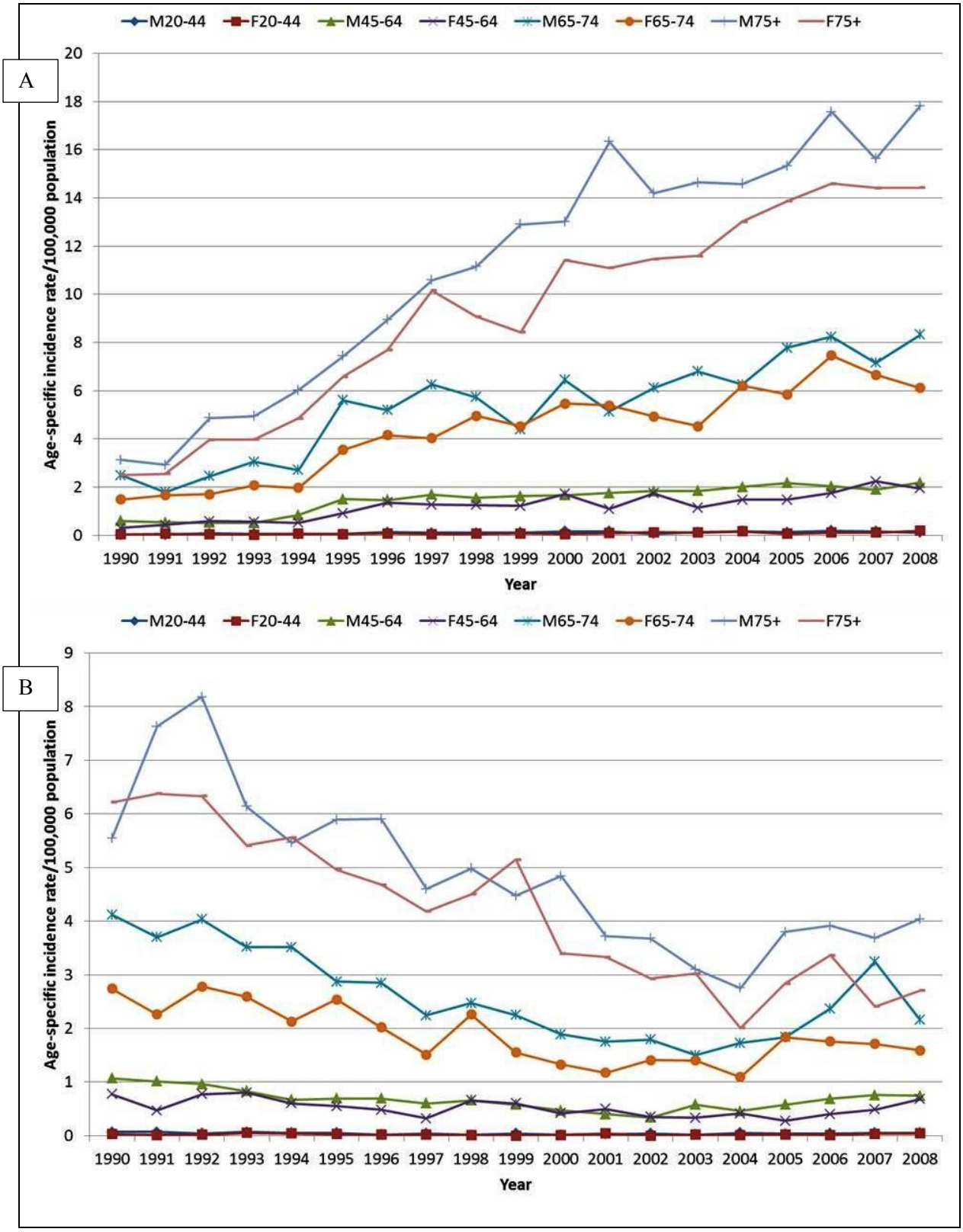

Figure 19. Age-specific incidence rates by gender per 100,000 population for bile duct cancers in England and Wales, 1990-2008. (A) C22.1/IHBD (excluding M8162/3 and (B) C24.0/EHCC (including M8162/3)

4.4 DISCUSSION

This study includes the first European investigation to analyse the impact of misclassification of hilar/Klatskin tumours on IHBD incidence rates; the first ever study to examine a whole national dataset. 'Klatskin' and 'hilar' CC are the same entity and should be coded as extrahepatic tumours, yet the main finding of this investigation is that of confusion and inconsistency regarding the ICD topographical classification of CC. Discrepancies between coding guidelines in the first and second versions of the ICD-O may have resulted in the classification of anatomically-unspecified CC/Klatskin tumours as IHBD. These findings suggest that, in England and Wales, reported increasing rates for IHBD could be due to the incorrect classification of hilar/Klatskin CC as intrahepatic tumours rather than extrahepatic. The rising incidence of CC coded as intrahepatic in England and Wales has been sustained into the 21st century, as has the falling incidence of CC coded as extrahepatic.

After excluding 8162/3 (Klatskin tumours) from the IHBD (C22.1) group, there was still a marked increase in the ASIR from these cancers. This is because the misclassification of Klatskin tumours resulted in overestimation of IHBD incidence rates by only 1%. Yet, according to published studies, Klatskin/Hilar tumours account for the majority of all CC. Welzel and colleagues also acknowledged that the number of Klatskin tumours in the United States SEER database was low, at 8% (Welzel *et al*., 2006). I found that the proportion of Klatskin tumours registered in England and Wales was even lower; 0.9% during the period 1995-2004, when ICD-O-2 was in use. Even if the estimations that Klatskin tumours make up 60-70% of all CCs (Khan *et al*., 2002a;Klatskin, 1965b;Nakeeb *et al*., 1996) are overstated, the proportion of Klatskin tumours found in this study (0.9%) is undoubtedly a substantial under-

representation of the true number. Cancer registries in England and Wales do not code a tumour described as a ‘hilar’ CC with the designated Klatskin code, 8162/3. Thus, ‘hilar’ and ‘Klatskin’ CC are coded differently, even though they are the same entity, and it is therefore not currently possible to determine the true prevalence of all hilar/Klatskin tumours. As a result of this, it is also not possible to determine the true prevalence of IHBD and EHCC in England and Wales.

In experience, ‘hilar’ is preferred over ‘Klatskin’ as a clinical term in the UK. This could explain the low number of CC coded as ‘Klatskin’ in our data. Most hilar/CC pathology reports in the UK are not likely to specify “Klatskin”, but rather state “Hilar” or simply “Cholangiocarcinoma”, with no site specified.

Coding is not the only issue here. There are several potential weak points in the registration process which need to be addressed. Pathologists should be encouraged to seek clarification on the site of a reported CC to prevent unspecified extrahepatic CC being classified as IHBD, in concordance with current ICD rules. Clinicians need to be clearer too, when documenting medical notes and death certificates. Given that documentation and writing of death certificates in the England and Wales tends to be carried out by relatively junior members of the specialist team, clearer guidance on accurate documentation needs to come from senior specialists.

Conclusions and further work

Close surveillance of incidence trends for hepatobiliary tumours is recommended, particularly in light of recently reported dynamic changes. Trends should be adjusted for the potential misclassification of Klatskin tumours until the WHO establishes an accurate and consistent classification practice for CC. Rigorous classification of

hilar/Klatskin CC would permit more accurate monitoring of incidence trends of intrahepatic and extrahepatic CC. I recommend raising awareness amongst cancer registries that the terms ‘hilar’ CC and ‘Klatskin tumour’ are the same entity; otherwise the code 8162/3 for Klatskin tumours is not particularly useful. Consensus in coding practice should be reached on this important matter by the UK Association of Cancer Registries as well as all relevant international bodies. A proposal for the revision of the ICD-O need to be considered, ensuring that all ‘hilar/Klatskin’ tumours are coded topographically to extrahepatic tumours only, rather than as currently to intra- or extrahepatic. Moreover, the description of code 8162/3 should be changed from ‘Klatskin’ to ‘Hilar/Klatskin’. Alternatively, bile duct cancers could be sub-classified as intrahepatic, perihilar or distal with the term Klatskin being omitted altogether. Intrahepatic mass lesions impinging on common hepatic duct, the right and/or the left hepatic ducts should be termed perihilar; the terms intrahepatic versus extrahepatic are unhelpful in this situation. I suggest similar studies on cancer registry practices are carried out in other countries which have reported changes in CC rates. A consistent global classification of CC is required to accurately compare trends around the world. Finally, it is important to note that however CC are classified, the incidence of IHBD appear to be increasing overall and the reasons for this need to be investigated.

5. IMPACT OF VIRAL HEPATITIS ON HIV INFECTION IN AN AFRICAN COHORT

5.0 ABSTRACT

Background: Owing to shared routes of infection, hepatitis B and or C co-infecting HIV patients is/are common in many regions of sub Saharan Africa. However, large data from Africa characterising the rates and impact of hepatitis B (HBV) and hepatitis C (HCV) infections on response to long-term antiretroviral therapy (ART) of HIV infection are lacking. These parameters were determined in the present study.

Methods: This was a retrospective cohort study of 19,408 adults who were recruited between June 2004 and December 2010 in the AIDS Prevention Initiative in Nigeria (APIN) programme at Jos University Teaching Hospital. Serological assays; including HBsAg and HCV Ab were used to categorise hepatitis status of the patients. HBsAg was determined using Enzyme immunoassay (EIA) (Monolisa HBsAg Ultra3; Bio-Rad). HCV antibody was tested using third generation enzyme immunoassay (DIA.PRO Diagnostic, Bioprobes srl, Milan, Italy). HIV RNA levels were measured using Roche COBAS Amplicor HIV-1 monitor test version 1.5 (Roche Diagnostics, GmbH, Mannheim, Germany) with a detection limit of 400 copies/mL. Flow cytometry was used to determine CD4+ cell count (Partec, GmbH Munster, Germany). Comparison of categorical and continuous variables were achieved using Pearson's chi-squared and Kruskal Wallis tests respectively, using MedCalc Statistical Software version 12.7.5 (MedCalc Software bvba, Ostend, Belgium; http://www.medcalc.org; 2013)

Results: With an overall screening rate for hepatitis of over 90% for each virus; HBV, HCV and HBV/HCV were detected in 3,162(17.8%), 1,983(11.3%) and

453(2.5%) HIV infected adults respectively. The rate of liver disease was low, but highest among HIV mono-infected patients (29, 0.11%), followed by HBV co-infected patients (15, 0.08%). Patients with HBV co-infection and triple infection had higher log_{10} HIV RNA loads (HBV: 4.6 copies/mL vs HIV only: 4.5 copies/mL; *P* <0.0001) and more severe immune suppression (HBV: 645, 55.4%; HBV/HCV: 97, 56.7%) prior to initiation of antiretroviral therapy (ART) compared to HIV mono-infected patients (1852, 48.6%) (*P*<0.0001). Of 3,025 patients who were 4.4 years on HAART and whose CD4 cell counts results at baseline and end of follow up were available for analyses, CD4 increase was significantly lower in those with HBV co-infection (HBV: 144 cells/mm^3; HBV/HCV: 105 cells/mm^3) than in those with HCV co-infection (165 cells/mm^3) and also in those with human immunodeficiency virus (HIV) mono-infection (150 cells/mm^3) (*P* =0.0008).

Conclusion: High rates of HBV and HCV infections were found in this HIV cohort. CD4 recovery was significantly diminished in patients with HBV co-infection. Further studies would be required to document the impact of these co-infections on the survival of HIV infected patients undergoing ART.

5.1 BACKGROUND

Studies of the prevalence of hepatitis in HIV infected individuals confirm that the rates of hepatitis B (HBV) in HIV-infected patients vary widely with tendency towards higher values compared to HBV prevalence in the general population (Adekunle *et al*., 2011;Adesina *et al*., 2010). In the north central region of the Federal Republic of Nigeria, the prevalence of hepatitis B and hepatitis C in HIV-infected people were 27.8% and 18.3% respectively, and triple infection (HBV/HCV/HIV) was found in 7.2% of 180 HIV-infected patients (Forbi *et al*., 2007). HBV prevalence in population studies in Nigeria is between 10 and 20% (Ladep and Taylor-Robinson 2007).

Although it is widely recommended that HIV-infected patients are screened for hepatitis before antiretroviral therapy, no data from Nigeria are accessible to ascertain adherence to this guideline. Reports from Thailand confirm that compliance to hepatitis screening in HIV patients prior to initiating ARVs was poor (between 55-69%) (Kiertiburanakul *et al*., 2011;Sungkanuparph *et al*., 2008). Inadequate information on hepatitis in HIV patients may underpin one reason for national health schemes in many developing countries not offering integrated hepatitis services in HIV-infected persons. As a consequence, patients co-infected with HBV and HCV are being ignored in regards to timing of antiretroviral therapy, screening for cirrhosis of the liver and hepatocellular carcinoma, as well as in the choice of ARV regimens that have the potential to optimise their care.

Rising trends in the prevalence of HBV and HCV among HIV-infected individuals during the last decade have been reported in a study in the USA. That study, involving about 30,000 HIV-infected patients recorded a low, but significantly increasing proportion of patients being screened for hepatitis; from 20% in 1998 to

60% in 2004 (Buskin *et al*., 2011). The researchers found that the rate of HBV and HCV increased from 7 to 8.5% and 9 to 24% respectively. To date, no report of trends in the rate of hepatitis in patients infected with HIV from sub-Saharan African countries has been published.

The choice of ART regimen can be critical in achieving good treatment outcomes; and knowledge of hepatitis co-infection is vital in this regard. Lamivudine resistance in HIV/HBV co-infected patients on ART has been described in some studies within the West African sub-region (Kouanfack *et al*., 2012;Stewart *et al*., 2011). A Francophone study has recently demonstrated the advantage of treating HBV-HIV co-infected patients with Tenofovir-containing ARV regimen, particularly for wild type precore mutant and lamivudine-resistant HBV (Benhamou *et al*., 2006). Guidelines for the choice of ART regimens generally recommend screening for hepatitis, but not routinely undertaken and/or largely depends on availability of resources. Even where screening for hepatitis takes place, a large number of HIV physicians base the choice of ART on available drugs rather than on informed co-morbid conditions.

The importance of well-designed research to answer these questions cannot be overemphasised in order to advice adequate provision of resources for the optimisation of care for HIV/hepatitis co-infected individuals in Africa. Thus, I aimed to determine the rate of hepatitis screening in HIV infected patients, magnitude of hepatitis infection in this large cohort, impact of hepatitis co-infection on baseline HIV parameters: HIV suppression and CD4+ cell increase following HAART.

5.2 PATIENTS AND METHODS

5.2.1 Study Population

The AIDS Prevention Initiative in Nigeria (APIN) and Harvard School of Public Health HIV program, supported by a grant from the United States President's Emergency Plan for AIDS Relief (PEPFAR) started to provide antiretroviral therapy, at no cost to patients in Nigeria from 2004 until the time of writing this report. This programme is run on a community-based model (although the major sites in Nigeria are located within tertiary health centres), in which integrated community prevention outreaches, on-site HIV screening, counselling, provision of medications, follow up, monitoring and evaluation of all activities are embarked upon. Jos University Teaching Hospital (JUTH) site is one of several centres in Nigeria, with latest HIV prevalence of 4.4% (Agaba *et al*., 2011). JUTH has a specialised centre of care for HIV infection where patients are seen at the outpatient facility at planned intervals of 4 to 12 weeks.

The initial first line ARVs in this population included Stavudine/Zidovudine, Lamivudine and Efavirenz/Nevirapine. However, from 2006, Truvada® (Tenofovir plus Emtricitabine) started to be administered to HIV patients going on ART in the programme. From June 2004 to December 2010, approximately 19,408 HIV-infected individuals had been recruited in the JUTH/APIN/Harvard programme and were initiated on ARVs and anti-tuberculosis drugs, if indicated.

For the initial part of the study, all subjects whose HIV status were confirmed by Western blot assay and enrolled in the programme between 2004 and 2010 were included. Information on age, gender, educational attainment, status of hepatitis B and C were obtained. HIV RNA levels and CD4+ cell counts at baseline and most

recent assays were also included as were information on the last day of follow-up, death or discontinuation of therapy.

Subjects were defined as having HBV and HCV infection if they tested positive for hepatitis B surface antigen (HBsAg) and hepatitis C antibody (HCV Ab), respectively on baseline blood samples. HIV RNA levels and CD4+ cell count were determined at baseline for patients and at 3 monthly intervals until the end-point of the study. Hepatotoxicity was defined based on the recommendation of the AIDS Clinical Trials Group(AIDS Clinical Trial Group 1996); as elevated alanine aminotransferase (ALT) values ≥5 folds of upper limit of normal (ULN) (41IU/mL for JUTH) if baseline ALT was within normal range or of ≥3.5 folds of baseline, if ALT at baseline was higher than 41IU/mL prior to commencement of ART or anti-tuberculosis medications.

Diagnosis of tuberculosis was established based on sputum smear positivity on at least two of three sputa collected according to an established local protocol. Patients who are coughing are given three universal containers. They are required to cough up sputum into the container, the first thing in the morning. On arrival to hospital, they produced the second sputum. The third sputum was brought on the second day. This protocol was practical for those patients that lived close to the research centre at JUTH. In view of the limitations of resources and the fact that most patients infected with HIV are likely to have smear-negative TB, chest X-ray findings, typical clinical presentation (cough for more than two weeks, fever, night sweating, weight loss and history of prolonged contact with a person known or suspected to have TB), according to WHO guidelines for resource-limited settings was used to define a TB case (World Health Organization *et al*., 2007).

Recruited patients gave written informed consents approved by the ethical committee at JUTH and the institutional review board (IRB) at the Harvard School of Public Health. For the present work, I obtained a further approval for secondary use of data.

5.2.2 Study Design

This was a retrospective cohort study. The pro-forma utilised in the analyses is summarised in Figure 20. The number of HIV-infected individuals who were screened for hepatitis B and hepatitis C were divided by the total number of patients recruited to ascertain the proportion of hepatitis screening. The rates of HBV and HCV were calculated from the population that underwent serological testing. All patients who had hepatotoxicity, liver cirrhosis and hepatocellular carcinoma were categorised into a single group (liver disease). As there was overlap of the morbidities, further categorisation of the cumulative rates of liver related morbidities was undertaken.

Case-controlled analyses of the impact of hepatitis co-infections on baseline HIV viral load and CD4+ cell counts were also embarked upon.

5.2.3 Laboratory testing

Before recruitment into the APIN programme, subjects were screened for HIV, using enzyme linked immunoassay (ELISA) and subsequently confirmed by Western Blot assay. HBsAg was determined using Enzyme immunoassay (EIA) (Monolisa HBsAg Ultra3; Bio-Rad). HCV antibody was tested using third generation enzyme immunoassay (DIA.PRO Diagnostic, Bioprobes srl, Milan, Italy). HIV RNA levels

were measured using Roche COBAS Amplicor HIV-1 monitor test version 1.5 (Roche Diagnostics, GmbH, Mannheim, Germany) with a detection limit of 400 copies/mL. Flow cytometry was used to determine CD4+ cell count (Partec, GmbH Munster, Germany).

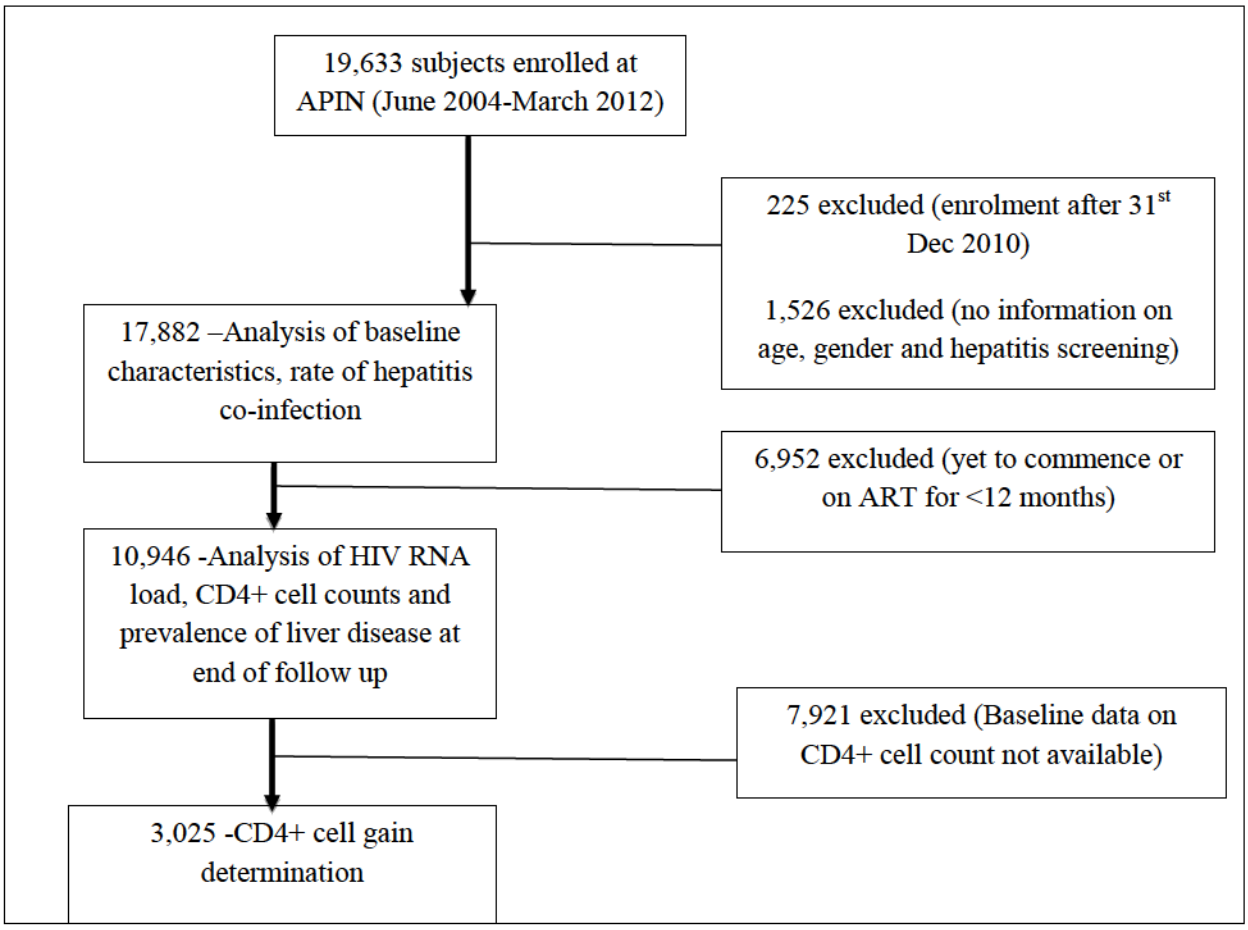

Figure 20. Flow-chart representation of analyses on HIV-infected individuals at APIN, Jos University Teaching Hospital, Federal Republic of Nigeria

5.2.4 Statistical Analyses

As the diagnoses of HBV, HCV and liver diseases were likely to overlap, the cumulative prevalence of liver morbidities by overlapping diagnoses were calculated; categorised into HBV only, HCV only, HBV/HCV, and liver disease. The relationships in the demographics of the patients and liver morbidities, as well as baseline HIV parameters were determined. The obtained characteristics of HBV, HCV, HBV/HCV and HIV only subjects were compared against each other at baseline using Chi-squared and Kruskal Wallis tests for categorical and continuous variables respectively. Analyses were accomplished using MedCalc Statistical Software

version 12.7.7 (MedCalc Software bvba, Ostend, Belgium; http://www.medcalc.org; 2013). *P* values of <0.05 were considered statistically significant.

5.3 RESULTS

Between June 2004 and Dec 2010, 19,408 HIV subjects had been enrolled. Median follow up period was 53 months (interquartile range: 31-72 months). Table 8 presents a summary of the main characteristics of the cohort at baseline. Subjects diagnosed with hepatitis B were more likely to be young (median age: 32; p<0.001), male and to have had high HIV RNA loads and CD4+ cell count below 200/mm^3. Hepatitis C co-infected individuals were more likely to be males, older (median age: 36 yrs), have had low level of education and high HIV RNA in their plasma.

5.3.1 Hepatitis screening and prevalence of co-infections

At the beginning of the study period (year 2004), 99.3% and 99.5% of recruited HIV infected individuals underwent HBV and HCV screening, respectively. There has been a significant decline in the rates of screening for HBV and HCV during approximately 7 year study period to 73.0% and 87.6% respectively in 2010 (*P*<0001) (Figure **21**). Overall, the prevalence of HBsAg was 20.7%. A significant increase in the rate of HBV from 14.4% in 2004 to 21.0% in 2010 was observed (*P*<0.001) and although, fluctuating rates of HCV Ab was recorded among those that were screened, an increasing pattern was noted. The prevalence of HCV Ab was 10.6% in 2004, increasing to 11.7% in 2010. Higher rates of HBV infection was found in men than women rising from 17% in 2004 to 25% in 2010.

Table 8 Baseline characteristics by hepatitis status of patients at APIN, 2004-2010

Characteristic	Total, *n* (%)	HBV	HCV	Triple infection	HIV only
Gender					
Male	6222(34.8)	1214(38.1) [b]	764(37.9) [b]	178(39.3) [b]	4066(33.2)
Female	11660(65.2)	1971(61.9) [b]	1250(62.1)[b]	275(60.7) [b]	8164(66.8)
Age Group					
15-29	5870(32.8)	1097(34.4) [b]	463(23.0) [b]	120(26.5)	4190(34.3)
30-39	7106(39.7)	1309(41.1) [b]	786(39.0)[b]	206(45.5)	4805(39.4)
40-49	3619(20.2)	615(19.3) [b]	533(26.5) [b]	94(20.8)	2377(19.5)
≥50	1287(7.2)	164(5.1) [b]	232(11.5) [b]	33(7.3)	828(6.8)
TB diagnosis					
Present	2552(14.3)	470(14.8)	300(14.9)	68(15.0)	1714(14.0)
Absent	15330(85.7)	2715(85.2)	1714(85.0)	385(85.0)	10516(86.0)
Education status					
None	3230(18.8)	527(17.2)	471(24.3)	87(19.8)	2145(18.3)
Primary	3487(20.3)	623(20.3)	440(22.7)	96(21.8)	2328(19.9)
Secondary	5208(30.4)	962(31.4)	536(27.6)	146(33.2)	3564(30.5)
Tertiary	5219(30.4)	954(31.1)	494(25.4)	111(25.2)	3660(31.3)
CD4; cells/mm^3					
<200	2937(50.7)	645(55.4) [b]	343(52.5)	97(56.7) [b]	1852(48.6)
200-499	2214(38.2)	410(35.3) [b]	249(38.1)	57(33.3) [b]	1498(39.3)
≥500	646(11.1)	108(9.3) [b]	61(9.3)	17(9.9) [b]	460(12.1)
HIV; copies/mL					
Undetect; <400	2194(12.4)	351(11.2) [b]	221(11.1) [b]	29(6.7) [b]	1583(13.1)
Low; 400-9,999	3581(20.3)	562(17.9) [b]	364(18.2) [b]	99(22.8) [b]	2556(21.1)
Interm;10,000-29,999	2774(15.7)	523(16.7) [b]	321(16.1) [b]	72(16.6) [b]	1858(15.4)
High; ≥30,000	9115(51.6)	1700(54.2) [b]	1090(53.9) [b]	234(53.9) [b]	6091(50.4)

[b]$P < 0.01$ *vs* HIV only; Undetect: undetectable; Interm: intermediate

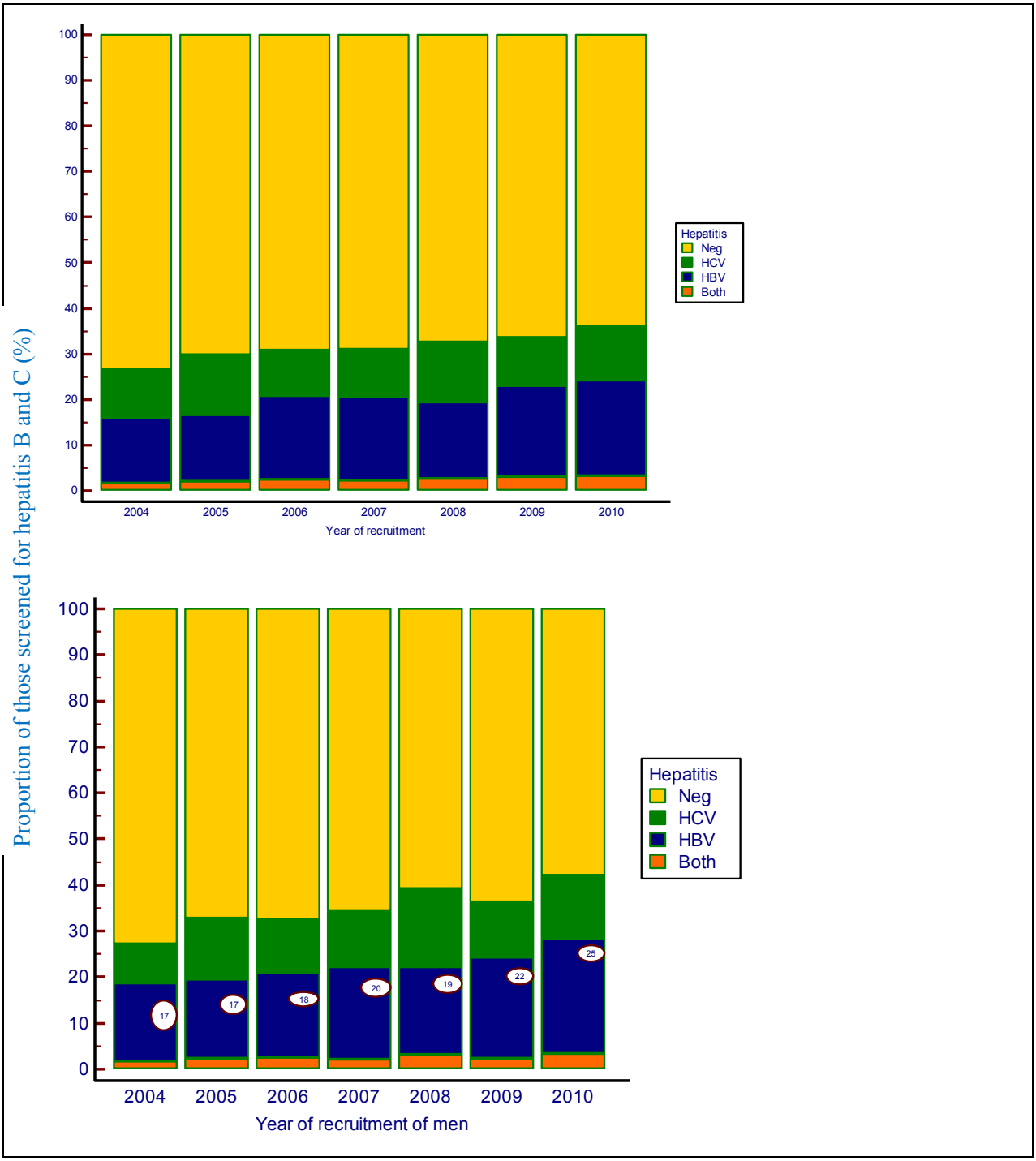

Figure 21. Rates of hepatitis co-infection of HIV infected individuals in both genders and men among AIDS Prevention Initiative in Nigeria cohort, JUTH; 2004-2010.

5.3.2 Overlapping Diagnosis

Cumulatively, 3,185(17.8%) patients were positive for HBsAg and 2,014 (11.3%) patients had HCVAb. 453 (2.5%) patients had evidence of combined HBV and HCV infections (Figure 22). Liver disease was diagnosed in 50 (0.3%) patients. Of these, 15 had HBV, 6 had HCV and 29 had no evidence of hepatitis co-infection. None of those with triple infection had a diagnosis of liver disease. Diagnoses of liver disease were achieved via conventional means, including: assessment of sequential serum liver enzyme measurements, liver ultrasound, and serum alpha fetoprotein levels.

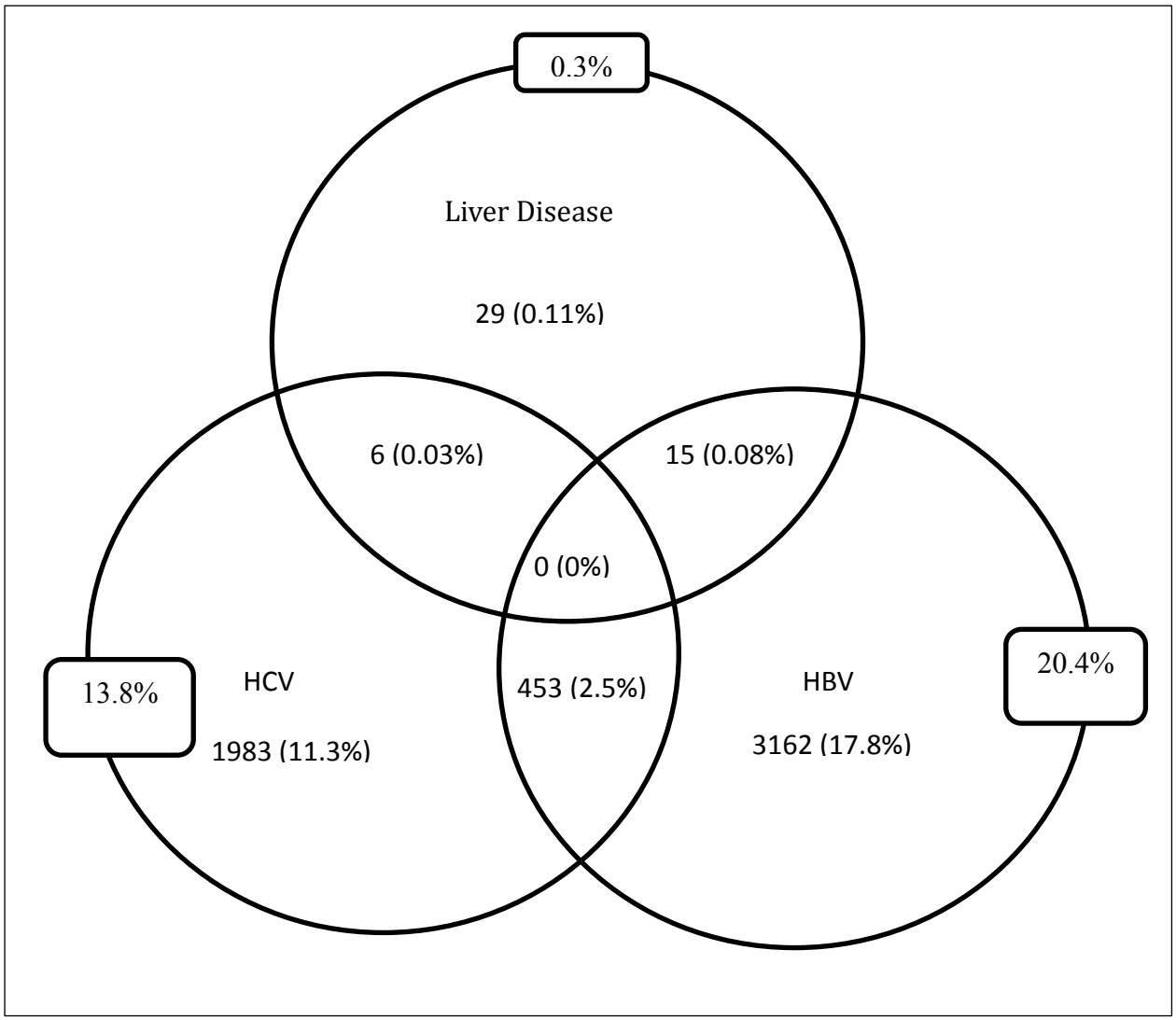

Figure 22. Prevalence of hepatitis B, hepatitis C, hepatitis B &C and liver disease diagnoses among HIV infected individuals (AIDS Prevention Initiative in Nigeria cohort) at Jos University Teaching Hospital, 2004-2010

5.3.3 Impact of Hepatitis co-infection on outcome of ART

Higher proportion of HBV (645, 55.4%) and HBV/HCV (97, 56.7%) infected patients had CD4+ cell counts below 200 cells/mm^3 at baseline compared with HCV (343, 52.5%) and HIV-only (1852, 48.6%) patients (*P*<0.0001). The median HIV RNA at baseline were Log_{10} 4.6 copies/mL each for HBV and HCV patients; and 4.5 copies/mL for HIV mono-infected patients (*P*<0.0001). At the end of follow up on ART (median duration: 4.4yrs [interquartile range: 2.6-6yrs]), no significant difference in HIV RNA load suppression was observed in all study groups. However, there was a significantly lower CD4+ cell increase among those individuals co-infected by HBV/HCV (105 cells/mm^3) and HBV (144 cells/mm^3) than in HCV (165 cells/mm^3) and HIV-only (150 cells/mm^3) patient groups (*P*=0.008) (Figure **23**).

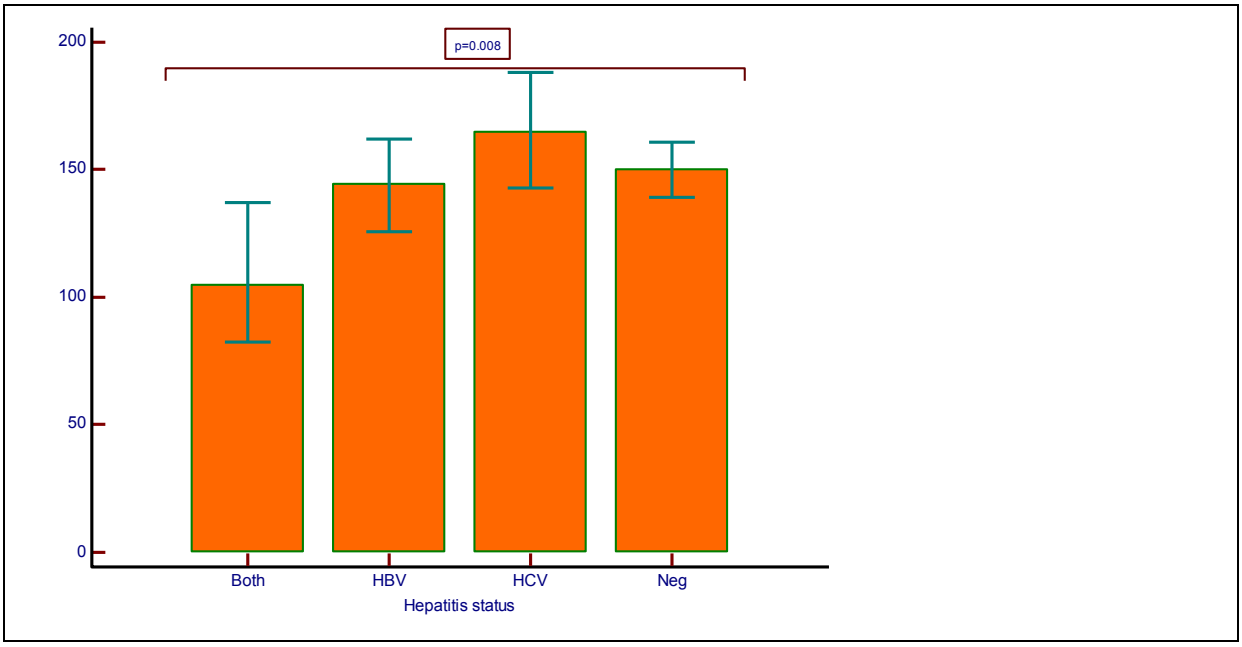

Figure 23. Median CD4 increase by hepatitis status following ART (AIDS Prevention Initiative Nigeria cohort) at Jos University Teaching Hospital, 2004-2010

5.4 DISCUSSION

In this large cohort of HIV-infected sub-Saharan African patients, I found that whereas chronic HBV and HCV were frequent diagnoses, liver disease was not commonly reported; although it was investigated only when there were overt clinical symptoms. Overall, one out of every five patients had HBV and more than a tenth had HCV. Almost every patient that who was recruited at the beginning of the study was screened for HBV and HCV. This is rather remarkable for a resource limited setting and much higher than obtainable in some cohorts elsewhere (Kiertiburanakul *et al*., 2011;Sungkanuparph *et al*., 2008). The fact that this study site benefitted from grants for research and involved the services of specialists may explain the high hepatitis screening rate. However, this initial enthusiasm was not sustained, as there has been a significant decline in the rates of screening for hepatitis during the study period. Interestingly, diagnoses of HBV and HCV showed significant rising trends. I surmise that there may be a selection bias in this regard as people tend to go to tertiary care centres to seek treatment.

My findings corroborate the reports of other researchers who had observed higher prevalence of HBV and HCV infection among HIV-infected patients than in the general population (Chun *et al*., 2010). Studies of prevalence of HBV in the general population of people living within the study area during the period between 2002 and 2007 had found rates of between 10.3% and 15.1% (Egah *et al*., 2007;Jombo *et al*., 2005;Sirisena *et al*., 2002;Uneke *et al*., 2005). These confirm that the rates of hepatitides are higher in the HIV patients than in the general population of Nigeria. The fact that HIV and hepatitis viruses share the same routes of transmission supports this explanation. However, it remains unknown whether hepatitis infection in this cohort occurs at the same time as HIV infection or predates it.

The precise modes of transmission of HBV and HCV in this cohort are not known although most of the subjects incriminated heterosexual route for the acquisition of HIV. However, it has been reported that transmission of HBV most commonly occurs in early childhood among African populations (Lesi *et al*., 2007), compared to high transmission rates among adults in industrialised countries (Puoti *et al*., 2008). Whereas intravenous drug use is the major route of transmission of HIV and indeed hepatitis viruses in western industrialised countries, heterosexual and horizontal routes, as well as indiscriminate injections (unsterile needles) are thought to be the prevalent modes of transmission of these viral infections in African communities (Simonsen *et al*., 1999). Many patients may have iatrogenic transmission from poor sterilisation during routine medical, obstetric, dental and surgical procedures. Most HIV/hepatitis co-infected patients in Nigeria are postulated to have become infected by hepatitis viruses before HIV (Lesi *et al*., 2007). Longitudinal studies will be required to appropriately determine patients that may have acquired hepatitis before, at the same time or after HIV infection. Such a study has the advantage of providing additional information for reinforcing prevention methods, for example HBV vaccination, not only for the present cohort, but for HIV-infected patients in HBV endemic regions.

The incidence of liver disease in my study was not assiduously documented, although observed to be common. Only 11 patients were documented to have had PLC. Eight of these patients were screened for hepatitis. While four were HBsAg positive, 4 were negative to both HBV and HCV. As population-based cancer registries are not routinely available and/or reliable in Nigeria owing to poor registration of diseases and deaths, it was not possible to compare the incidence of PLC in the present cohort with those from the general population. However, studies

in United States have confirmed that PLC occurs about 6 times more commonly in HIV-infected individuals than in the general population (Engels *et al*., 2008;Shiels *et al*., 2009). With such a high rate of HBV and HCV infection in this African cohort, there is a chance that a large number of PLC cases were missed or will yet manifest. It should be noted that prior to free provision of ART to HIV patients in Nigeria from 2004, the cost of these medications was prohibitive and the incidence of HIV mirrored its mortality (Idoko, 2012). It is thus likely that most patients would have died earlier than they could present with hepatocellular carcinoma (HCC). Furthermore, with prolonged ART, many of these patients will survive longer and HCC could become more frequently diagnosed.

Unfortunately, the study design did not allow for prospective evaluation of hepatitis status. In addition, the patients were treated with ARVs (Truvada) that in some cases may have been both active on hepatitis and HIV infection. The baseline evaluation and hepatitis status seems to indicate that other non-infectious causes of hepatic disease may need to be considered. It will be anticipated that HIV hepatitis co-infected patients would have higher incidence of liver disease. This would have been the case if the definition of liver disease were restricted to end-stage liver disease (fibrosis, cirrhosis and liver cancer). However, I included hepatotoxicity of ARVs in the definition. This would explain, in part the higher incidence of liver disease in HIV mono-infected patients. Chronic liver disease was small in the cohort, perhaps due to under reporting, or perhaps a high threshold for recording cases in the database. Reasons for the apparent rarity of liver disease in HIV/hepatitis co-infected patients and higher cases of liver disease in HIV mono-infected than hepatitis co-infected patients require further studies.

At baseline, patients with hepatitis co-infection had higher HIV RNA than patients with HIV mono-infection. Correspondingly, a higher proportion of patients co-infected with HBV had CD4+ cell counts below 200 /mL compared to HIV mono-infected individuals. The finding of higher HIV RNA at baseline corroborates earlier findings in a study of a small number of patients (1,564) from the same study site (Idoko *et al*., 2009), as well as another study from China (Sheng *et al*., 2004). Following ART, the gain in CD4+ cell count was significantly diminished in those patients who had hepatitis B co-infection compared to those with HIV mono-infection. In contrast to these findings, two studies that assessed the impact of hepatitis B on response to ART found no difference in CD4+ cell gain (Konopnicki *et al*., 2005;Law *et al*., 2004). The underlying reasons for the differential outcomes are not obvious. Differences in environment are unlikely, as my findings are in contrast to the observation of a study of South African patients (Hoffmann *et al*., 2008). I note however, that whereas the Republic of South Africa study had a shorter duration of follow up (1.5 years), my cohort was followed for a longer duration on ART (4.4 years). Nevertheless, results of hepatitis B and long-term HIV outcomes (7 years) in the USA found no difference in the HIV load suppression and CD4+ cell gain. Be that as it may, as the natural history of HBV is likely to differ between American and African populations, owing to differential age at acquisition of hepatitis infections, studies comparing African patients on long-term ART would provide a better assessment.

The present findings suggest that CD4+ cell loss by HIV is accentuated by HBV, despite on-going HIV treatment. A few studies have highlighted that active HBV infection is associated with T-lymphocyte exhaustion (Dunn *et al*., 2009;Iser *et al*., 2011). This has been further strengthened by the fact that inhibition of HBV DNA replication using anti-HBV drugs resulted in immune restoration (You *et al*.,

2008;Zoutendijk *et al*., 2012). One would expect such an effect to be universal. However, variable outcomes of ART in regards to HIV load suppression and/or increases in CD4+ cell count in HBV co-infected versus HIV mono-infected patients have been reported. While some studies found no differences between HBV and HIV mono-infected groups (Hoffmann *et al*., 2008;Hoffmann *et al*., 2009a), others found non-sustained differences in CD4+ cell increases (Law *et al*., 2004;Sagoe *et al*. 2012). Studies of HIV treatment outcomes of HBV co-infected and HIV mono-infected African patients comparing HBV suppressive agents versus regimens that are non-HBV suppressing will be required to adequately characterise the importance of HBV in the era of ART.

This study was not without limitations. First, the use of HBsAg positivity as the sole indicator of chronic HBV infection may be misleading. The definition of chronic HBV would require a positive HBsAg assay carried out consecutively, at least 6 months apart. As single HBsAg was utilised to define cases of HBV in this current study, cases of misclassification might have occurred. Also, delineating HBV cases by their HBV DNA loads and HBeAg status would have provided more meaningful analyses. However, as it is generally known that HBV infection in Africans occur more commonly in childhood, the chance of falsely misclassifying HBV is low. Restricted by the terms of IRB approval to utilise data only, additional HBeAg and HBV DNA analyses could not be performed.

Another issue that could have led to misclassification to hepatitis status is reliance on anti-HCV Ab results to define active HCV infection. Earlier data (unpublished) from a sub-group of the current cohort had found HCV viraemia of 33% in those that were HCV Ab positive. It is thus possible that of the patients that were classified

“HCV”; only a third may actually be HCV viraemic. More studies with better characterisation of HCV status in this cohort would be required.

Missing data were another issue I encountered. In some of the patients, HBsAg and HCV Ab and baseline CD4+ data results were unavailable. Also, reliance on diagnosis of liver diseases (hepatotoxicity, cirrhosis and PLC) from clinical notes of the patients, where available, could have hampered the interpretation of these data. As a result of these, analysis of the rates of hepatitis was accomplished among those that had available hepatitis results. Also, CD4+ cell gain was analysed for 3,012 patients for whom there were baseline and follow up results and who were on ART.

In conclusion, high and increasing rates of HBV, HCV and HBV/HCV co-infections were found in this large HIV infected cohort of Africans. The prevalence of liver disease, particularly liver cancer was low; mostly reported among HBV/HIV and HIV-only individuals. HBV co-infection was associated with high HIV RNA load and decreased CD4+ cell counts at baseline and attenuated immunological recovery after a median follow up duration on ART of 4.4 years. These findings underscore the urgent need to maintain a strict hepatitis screening policy among HIV-infected patients undergoing ART, as well as inclusion of ART regimens with potent anti-HBV activities in HBV-endemic regions of the world. Longitudinal studies in African patients to ascertain super-infection of HIV by hepatitis, assessments of impact of HBV-suppressive versus HBV non-suppressive ART regimens and predictive value of hepatitis on the mortality of the present cohort will form a significant contribution to future research.

6. IMPACT OF CHRONIC HEPATITIS B INFECTION ON SURVIVAL OF HIV-INFECTED PATIENTS

6.0 ABSTRACT

Background: Hepatitis B has been reported to be high in HIV-infected African populations. However, the impact of HBV infection on the survival of HIV-infected Africans on long-term highly active antiretroviral therapy (HAART) remains poorly characterised. I investigated the impact of HBV/HIV co-infection on survival of HIV infected patients undergoing antiretroviral therapy in a West African population.

Methods: This was a clinic-based cohort study of HIV-infected adults enrolled in Nigeria, West Africa. Study subjects (9,758) were screened for hepatitis B and hepatitis C at ART initiation. Kaplan-Meier survival and Cox proportional hazards models were used to estimate probability of survival and to identify predictors of mortality respectively, based on hepatitis B surface antigen status. All patients had signed an informed, written consent before enrolment into the study; and additionally, permission for secondary use of data from the Harvard institutional review board was obtained.

Results: Patients were followed up for a median of 41 months (interquartile range: 30-62 months) during which, 181 (1.9%) patients died. Most of the deaths; 143 (79.0%) occurred prior to availability of Tenofovir. Among those that were on ART, hepatitis B co-infected patients experienced a significantly lower survival than HIV mono-infected patients at 74 months of follow up (94% vs. 97%; p=0.0097). Generally, hepatitis B co-infection: HBsAg-positive/HIV-positive (Hazards Rate [HR]; 1.5: 95% CI 1.09-2.11), co-morbid tuberculosis (HR; 2.2: 95% CI 1.57-2.96) and male gender (HR; 1.5: 95% CI 1.08-2.00) were significantly predictive of mortality.

Categorising the patients based on use of Tenofovir, HBV infection failed to become a predictor of mortality among those on Tenofovir-containing HAART.

Conclusions: HBsAg-positive status was associated with reduced survival and was an independent predictor of mortality in this African HIV cohort on ART. However, Tenofovir annulled the impact of HBV on mortality of HIV patients in my study cohort.

6.1 BACKGROUND

Viral hepatitis infection is the leading cause of cirrhosis and primary liver cancer in Africa (de *et al*., 2012), where HBsAg prevalence is up to 20% in the general population (Ott *et al*., 2012). Owing to shared routes of transmission, hepatitis infection rates among HIV-infected patients are significantly higher than in the general population (Forbi *et al*., 2007;Mayaphi *et al*., 2012).

Since the global scale-up of ART, numerous studies have shown that the efficacy of ART in suppression of HIV in Africa is comparable to that obtained in resource-rich countries (Etard *et al*., 2006;Lawn *et al*., 2005;Zachariah *et al*., 2006), though there are conflicting data on whether HBV co-infection affects HIV suppression (Hoffmann *et al*., 2008;Hoffmann *et al*., 2009a;Matthews *et al*., 2011). In the developed world setting, liver disease has emerged as a leading cause of death in the era of ART (Konopnicki *et al*., 2005;Shafran, 2007;Tuma *et al*., 2010a) and hepatitis plays an important role in the progression to cirrhosis in hepatitis B and hepatitis C patients, who are co-infected with HIV (Tuma *et al*., 2010b). This pattern has continued despite increasing availability and administration of HBV-active ART.

The role of viral hepatitis and consequent chronic liver disease on mortality has not been widely studied in HIV-infected African populations (Hoffmann and Thio, 2007;Rockstroh *et al*., 2011), but some studies have noted high death rates during the initial period of ART (Johannessen *et al*., 2008;Mzileni *et al*., 2008). However, most of the published studies have been small (Etard *et al*., 2006;Lawn *et al*., 2005;Zachariah *et al*., 2006). Knowledge of the prognostic effect of liver-related conditions, such as hepatitis B, which is highly endemic in the West African region,

would help inform guidelines on screening and treatment with a potential to reduce excess mortality in HIV-infected patients.

The Federal Republic of Nigeria is categorised as a low income country, with a population of over 150 million and an estimated HIV prevalence of 4.4% (Agaba *et al*., 2011) and HBsAg prevalence rates of up to 20% (Ladep & Taylor-Robinson, 2007a). The aims of the present study were:

I. to assess the impact of hepatitis B infection on the survival of HIV-infected individuals; and
II. to identify predictors of mortality in a large cohort of HIV patients on ART in Nigeria.

6.2 METHODS

6.2.1 Study setting and patients

The study was conducted as part of the "AIDS Prevention Initiative in Nigeria" (APIN) programme, affiliated to the Jos University Teaching Hospital (JUTH), Jos, Plateau State in the north-central region of Nigeria. Most patients enrolled into the JUTH/APIN programme were detected through Voluntary Counselling and Testing (VCT) services in adjacent communities. Hospitalised patients were investigated on clinical suspicion or referred from nearby health centres. Subjects found to be reactive to HIV by ELISA were confirmed by Western blot assay prior to enrolment. Serum liver function tests (LFTs), full blood count, HIV RNA and CD4 cell counts were measured in all recruited patients at baseline who were also screened for tuberculosis (TB), prior to commencement of HAART, following the Nigeria national HIV treatment guidelines (www.naca.gov.ng). From June 2004 to December 2010, 19,408 HIV-infected individuals had been recruited in the JUTH/APIN programme

and were being monitored, on ART and/or on anti-tuberculosis drugs. This number estimates at least 13% of all HIV-infected patients within the catchment area (Agaba *et al*., 2011). Onsite and offsite training was provided to personnel working at JUTH/APIN by HIV specialists from the Harvard School of Public Health (Boston, Massachusetts), Northwestern University (Chicago, Illinois) and Johns Hopkins University (Baltimore, Maryland), USA.

All patients were subjected to pre-treatment counselling. A patient tracking team; responsible for providing support to all enrolled patients (in terms of adherence fostering and to follow-up missing patients) was put in place in 2006. Treatment support groups (Hope Support), comprising mostly of HIV-infected patients, were set up within the clinic and surrounding communities as well.

Ethical approval for the present study was obtained from the Ethics Committee of JUTH and an additional Institutional Review Board (IRB) of Harvard School of Public Health, USA.

6.2.2 Laboratory Testing

Before recruitment into the APIN programme, subjects were screened for HIV using Rapid HIV test kits and subsequently confirmed by Western Blot assay. HBsAg was determined using Enzyme immunoassay (EIA) (Monolisa HBsAg Ultra3; BioRad, Hercules, CA, USA). HCV antibody was tested using third generation enzyme immunoassay (DIA.PRO Diagnostic, Bioprobes srl, Milan, Italy). HIV RNA levels were measured using Roche COBAS Amplicor HIV-1 monitor test version 1.5 (Roche Diagnostics, GmbH, Mannheim, Germany) with a detection limit of 400 copies/mL. Flow cytometry was used to determine $CD4^+$ T-cell count (Partec, GmbH, Munster, Germany).

6.2.3 Recruitment, treatment, monitoring and endpoints

Initial first-line antiretrovirals (ARV) in 2004 included Stavudine/Zidovudine, Lamivudine and Efavirenz/Nevirapine. Abacavir and Truvada® (Tenofovir plus Emtricitabine) have been administered since 2007 at APIN. Additionally, second-line treatment with protease inhibitors, in cases of treatment failure also became available since early 2007. However, during 2004 to 2006, Tenofovir and second-line agents were not available and HAART was not individualised. During 2007-2010, those patients commencing HAART that were co-infected with HBV, as well as those requiring second line agents, for virological failure were given ARVs containing Tenofovir.

Patients with CD4 cell counts below 200 cells/mm^3 were prescribed co-trimoxazole 960mg once a day, as prophylaxis for *Pneumocystis jiroveci* pneumonia (PCP). After an initial 4 weeks of fortnightly dispensing, ARVs were provided on a monthly basis to those patients on ART. Paper-based format of data entry was ensured by nurses, physicians and trained clinical officers and same-day computerised data entry was carried out by Data Officers who were supervised by a Data Manager. Personal information, medical history, physical examination, laboratory investigation and chest X ray reports comprised the initial records. Follow-up blood tests performed every 3 months, including LFTs, FBC, HIVRNA and CD4 cell count. Drug-related hepatotoxicity was defined as alanine aminotransferase (ALT) values ≥5 fold over the upper limit of the normal range (ULN) (41iu/mL for JUTH), or if ≥3.5 fold over ULN, if the baseline ALT was above ULN.

As HBV DNA and HCV RNA assays were not available routinely for this cohort, subjects were defined as having HBV and HCV infection if they tested positive for

hepatitis B surface antigen (HBsAg) and hepatitis C antibody (HCV antibody), respectively, on baseline blood samples.

The main end-point of my study was all-cause mortality. Most deaths were reported through the activities of the Tracking team and Hope Support groups. Other outcomes that were censored included those that stopped treatment (detected by pharmacy records), transfer to another health centre or lost to follow-up.

6.2.4 Statistical analyses

The profile of how the patients were selected for analyses is presented in Figure 24. Patients were included only if they had hepatitis B and hepatitis C screening performed on their sera at baseline. Apart from over 7000 patients who did not have one or both HBV and HCV screening, we additionally excluded those that were recruited after December 2010, and those with incomplete information (gender, age or under 15 yrs).

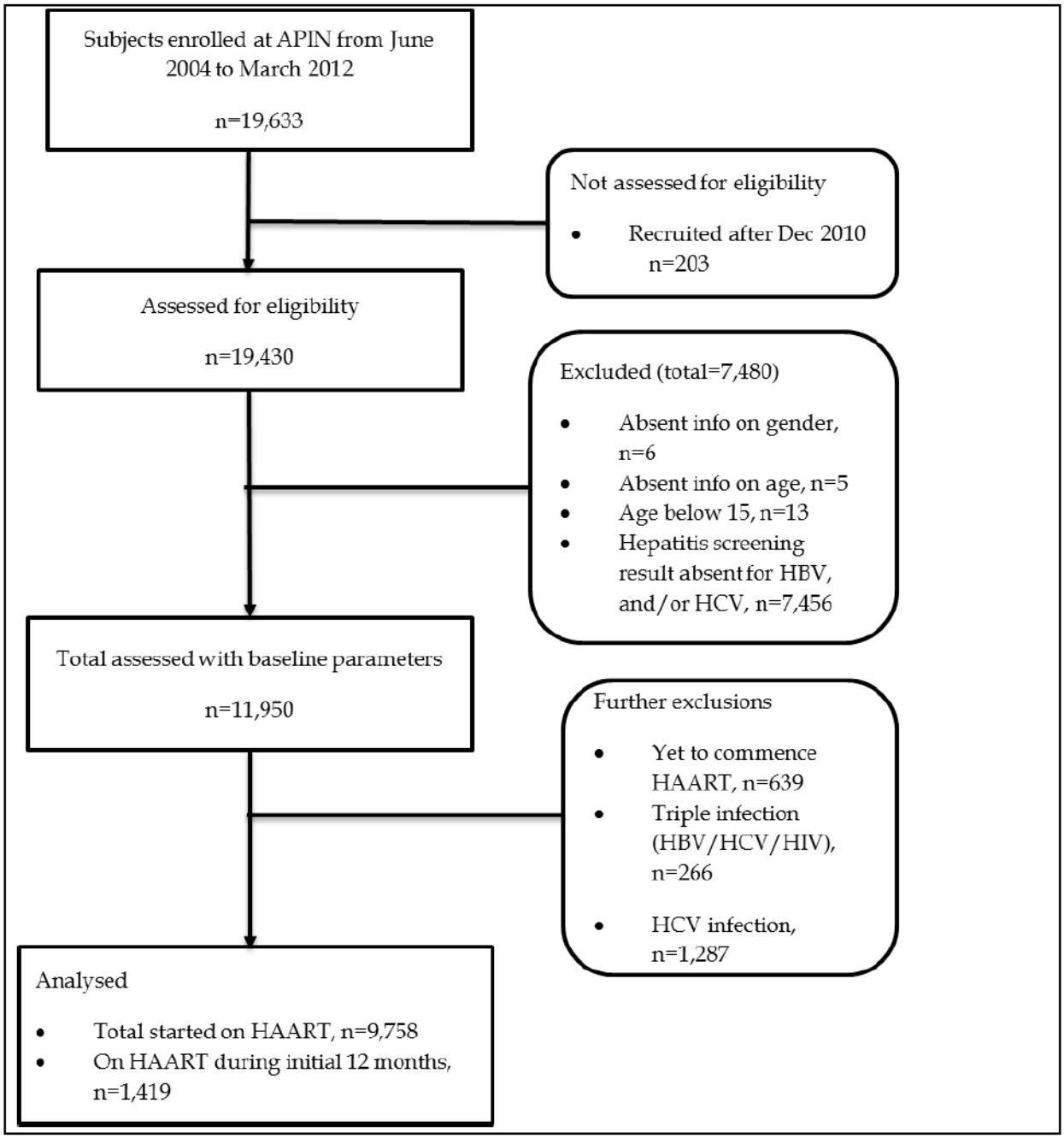

Figure 24. Profile of study cohort, AIDS Prevention Initiative in Nigeria, Jos University Teaching Hospital, in the Federal Republic of Nigeria, 2004-2010

Kaplan-Meier models were used to estimate survival of the patient groups, while a Cox proportional hazard modelling was applied to identify independent predictors of mortality. Univariate analysis was embarked upon initially; and when a statistically

significant association was detected, the variable was fitted into the multivariate model and hazard ratios calculated. Subsequently, we analysed the hazards of death for those patients on TDF-containing HAART, as well as for those not on TDF-containing HAART. Analyses were accomplished using MedCalc Statistical Software version 12.7.7 (MedCalc Software bvba, Ostend, Belgium; http://www.medcalc.org; 2013). P values less than 0.05 were considered statistically significant.

6.3 RESULTS

6.3.1 Baseline characteristics

Of the 9,748 adults that were commenced on HAART, 6,523 (66.9%) were women. The median age of the study population was 33 years (range 15-80 yrs.) and 5,915 (60.7%) of the patients had commenced HAART during the initial period, 2004-2006. The median follow-up duration was 41 months (Interquartile range 30-62 months).

Summary of the baseline characteristics of patients recruited in the study is presented in Table 9. TB treatment was administered to 1,641 (16.8%) patients. For those that had CD4 results at baseline, there was a significantly higher median CD4 cell count in women than in men (M: 146 (95% CI=136-156) cells/mm^3; F: 188 (95%CI=179-195) cells/mm^3; p<0.0001). The median baseline HIV RNA load was log_{10} 4.52 (95% CI=4.50-4.55)(M:4.6(95%CI=4.6-4.7) copies/mL; F:4.5(95%CI=4.4-4.5) copies/mL; p<0.0001). HBsAg-positive rate was common in this study cohort; 1951 (20.0%). Liver disease was documented in only 23 (0.2%) of the patients.

Table 9. Baseline characteristics and associated deaths among HIV-infected patients in Nigeria, 2004-2010

Characteristic	Total, n=9,758	Deaths, n(%)	p value	Male, n=3,229	Deaths, n(%)	Female, n=6,530	Deaths, n(%)
Age-group (years)			0.008*				
15-29	3,147	45(1.4)		393	4(4.0)	2,754	41(1.5)
30-39	3,967	73(1.8)		1,378	31(2.2)	2,589	42(1.6)
40-49	2,001	47(2.3)		1,069	35(3.3)	932	12(1.3)
50+	644	16(2.5)		389	9(2.3)	255	7(2.7)
HBsAg status			0.034				
HBsAg +ve	1,950	48(2.5)		721	25(3.5)	1,230	23(1.9)
HBsAg –ve	7,808	133(1.7)		2,508	54(2.2)	5,300	79(1.5)
Liver disease			0.9				
Present	23	0(0.0)		5	0(0.0)	18	0(0.0)
Absent	9,728	181(1.9)		3,220	79(2.5)	6,508	102(1.9)
TB diagnosis			<0.0001				
Present	1,640	55(3.4)		743	30(4.0)	898	25(2.8)
Absent	8,118	126(1.6)		2,486	49(2.0)	5,632	77(1.4)
HIV RNA at baseline (viral copies/mL)			0.06*				
<400	1,267	25(2.0)		408	11(2.7)	859	14(1.6)
400-9,999	1,950	23(1.2)		565	9(1.6)	1,385	14(1.0)
10,000-29,999	1,523	22(1.4)		427	10(2.3)	1,096	12(1.1)
≥30,000	5,019	111(2.2)		1,829	49(2.7)	3,190	62(1.9)
CD4 cell count at baseline (cells/mL)			0.003*				
<200	1,599	24(1.5)		605	7(1.2)	994	17(1.7)
200-499	1,050	4(0.4)		295	1(0.3)	755	3(0.4)
≥500	136	0(0.0)		24	0(0.0)	112	0(0.0)

Period of enrolment			<0.0001				
2004-2006	5,920	143(2.4)		1,963	67(3.4)	3,958	76(1.9)
2007-2010	3,838	38(1.0)		1,266	12(0.9)	2,572	26(1.0)

*p values indicate comparison for trend

6.3.2 Survival analyses

Of those patients that were on HAART, 181 patients (1.9%) died during the follow-up period. One hundred and forty-three (79.0%) of those that died, started ART during the initial period (2004-2006). Patients who were co-infected with HBV had a lower survival rate, compared to patients negative for HBsAg (Figure 25). The survival probability of HBsAg-positive versus HBsAg-negative patients at 78 months of follow-up was 94% and 97%, respectively (p=0.009). This was the case particularly for men; as HBV infection did not result in survival differences in women. HBsAg-positive men had lower survival, compared to HIV mono-infected men (HIV-positive/HBsAg-positive: 88.5%; HIV-positive/HBsAg-negative: 96.5%; p=0.04).

Fitting the non AIDS-related factors into the Cox regression multivariate model, independent predictors of death for duration of follow up were found to be male sex, co-morbid tuberculosis and HBV infection. Male gender, age ≥40 years at recruitment, co-morbid tuberculosis, HBsAg positivity, and recruitment during the initial phase of the study were all significantly associated with death (Table 10). The hazard of mortality was significantly reduced in patients who started ART during 2007-2010 (HR: 0.64 [95% CI: 0.42-0.88]), compared to those enrolled during the earlier phase of the programme (p=0.0081). Of note, HBsAg-positivity, male gender and more recent enrolment ceased to be significant in those that were on TDF-containing HAART.

As the majority of the deaths occurred during the initial follow-up period, I performed an additional Cox proportional hazard analysis of death during 12 months from recruitment into the study. Age ≥ 40 yrs at recruitment, HBV infection (HBsAg-positive) and enrolment during 2004-2006 were significant predictors of mortality during the early follow-up period.

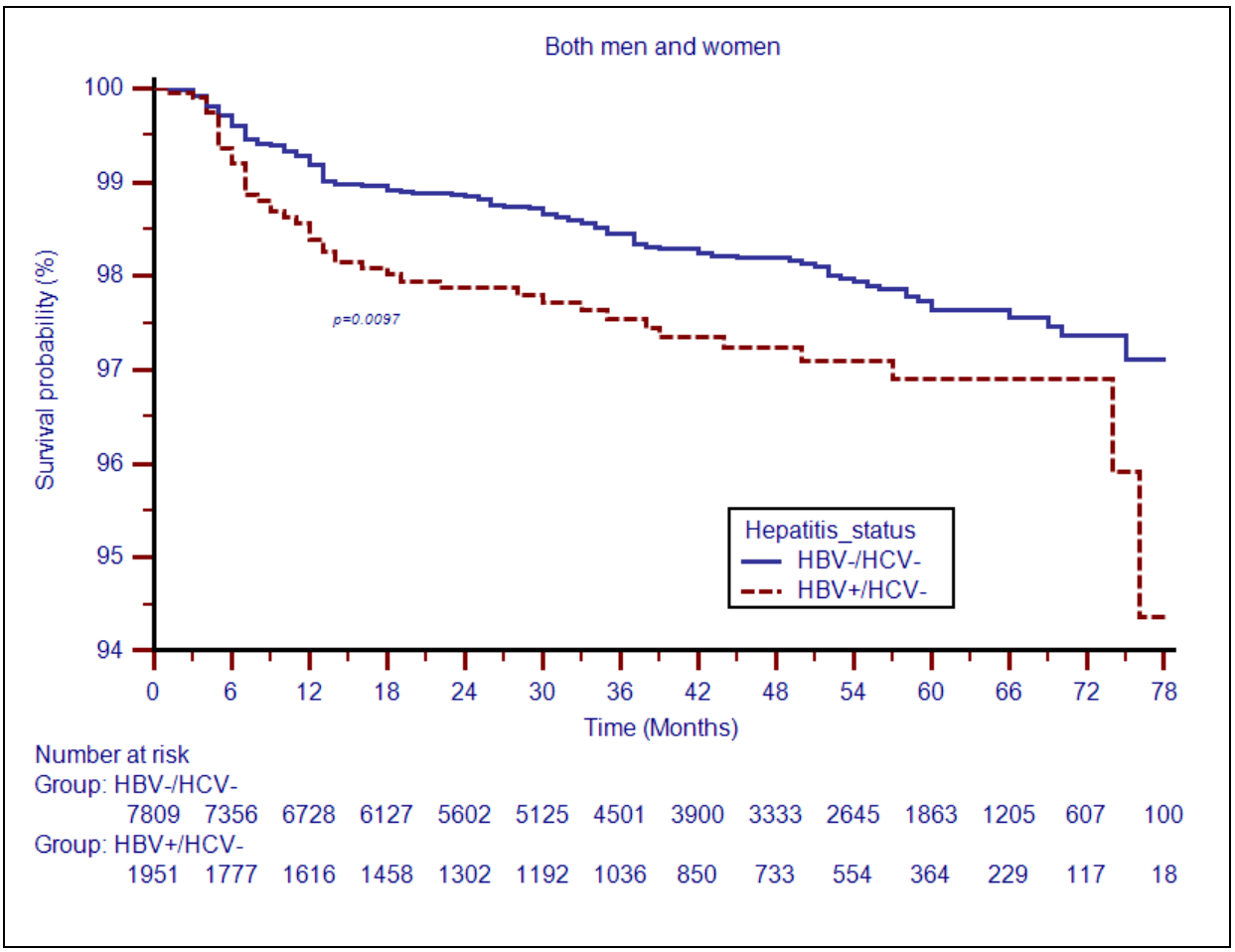

Figure 25 Kaplan-Meier survival curves according to hepatitis co-infection status for: both sexes; and men; APIN 2004-2010; HBV: Hepatitis B virus; HCV: hepatitis C virus

Table 10. Multivariate analyses of predictors of mortality in HIV-infected individuals, AIDS Prevention Initiative in Nigeria JUTH, 2004-2010

HAART	Covariate	HR (95% CI)	SE	Coefficient	p value
All time	Male	1.5(1.08-2.00)	0.15	0.4	0.0134
	HBsAg +ve	1.5(1.09-2.11)	0.17	0.4	0.0129
	Latter recruitment (2007-2010)	0.6(0.42-0.88)	0.19	-0.5	0.0081
	TB diagnosis	2.2(1.57-2.96)	0.16	0.8	<0.0001
Initial 12 months					
	HBsAg +ve	2.1(1.32-3.21)	0.23	0.7	0.0015
	40-49 years	1.9(1.16-3.07)	0.25	0.6	0.011
	≥50 years	2.5(1.31-4.80)	0.33	0.9	0.0057
	Earlier recruitment (2004-2006)	6.3(3.86-10.31)	0.25	1.8	<0.0001
None-TDF-based HAART	Male	1.8(1.16-2.68)	0.21	0.6	0.0084
	HBsAg +ve	4.3(2.60-6.95)	0.25	1.4	<0.0001
	Latter recruitment (2007-2010)	0.3(0.14-0.57)	0.36	-1.2	0.0004
TDF-based HAART	TB diagnosis	2.9(1.94-4.39)	0.21	1.1	<0.0001

6.3.3 Virological suppression

Owing to lower survival associated with HBV infection, the impact of HBsAg-positive status on HIV RNA suppression, following long-term HAART was explored. Thus, I analysed HIV viral load on 8,515 patients, of which 1,643 were HBsAg-positive and 6,872 were HBsAg-negative. HBV co-infection was associated with higher HIV RNA log_{10} copies (HBsAg-positive: 4.59(95%CI=4.53-4.65) vs HBsAg-negative: 4.49(95%CI=4.47-4.52); p<0.0001) at baseline. However, the median HIV RNA load at ≥12 months on HAART was not different between the study groups (HBsAg-positive: 2.3(95%CI=2.3-2.3) vs HBsAg-negative: 2.3(95%CI=2.3-2.3) (Figure **26**).

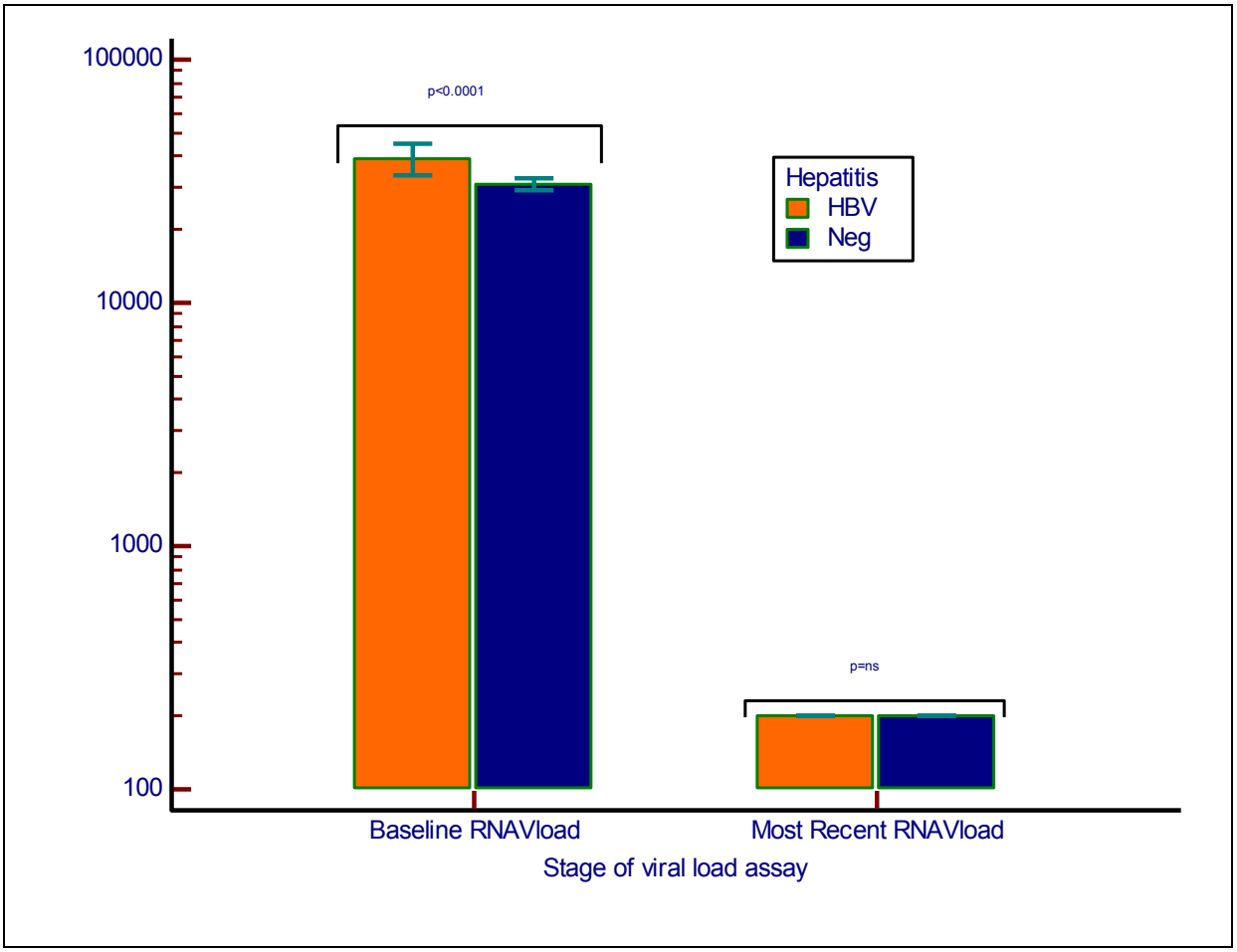

Figure 26 Median HIV RNA load at baseline and at ≥12 months on HAART of HBV and HIV mono-infected groups; JUTH/APIN 2004-2010; HBV: hepatitis B co-infection; Neg: HIV mono-infection

6.4 DISCUSSION

In this African HIV-infected cohort of 9,758 patients on HAART, followed up for a median period of 41 months, the impact of HBV infection on survival and independent predictors of mortality of HIV-infected patients undergoing antiretroviral therapy were determined. HIV/HBV co-infection was found to be significantly associated with a lower probability of survival, compared to HIV mono-infection. On the other hand, relatively recent recruitment into the APIN programme was significantly associated with reduced rate of death in both groups. Apart from HBV co-infection, tuberculosis and male sex were also significantly associated with mortality.

Two studies from South Africa found conflicting outcomes regarding the impact of HBV co-infection on HIV mortality. Hoffman and colleagues in 2008 reported similar probability of deaths in HBV/HIV and HIV mono-infected patients over a 72-week period. In contrast, Matthews and colleagues (2011) observed a significantly higher mortality in HBV co-infected patients, compared to HIV mono-infected 1,771 patients in South Africa over 48 weeks follow-up duration (Matthews *et al*., 2011).

The findings in the present study corroborated those of a large study of HIV-infected men in the US in which non AIDS-related mortality was highest among HIV/HBV-infected patients, despite being on HBV-active HAART (Hoffmann *et al*., 2009b) and in which no association between HBV infection and higher rates of HIV virologic failure was found. Similarly, in South African patients, HIV/HBV co-infection was not associated with lower HIV RNA suppression during ART (Hoffmann *et al*., 2008;Matthews *et al*., 2011).

The reasons for the negative impact of HBV infection on mortality are not fully understood, but it is unlikely to be related to accentuation of HIV replication, as similar found similar levels of virologic suppression in co-infected versus HIV mono-infected patients was observed. HBV infection in this population frequently occurs early in life and is postulated to precede HIV infection (Lesi, Kehinde, Oguh, & Amira, 2007) by which time liver damage may be irreversible and non-responsive to ART. Inflammatory response commonly occurs during the early phase of ART(Hirsch *et al*., 2004) and could be contributing to the flaring of HBV infection(Crane *et al*., 2009).

I noted higher mortality during the early phase of ART in this study population. It is possible that this effect is a result of patients with more advanced disease entering the programme and this is supported by the change in overall mortality over time. However, analysis restricted to only those who are taking Tenofovir suggests a beneficial impact in those with HBV.

Tuberculosis is a common co-morbidity of HIV-infected patients and was an independent predictor of mortality in the present study, in agreement with the results of other studies across Africa (Lawn *et al*., 2008;Lim *et al.,* 2012;Richter *et al*., 1995). It is known that the relative risk of anti-tuberculous therapy-induced hepatotoxicity is higher in HBV/HIV co-infected patients than in HIV mono-infected individuals (de *et al*., 2010). However, I did not identify any significant association between death and decompensated liver disease. Notably, none of the patients who had hepatotoxicity from ARVs died in this study. Nevertheless, symptomatic hepatotoxicity (particularly, jaundice) frequently prompts patients to seek alternative drugs, a common feature in African HIV populations (Chitturi and Farrell, 2000;Mudzviti *et al*., 2012), and/or which lead to treatment interruption and change to less potent regimens. Such

patients might have died at home or away from JUTH and therefore these data would not have been captured in this data set.

Male gender was a significant predictor of mortality in the study cohort. Men had lower CD4 cell count at HAART commencement than women. Indeed, the CD4 cell count at commencement of HAART has been found to correlate with response and ultimately, survival (Biadgilign *et al*., 2012). These data confirm this, as a significant trend of decreased death rate with increasing CD4 cell count prior to commencing ART was found. It is not clear the reason why men had higher HIV RNA load and lower CD4 cell count at baseline, but it is possible that antenatal HIV screening services as part of mother to child transmission of HIV (PMTCT) services might have enabled more women to be diagnosed at stages earlier than men.

Limitations encountered in the present study include the following: First, the causes of death were not well ascertained and autopsy rates in this population are low. Precise dates of death of several patients were not recorded and I depended on information from carers, Hope Support and Tracking teams. I was thus limited in describing disease-specific deaths. Whenever any of the patients had any serious illness, such as liver disease, they were referred to specialist consultation in the affiliated hospital (JUTH). As no linkage of records, exists currently between JUTH and APIN, considerable amounts of information that would have been relevant to our analyses may have been missed. It would have been quite informative to determine the contribution of factors associated with mortality in those who were yet to be on treatment for their HIV infection. This factor could not be examined as follow up documentation of deaths and routine investigations were undertaken only in those who were commenced on ART.

Second, the diagnosis of chronic HBV infection based on a single test for the detection of HBsAg could be misleading as the standard definition of chronic hepatitis B is based on two tests performed at least 6 months apart. A small chance of misclassification may have occurred due to this; however, because HBV transmission commonly occurs in childhood in Nigeria, it is unlikely that the cases identified as HBsAg positive had acute HBV. Unpublished confirmation of HCV and HBV viraemic status by molecular methods revealed that HCV viraemia in 314 anti-HCV positive patients was 31.2% while of 264 HBsAg positive individuals, HBV viraemia was documented in 86%. Characterising the patient groups by their viraemic status would have enhanced the power of this study.

In conclusion, HBV infection, tuberculosis and male gender were significantly associated with mortality during a 41-month median period, following commencement of ART therapy. Patients recruited during the latter period of enrolment, coinciding with increased access to Tenofovir-based treatment experienced less deaths. Indeed, HBV was not a significant predictor of mortality in those patients that were being administered Tenofovir-containing HAART. HBsAg-positive status was not associated with reduced HIV viral load suppression following HAART. Long-term follow-up and enhanced monitoring are needed to assess incidence of end-stage liver disease, hepatocellular carcinoma and disease-specific deaths in this cohort. These data support an active approach to identifying all HIV/HBV co-infected individuals in resource poor settings, if Tenofovir is not routinely offered as first line treatment(Lessells *et al*., 2008).

7. METABOLIC PROFILING OF HEPATOCELLULAR CARCINOMA

7.0 Definitions

Metabonomics is a study of the overall metabolic response to functional, drug and disease stimuli, while metabolomics is the analytical description of complex biological samples (Fiehn, 2001;Nicholson and Lindon, 2008). The former seeks to characterize and quantify all such small molecules in the sample. Metabonomics is able to provide robust information on cellular and organ function, complementing genetic, epigenetic and proteomic knowledge. Magnetic resonance spectroscopy (MRS) and mass spectrometry (MS) are the most commonly used methods for metabolite characterisation.

7.1 Tumour metabolism

Generally, tumour cells require increased amounts of energy (ATP) and substrates for synthesis of nucleotides, lipids and proteins for rapid proliferation. There is increasing evidence that this altered metabolism in tumour cells is both a cause and effect of carcinogenesis (Ward and Thompson, 2012). Cancer is thus visualised as a metabolic disease and the solution of two metabolic equations: first and foremost, to produce enough energy to survive when supplies and waste disposal are limited and second, to divert enough metabolic intermediate from energy products to biosynthetic pathways that provide the critical biomass needful to support cell proliferation.

7.1.1 Glycolysis in the tumour cell

To simulate an anaerobic condition, Warburg in 1924 placed a section of rat cancer tissue in Ringer's lactate that was saturated with nitrogen and observed that the tissue could be transplanted to a live donor if sugar was included in the solution, but

not if it was left plain (Warburg, 1958). Warburg later on discovered that even in the presence of sufficient oxygen, cancer cells preferentially metabolise glucose by glycolysis rather than oxidative phosphorylation, a more efficient pathway for energy production. He hypothesized that this increase in glycolysis in the presence of oxygen by cancer cells is a reflection of defective mitochondrial oxidative phosphorylation (Warburg, 1958). Indeed tumour cells take up high amount of glucose to fuel heightened glycolysis, the basis for which glucose-labeled positron emission tomography (PET) is used for tumour identification (Ariff *et al*., 2009). Apart from a faster rate of production of ATP, glycolysis also provides intermediates for the pentose phosphate pathway and subsequent biosynthesis of nucleic acids, both of which are of benefit to rapidly dividing tumour cells (Figure **27**).

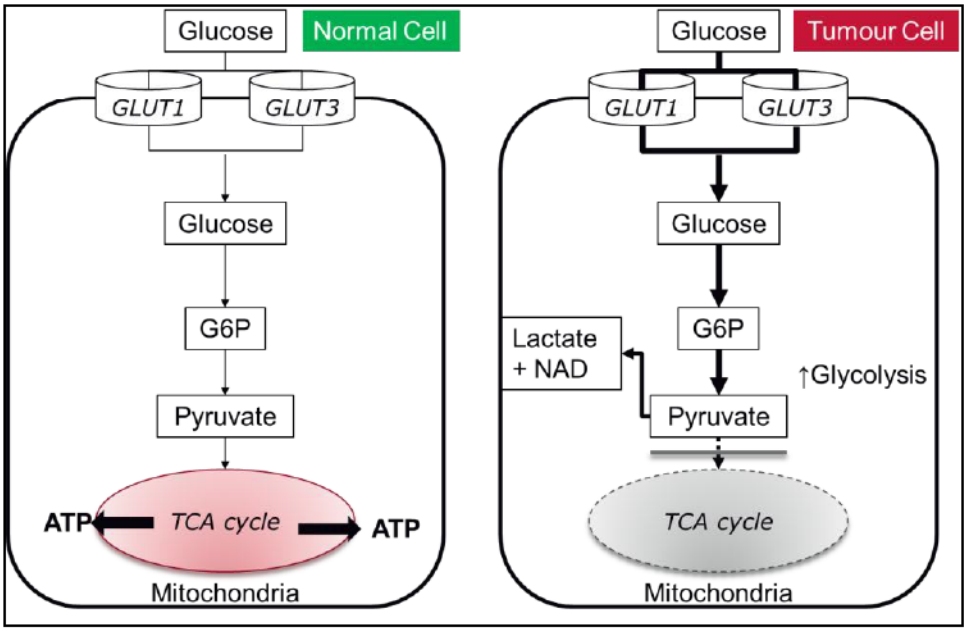

Figure 27. Altered glycolytic and mitochondrial metabolism in tumour cells

Key: G6P, glucose-6-phosphate; TCA, tricarboxylic acid cylcle; ATP, adenosine-tri-phosphate; NAD, nicotinamide adenine dinucleotide.

7.1.2 Mitochondrial metabolism and cancer

It is yet to be clearly defined the role that mitochondria play in tumour cell function. Primary defects in the mitochondrial membrane (small and absent cristae, as well as deficiency of β-F1 subunit of ATPase); the site of oxidative phosphorylation has been suggested by some researchers (Kroemer and Pouyssegur, 2008;Lopez-Rios *et al*., 2007). However, some groups have shown that despite a high glycolysis rate, oxygen consumption by cancer cells is not reduced (Weinberg and Chandel, 2009). Undoubtedly, HCC is a highly vascular tumour in the early stages and is certainly likely to be adequately supplied with oxygenated blood.

7.1.3 Molecular effectors of tumour metabolism

Hypoxia-inducible factor 1(HIF 1) and p53 are the commonest effectors that undergo mutations in HCC and may be responsible for some of the metabolic changes in HCC. A short introduction of these effectors follows.

7.1.3.1 HIF1

HIF1, a transcription factor which stimulates glycolysis in tumour cells is activated by diverse stress situations, including hypoxia, inflammation, metabolic and oxidative stressors (Harris, 2002;Kroemer & Pouyssegur, 2008;Weinberg & Chandel, 2009). This heterodimeric complex (consisting HIF 1α and HIF 1β) is stabilised at low oxygen levels, but is degraded by the proteosome in normoxic conditions. HIF1 stimulates glycolysis by increasing the expression of the rate limiting pro-glycolytic enzyme, hexokinase; as well as the expression of glucose transporter isoform 1 (GLUT 1) (Weinberg & Chandel, 2009). By inhibiting pyruvate dehydrogenase (PDH), HIF1 decreases the conversion of pyruvate to acetyl-coA (often required for production of electron donors, NADH and $FADH_{2;}$ both cofactors in oxidative phosphorylation). The importance of HIF1 in HCC pathogenesis cannot be

overemphasised as HIF1β deficient hepatoma cells in mice were found to have reduced growth rates and glycolytic intermediates compared to wild-type hepatoma cells (Griffiths *et al*., 2002).

The role of HIF1 in HCC is being further investigated and recent data involving animal models have demonstrated high HIF1 activity and GLUT1 (downstream counterpart) in liver cancer cells (Amann *et al*., 2009;Wang *et al*., 2009b;Yao *et al*., 2009). Vascular endothelial growth factor (VEGF) plays a vital role in the development of HCC (Armengol *et al*., 2004). HIF1 induces the expression of VEGF in hypoxic conditions thereby enhancing neovascularisation. This pro-angiogenic property leads to increased tumour growth but persistent central tumour hypoxia, thus allowing HIF 1 to remain active.

7.1.3.2 p53

The p53 gene is essential for a high quality of life and fidelity of replication of the organism. This tumour suppressor gene has been implicated in alterations in metabolism. p53 enhances the expression of cytochrome c oxidase 2 (SCO2), required for the assembly of the oxidative phosphorylation enzyme, cytochrome c oxidase (COX) (Matoba *et al*., 2006) but negatively regulates phosphoglycerate mutase (PGM) (Kondoh *et al*., 2005). Additionally, p53 transcriptionally activates the TP53-induced glycolysis and apoptosis regulator (TIGAR), which is also an inhibitor of phosphofructokinase activity. This inhibition of phosphofructokinase in turn lowers the level of the allosteric activitor of glycolysis, fructose 1,6-biphosphate (FBP) (Bensaad *et al*., 2006). Since wild-type p53 downregulates PGM, mutation of p53 can facilitate PGM and hence glycolysis, contributing to the Warburg phenomenon.

7.1.4 Metabolite effects on carcinogenesis

Lactate, thought to be a "waste" product of glycolysis, can stimulate HIF1 independently of hypoxia, thereby conditioning the tumour environment (Hsu and Sabatini 2008). Also, lactate has been suggested to suppress anticancer immune effectors (Koukourakis *et al*., 2006;Kroemer & Pouyssegur, 2008;Swietach *et al*., 2007).

TCA cycle intermediates; including fumarate and succinate can stimulate HIF1 production. Germ line mutations of TCA cycle genes lead to defective enzymes (fumarate hydratase and succinate dehydrogenase) (Gottlieb and Tomlinson, 2005) which result in the inability to metabolise their substrates. The sum effect of these changes is the accumulation of HIF1 as fumarate and succinate competitively inhibit α-ketoglutarate-dependent HIF-1 α-prolyl hydrolase, the enzyme that destroys HIF1.

7.2 Magnetic resonance spectroscopy and metabolic profiling of HCC

The behaviour of atoms when subjected to a magnetic field is referred to as nuclear magnetic resonance (NMR). Atoms with odd mass numbers such as hydrogen (^{1}H), phosphorus (^{31}P) and carbon (^{13}C) possess this property, often described as "spin" and behave as dipoles aligning along the axis of an applied magnetic field (Figure **28**). As protons relax following excitation, they generate radiofrequency signals which are detectable by receiver coils as "free induction decay". The frequency spectrum is expressed by the mathematical process of Fourier transformation. Being the most abundant atom in living organisms, studies of ^{1}H NMR spectra are mostly carried out and it not only provides information on metabolic differences between subjects, but additionally provide insight into cellular metabolic pathways.

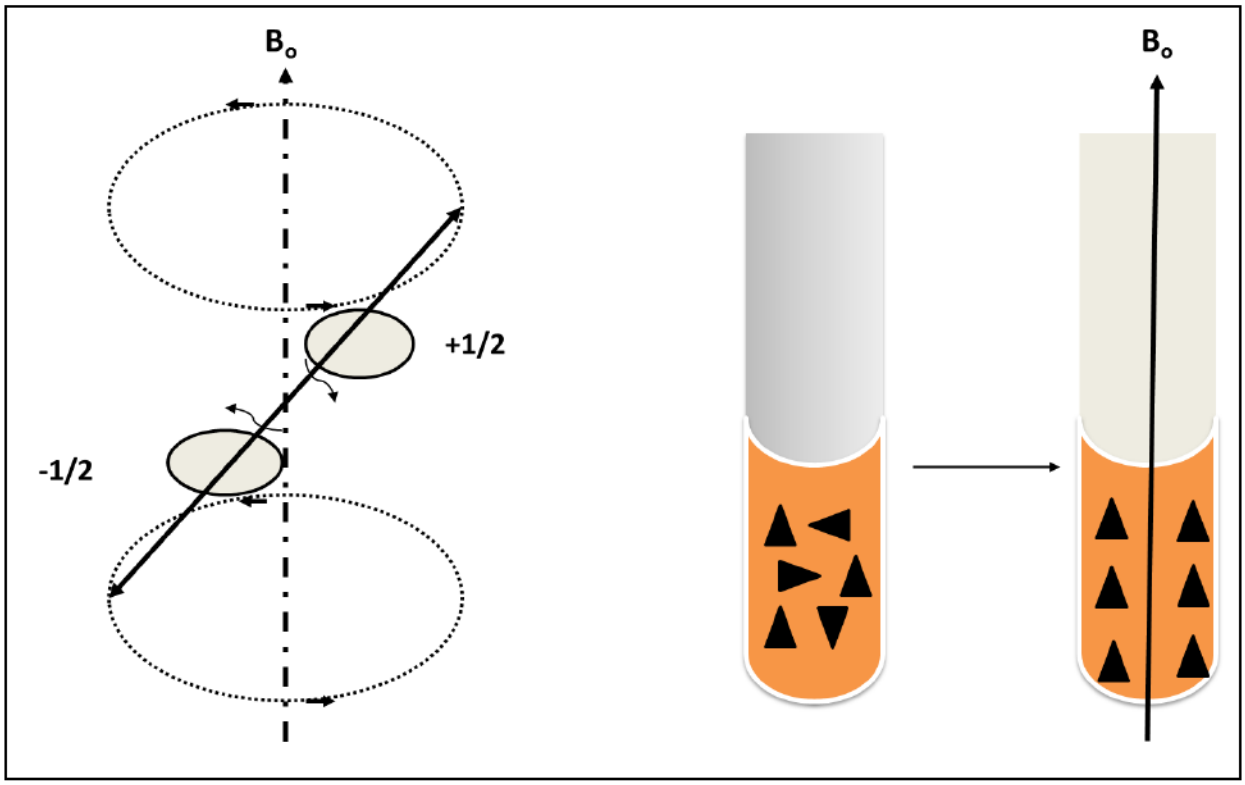

Figure 28. Spin attributes of protons

Protons possess "spin" and align along an applied magnetic field (B_O)

Metabolic profiles have been generated from biological substances including: intact tissue, bile (Khan *et al*., 2005a), serum (Williams *et al*., 2009) and urine (Shariff *et al*., 2010;Shariff *et al*., 2011). Yang and colleagues used *in vitro* magic angle spinning NMR and found that HCC tissues had high levels of lactate, glutamate, glycine, leucine, alanine, choline metabolites and reduced levels of triglycerides, glucose and glycogen compared to normal adjacent liver tissue (Yang et al. 2007).

7.3 Mass spectrometry and metabolic profiling of HCC

Mass spectrometry started to be used for metabolic profiling about 40 years ago (Pauling *et al*., 1971). It has a higher sensitivity for small molecules than 1H NMR

and complements the latter technique. Constituent fragments of, or primary metabolites are detected and distinguished by their molecular weights and ionic charges. Prior to analyses, biological fluids require separation using certain methods, among which are gas chromatography (GC) or liquid chromatography (LC). LC requires less extensive pre-treatment and derivation steps than GC and thus amenable to biofluid analyses. The samples are ionized after separation using electrospray ionization (ESI); often combined with LC-MS. ESI is highly sensitive and offers a soft ionization as well as an excellent quantitative analysis (Want *et al*., 2007). The particles are then detected by a time-of-flight (TOF) analyser, which allows the detection of analytes in the *m/z* 50-1000 range (Figure **29**). LC-MS has been used for metabolic profiling of HCC. Yin and colleagues identified dihydrosphingosine and phytosphingosine in serum of patients with HCC using reversed phase (RP) LC and hydrophilic interaction chromatography (HILIC) MS (Yin *et al*., 2009).

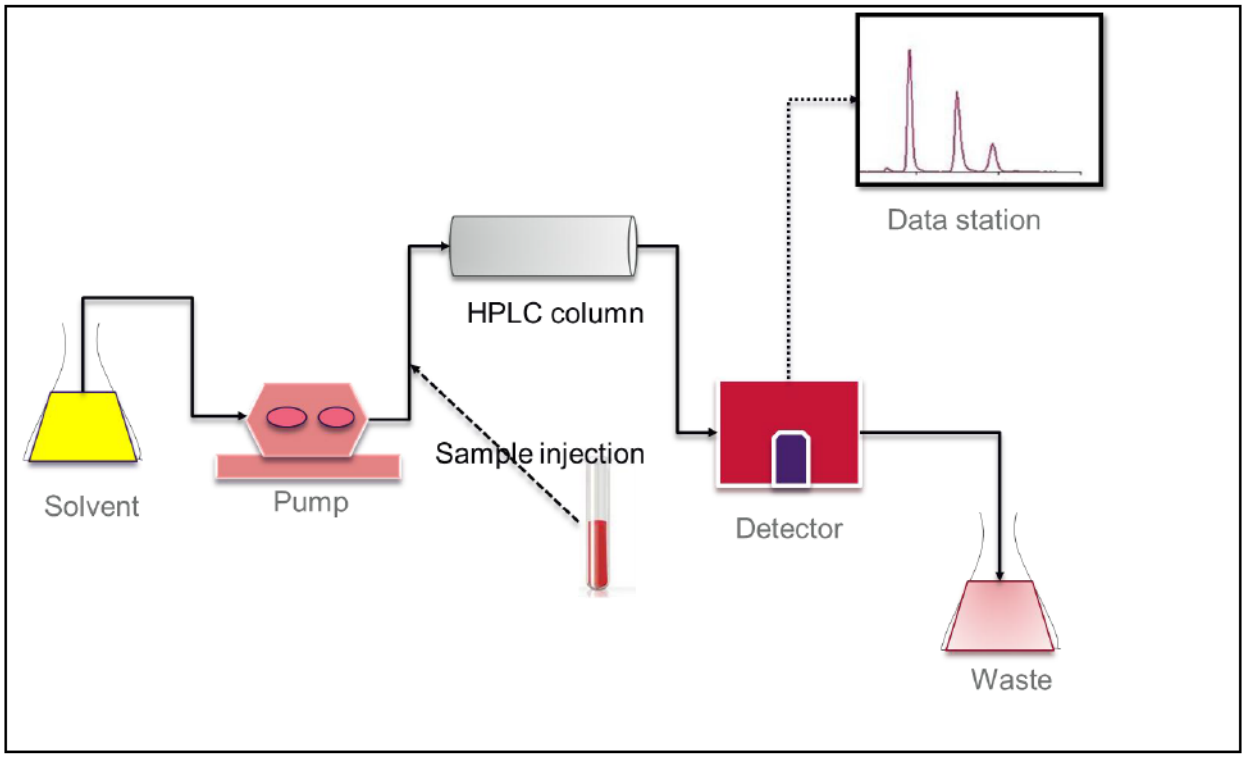

Figure 29. Principles of liquid chromatography mass spectrometry

Hypothesis

- HCC cells secrete metabolites into the systemic circulation that are detectable in urine.

Aims

- To determine urinary biomarkers of HCC;
- To compare the relative concentration of identified biomarkers with those of controls (cirrhosis, non-cirrhotic liver disease and healthy subjects); and
- To determine the diagnostic ability of identified metabolites.

8. BIOMARKER DISCOVERY OF HCC IN WEST AFRICAN PATIENTS USING PROTON NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

8.0 ABSTRACT

Background: The main clinically-used biomarker of hepatocellular carcinoma (HCC) in routine screening, surveillance and diagnosis, serum alpha fetoprotein (AFP), has a low sensitivity. Ultrasound imaging is impractical in severely resource-deprived communities. This study aimed to determine the diagnostic performance of urinary metabolites using proton NMR spectroscopy of patients from West Africa and to explore the correlation of identified metabolites with clinical stage of HCC.

Methods: Urine samples were collected at two sites in West Africa on the case-control platform of the "Prevention of Liver Fibrosis and Carcinoma in Africa" (PROLIFICA) study. 600MHz NMR was used to acquire one-dimensional spectral data from the urine samples, using a standard 1D NMR pulse sequence with presaturation of the water peak. Areas under the operating characteristic curves (AUC) of the relative concentration of metabolites identified, measured in 290 patients: 63 with HCC, 32 with cirrhosis (Cir), 107 with non-cirrhotic liver disease (DC) and 88 healthy volunteers (NC), were compared to serum AFP. Correlation of identified metabolites with Barcelona Clinic Liver Cancer (BCLC) stage of HCC was made.

Results: Multivariate modelling of the spectral data showed distinct urinary metabolic profiles of patients with HCC, compared to Cir, DC and NC with sensitivity (95%CI)/ specificity (95%CI) of 81%(77-95)/84%(64-96), 85%(73-94)/93%(86-97) and 97%(89-100)/99%(94-100), respectively. The metabolites that were significantly increased ($p<0.0001$) in urine of HCC patients, compared to non-HCC liver disease

controls (DC and Cir), were *N*-acetylglutamate, dimethylglycine, 1-methylnicotinamide, aspartate, methionine, acetylcarnitine, carnitine, 2-oxoglutarate, indole-3-acetate, and creatine. Urinary metabolite profiles performed better than AFP in discriminating HCC from other non-malignant liver conditions (AUC: HCC vs. DC; metabolite profile [0.9] vs. AFP [0.8], p=0.0001; HCC vs. Cir; metabolites [0.9] vs. AFP [0.7], p=0.0068). There was a significant positive correlation of *N*-acetylglutamate, acetylcarnitine, methionine, aspartate, dimethylglycine and creatine with BCLC stage of HCC.

Conclusions: NMR spectroscopy of urine of this West African population has identified metabolites that implicate altered energy-related pathways in the pathogenesis and progression of HCC. Additionally, the urinary metabolic profiles performed better diagnostically than serum AFP. These findings confirm that a panel of urinary metabolites may prove useful for screening HCC in at-risk populations.

8.1 BACKGROUND

Hepatocellular carcinoma (HCC), the commonest primary liver cancer, carries a poor prognosis being the third most common cause of cancer-related death (Parkin *et al*., 2005b;Parkin, 2006). The incidence to mortality ratio of HCC approaches unity in most developing countries, owing to very late diagnosis. It is responsible for approximately 700,000 deaths per year globally. There is a high incidence of HCC in sub-Saharan Africa and a steadily rising incidence in several developed countries (El-Serag *et al*., 2004;El-Serag, 2007;El-Serag & Rudolph, 2007;Khan *et al*., 2002b). Alpha-fetoprotein (AFP), a commonly used serum biomarker for diagnosis of HCC, lacks sufficient sensitivity for detection of HCC at stages amenable to curative therapies, besides being expensive (Daniele *et al*., 2004). Biomarkers for early detection and which may form targets for therapy of HCC are thus urgently needed.

Metabolic profiles, often affected by many pathological processes may assist in the development of novel diagnostic tests, and/or complement current tools in clinical use. Liver tissue (Yang *et al*., 2007), faecal material (Cao *et al*., 2011) and serum (Chen *et al*., 2011a;Chen *et al*., 2011b;Yin *et al*., 2009;Zhou *et al*., 2012) have been studied for molecular fingerprints of HCC using metabolomics. Most studies using urine have been undertaken in Chinese populations (Chen *et al*., 2011b;Wang *et al*., 2013b;Wu *et al*., 2009;Ye *et al*., 2012a), comparing HCC versus healthy controls. Metabolic biomarkers discriminating HCC from healthy controls are limited as they may represent fingerprints of decompensating liver disease (as most HCC occur in the background of cirrhosis) rather than those of malignancy, and hence may not be useful for screening of HCC in patients with cirrhosis (Bruix & Sherman, 2011). It has previously been shown that urinary biomarkers of HCC in a small pilot study of HBV-associated patients from Nigeria significantly discriminated HCC from cirrhosis

patients (Shariff *et al*., 2010). Shariff and colleagues have also been found similar metabolic changes in an HCV-associated HCC cohort from Egypt (Shariff *et al*., 2011), suggesting that the metabolic changes are as a result of the process of carcinogenesis, rather than aetiopathogenetically specific to HCC.

In this study, I aimed to determine urinary metabolites of HCC from a West African population (Gambia and Nigeria). A comparison of the diagnostic performance of the metabolite profiles with AFP was also ensured. Additionally, I examined the relationship between the intensities (relative concentrations) of identified metabolites with BCLC stage of HCC.

8.2 METHODS

8.2.1 Study design/subject selection

Samples for the present study were from subjects recruited in Nigeria and Gambia, who are formally consented to participate in the Prevention of Liver Fibrosis and Carcinoma in Africa (PROLIFICA) project. PROLIFICA is a European Union Framework 7-funded project (PROLIFICA: www.prolifica.eu). The case-control platform involved identifying cases (HCC, chiefly arising on the background of chronic hepatitis B viral infection) and three classes of control, namely:

i. mainly hepatitis B-related cirrhosis,

ii. mainly non-cirrhotic chronic hepatitis B-related liver disease, and

iii. healthy volunteers from the same African populations.

Urine samples were collected for metabonomic studies using urinary proton (^{1}H) nuclear magnetic resonance spectroscopy (NMR) at Imperial College London. Samples collected from patients, diagnosed with HCC according to the European

Association for the Study of the Liver (EASL) guidelines were stored at -80^{o}C until air-transported on dry ice to Imperial College London. Ethics committees of JUTH, Nigeria and MRC, Gambia; as well as Imperial College London granted approval for the study.

In particular, the EASL guidelines utilised in the recruitment of HCC cases (*n*=63) was based on two imaging techniques showing early arterial enhancement and rapid venous washout; or one characteristic imaging with serum AFP >400 ng/mL (Anon 2012). Two groups of disease control (cirrhosis; *n*=32 and non-cirrhotic liver disease; *n*=107) and a group of healthy subjects (*n*=88) provided urine samples for comparisons. All controls were subjected to liver ultrasound scan and underwent serological testing for HBsAg, anti-HCV and HIV screening. Staging of HCC was performed according to the Barcelona Clinic Liver Cancer (BCLC) criteria (Llovet et al. 1999).

8.2.2 Urine collection methodology and handling

Approximately 5mL of non-fasted urine samples were collected into universal containers after obtaining informed written consent and administration of dietary questionnaires. The questionnaires captured information on the 72-hour as well as 24-hour dietary recall as well as drugs the subjects would have taken. The collected samples were immediately placed in cold-boxes prior to transportation to storage facilities. It was ensured all urine samples were aliquoted into storage (-80 degrees freezer) eppendorfs within one hour of collection. In situation where this was not immediately achievable, the samples were stored in -20 degrees freezers before transportation to the laboratory. These were transported, accompanied by laboratory personnel to the laboratory in sealed cold boxes.

Shipments of samples from the participating sites to Imperial College London were on dry ice. However, after one failed shipment from Nigeria, I developed a visual protocol (see Appendix F) for sample handling and transportation that has ensured successful shipment of studied samples. The urine samples had to be accompanied in order to ensure smooth transition through customs and immigration checkpoints.

8.2.3 Sample preparation

Prior to spectral acquisition, samples were thawed at room temperature. Sample preparation and acquisition methods were performed according to previously published methods (Beckonert *et al*., 2007). 400 µL urine sample was mixed with 200 µL of phosphate buffer solution (0.2 M Na_2HPO_4/0.04 M NaH_2PO_4, pH = 7.4 plus 0.1% sodium azide, 1 mM 3-trimethylsilyl-1-[2,2,3,3,-$^2H_4$] propionate (TSP)) to stabilize the urinary pH. The samples were allowed to stand for 10 min prior to centrifugation at 13000 rpm for 10 min in order to remove insoluble material. 400 µL of the supernatants from each urine sample was aliquoted into 5 mm NMR tubes (Wilmad LabGlass^{TM}, New Jersey, USA) for proton nuclear magnetic resonance (^{1}H NMR) studies.

8.2.4 ^{1}H NMR spectral acquisition and processing

^{1}H NMR spectra were acquired with a 600 MHz spectrometer (Bruker Avance) operating at 600.13 MHz for ^{1}H at 300 K. It was equipped with a 5 mm broad-band inverse configuration probe. Samples were randomly analysed in automation with a B-ACS 60 sample changer system. Urine samples were analysed using water suppressed 1D NMR spectrum using the NOESYPRESAT pulse sequence (256 transients). Irradiation of the solvent (water) resonance was applied during presaturation delay (2.0 s) for all spectra and for the water suppressed 1D NMR spectra also during the mixing time (0.1 s). The pulse sequence parameters

including the 90° pulse (~ 10 µs), pulse frequency (~ 4.8 ppm), receiver gain (~ 200), and pulse powers were optimised for each sample set run. The spectral width was 20 ppm for all spectra. The NMR data were processed with an exponential line broadening of 1.0 Hz prior to Fourier transformation, which were collected with approximately 32 k real data points. Data [-1.0 to 10.0 ppm] were imported into MATLAB 7.0 software (MathWorks, Natick, MA), where they were automatically phased, baseline corrected and referenced to the TSP peak (0.00 ppm), using scripts written in-house. To reduce analytical variation between samples the residual water signal (4.70 – 5.00 ppm) was truncated from the data set. Normalisation to total area was performed by calculating, for each spectrum individually, the ratio between each variable and the sum of each spectrum after removal of regions specified above. Assignment of endogenous urinary metabolites was made by reference to published literature data (Bollard *et al*., 2005;Nicholson *et al*., 1995).

8.2.5 Multivariate statistical analyses

The acquired spectra were divided into small regions of 0.02±0.01ppm, representing specific metabolites. These regions were aligned (controls mild shifts in the spectra) and normalised (controls differential dilution of samples). A matrix of samples against variables (ppm bins) was thus generated and transferred to SIMCA 13.0 (statistical software) for unsupervised principal components analysis (PCA) and supervised partial least squares discriminant analysis (PLS-DA). Using these techniques, outliers were identified; with some being removed from the model (especially those due to experimental artefacts). The models were subjected to “leave-one-out” validation, a technique in which each sample in turn was automatically excluded from the analysis, a model created from the remainder of the samples and the class

membership of the excluded sample predicted. This technique was applied to every 7th sample until each sample was excluded once.

8.2.6 Univariate statistical analysis

The metabolites that contributed most to the OPLS-DA separation were identified through analysis of the loading and S-line plots using MATLAB 7.0 and SIMCA 13.0, respectively. Identified metabolites were confirmed using an in-house statistical total correlation spectroscopy (STOCSY) tool. Intensities of the metabolites were expressed as concentration relative to creatinine (controls for differential kidney function of the subjects). Using MedCalc Statistical Software version 12.7.7 (MedCalc Software bvba, Ostend, Belgium; http://www.medcalc.org; 2013), Mann-Whitney U test of significance was applied to study the differences in the median values of identified metabolites between HCC (cases) and the three classes of controls.

The ability of single metabolites and each model to discriminate HCC from cirrhosis, non-cirrhotic liver disease and healthy control subjects were examined for sensitivity and specificity based on measurement of Area under the Receiver Operating Characteristic (AUROC) curves. The AUROC curves were modelled to compare the diagnostic performance of these metabolite panels with serum alpha fetoprotein; each for HCC versus cirrhosis, HCC versus non-cirrhotic liver disease and HCC versus healthy.

Finally, the regression coefficients of each of the significant metabolites were correlated to the clinical stage of HCC by BCLC staging system.

8.3 RESULTS

8.3.1 Study population

290 West African subjects were recruited at study sites in Nigeria (Jos University Teaching Hospital) and Gambia (Medical Research Council, Fajara, Gambia). These consisted of 63 patients with HCC; 32 with cirrhosis (Cir); 107 non-cirrhotic liver disease (DC) and 88 healthy (NC) subjects (Table 11). Patients with HCC were significantly older than the other classes and comprised mostly of males (79%). Most of the HCC patients had advance disease at recruitment. HBV constitutes the most common aetiological factor for HCC in this study population. 25(39.7%) had HBV, 12(19%) with HCV, whereas a substantial proportion of HCC subjects [16(25.4%)] had neither HBV nor HCV.

Table 11. Clinical and baseline laboratory characteristics of patients and control subjects recruited at Jos University Teaching Hospital, Nigeria and Medical Research Council, Gambia

	HCC subjects (n=63) Nig(n=63); Gam(n=0)	**Cirrhosis subjects (n=32) Nig(n=32); Gam(n=0)**	**Non-cirrhotic liver disease subjects (n=107) Nig(n=53); Gam(n=54)**	**Healthy controls (n=88) Nig(n=16); Gam(n=72)**
Age ; yrs(Median, range)	46(26-80)	39(21-58)	37(22-82)	41(26-98)
Male/Female, n (%)	50/13(79.4/20.6)	25/7(78.1/21.9)	58/49(54.2/45.8)	36/52(40.9/59.1)
BCLC stage†				
Stage A, n (%)	2(4.3)	/	/	/
Stage B-C, n (%)	26(55.3)	/	/	/
Stage D, n (%)	19(40.4)	/	/	/
Body Mass Index; Kg/m^2	NA	NA	21.5(14.9-34.1)	22.0(16.2-44.9)
Serum AFP values (median, range); ng/mL	201(0-1,085)	150(0.2-853)	7(0.2-902)	15(1.6-387)
Serum albumin, g/L	26.0(4.0-49.0)	23.0(10.0-47.0)	41.5(4.4-57.0)	42.0(22.0-51.0)
Serum ALT, IU/L	72.0(4.0-1,336)	41.0(8.0-122.0)	28.0(2.0-498.0)	28.0(13.0-65.0)
Serum creatinine, µmol/L	83.5(1.0-273.0)	85.0(5.4-899.0)	79.0(9.2-1000.0)	70.0(46.0-116.0)
Serum bilirubin, µmol/L	19.2(0.7-241.5)	54.5(2.4-884.3)	11.0(0.6-83.4)	12.0(7.0-28.0)
Aetiology of liver disease				
HBV, n (%)	25(39.7)	23(71.9)	103(96.3)	/
HCV, n (%)	12(19.0)	2(6.2)	1(0.9)	/
HBV/HCV, n (%)	2(3.2)	1(3.1)	1(0.9)	/
Neg hepatitis, n (%)	16(25.4)	3(9.4)	0(0.0)	88(100)
Unknown, n (%)	8(12.7)	3(9.4)	2(1.9)	/

† Unable to classify 15subjects owing to insufficient information; Nig: Nigeria; Gam: Gambia

8.3.2 Multivariate analysis

Principal components analysis of samples demonstrated class clustering (Figure 30). PCA scores plots of patients by country of recruitment failed to demonstrate any clustering or significant outliers (Figure 31). OPLS-DA of various class combinations, including: HCC vs. Cirrhosis, HCC vs. DC and HCC vs. NC enabled identification of discriminatory metabolites of HCC (Figure **32**).

8.2.2.1 Diagnostic performance of models

The sensitivities (95% CI) of urinary model to discriminate HCC from NC, DC and Cir were 95.0% (86.1-99.0), 88.3% (77.4-95.2) and 83.3% (71.5-91.7) respectively, whereas that for AFP were 48.3% (35.2-61.6), 50.8% (37.3-63.9) and 49.2% (36.1 - 62.3) respectively (Table 12). Similarly, the specificities of urinary model were 92.13% (84.5-96.8), 88.5% (80.7-93.9) and 83.8% (66.3-94.5) for HCC vs. NC, HCC vs. DC and HCC vs. Cir respectively. There were comparatively lower specificities of AFP to discriminate HCC from these classes [71.4% (41.9-91.6), 86.0% (77.3-92.3) and 77.4% (58.9 - 90.4) respectively].

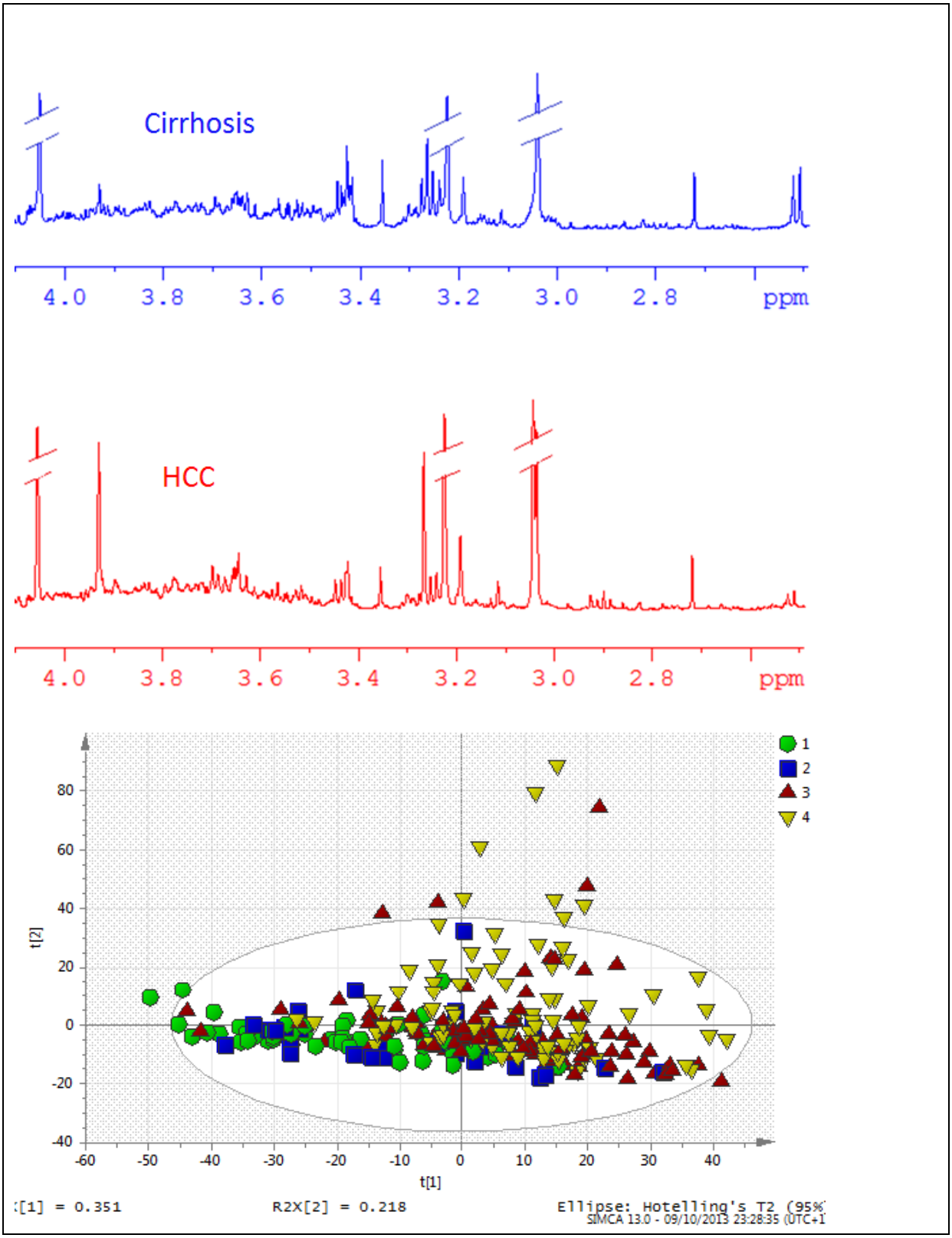

Figure 30. Representative spectra (top) and principal components analysis (PCA) plot (bottom) coloured by class of subjects (**Green**: hepatocellular carcinoma; **Blue**: cirrhosis; **Red**: non-cirrhotic liver disease and **Yellow**: Healthy volunteers)

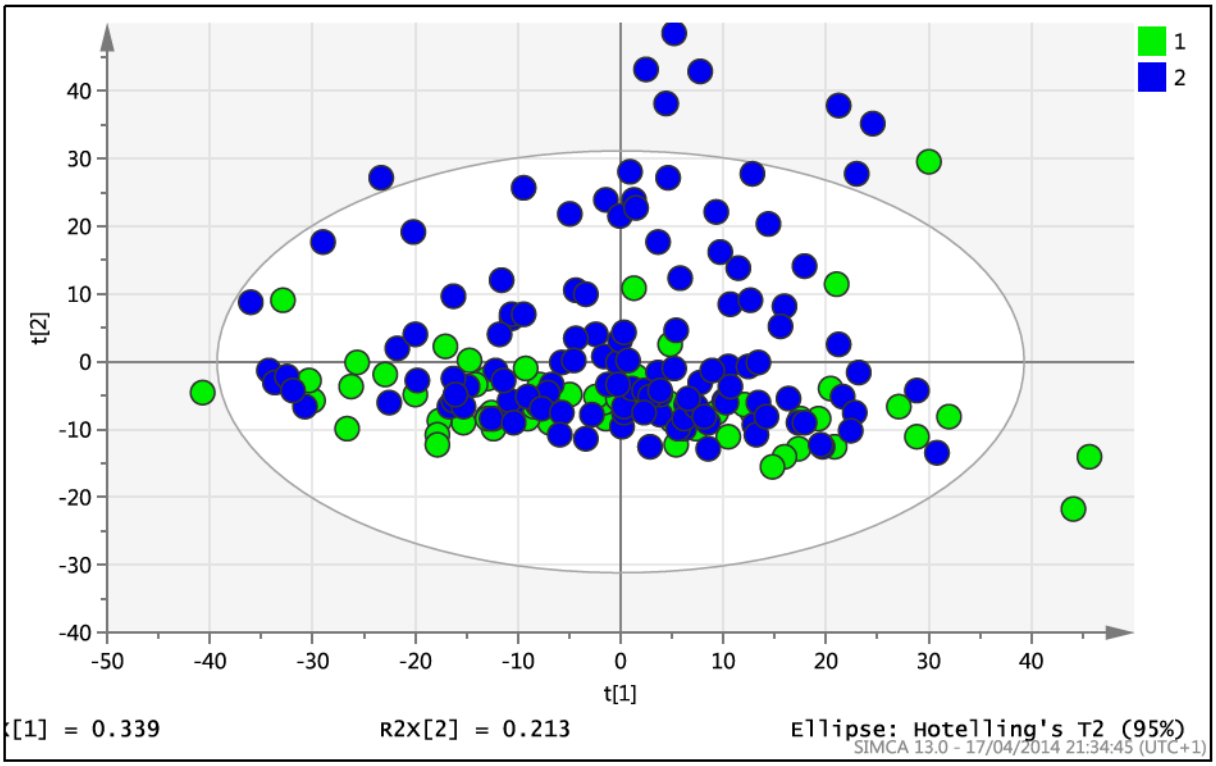

Figure 31. PCA scores plot of subjects by recruitment site; Nigeria (green circles) and Gambia (blue circles)

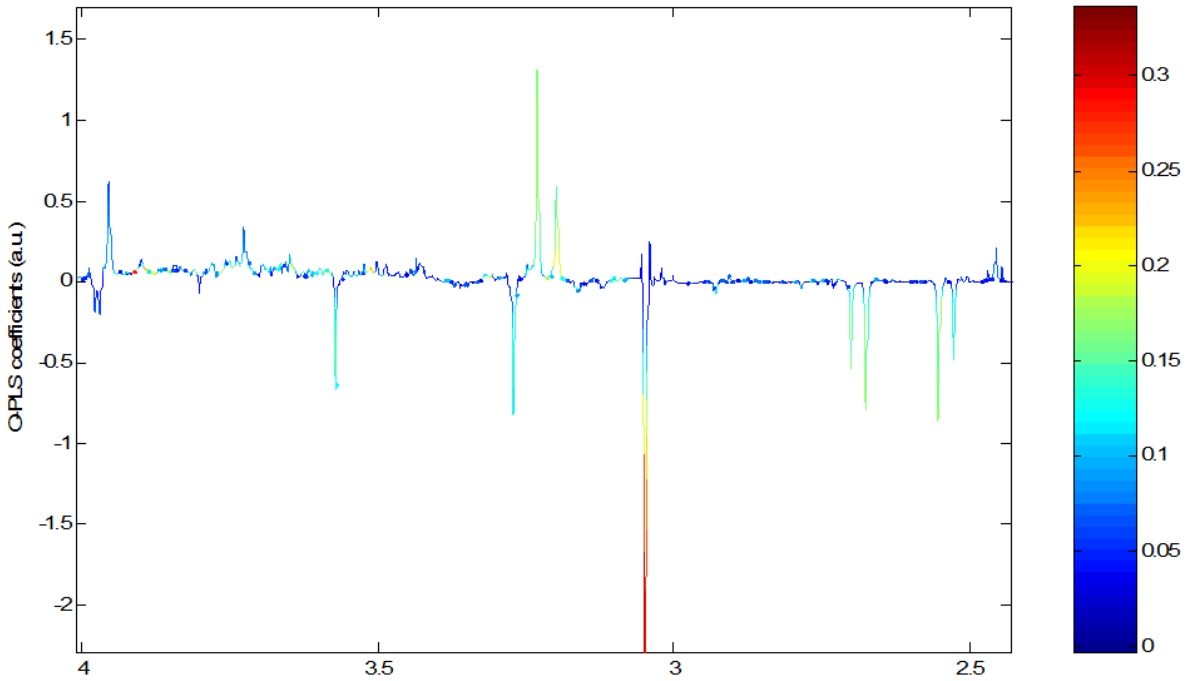

Figure 32. Loadings plot (MATLAB) displaying region of interest which was used to identify discriminating metabolites (HCC vs. DC). Peaks above the horizontal line indicate metabolites with higher concentration in HCC than DC whereas those below the line; higher in DC than HCC.

Table 12. Diagnostic accuracy of urinary model based on NMR spectroscopy versus serum AFP for HCC versus controls

Comparison class		AUC (95% CI)	SE	AUC difference(95% CI)	SE	P value
HCC vs. NC	Model	0.89(0.80-0.98)	0.03	0.16(0.05-0.32)	0.08	0.034
	AFP	0.73(0.58-0.88)	0.08			
HCC vs. DC	Model	0.93(0.89-0.97)	0.02	0.16(0.08-0.24)	0.04	0.0001
	AFP	0.78(0.70-0.85)	0.04			
HCC vs. Cir	Model	0.86(0.78-0.94)	0.04	0.20(0.06-0.35)	0.07	0.0061
	AFP	0.68(0.56-0.79)	0.06			

8.3.3 Metabolite Assignments

Using statistical total correlation spectroscopy (STOCSY) tool in MATLAB, a combination of loadings plot (Figure **32**) and VIP plots, as well as online database (www.hmdb.ca), it was possible to identify metabolites that were most influential in discriminating HCC from controls. Of the resonances identified by a combination of S-line and VIP plots, it was not possible to identify 13.3% of these using the online databases.

The intensities (relative concentrations) of several metabolites including: acetylcarnitine, dimethylglycine, carnitine, creatine, indole-3-acetate, creatine, methionine, *N*-acetylglutamate and 2-Oxoglutarate were significantly increased in the urine of HCC subjects compared to controls (Table 13).

Table 13. Pattern of alterations of urinary metabolites in HCC patients compared to controls

Metabolite	Chemical shift (ppm)	HCC vs Healthy			HCC vs Liver disease			HCC vs Cirrhosis		
		VIP	FC	p values	VIP	FC	p values	VIP	FC	p values
Beta-hydroxybutyrate	1.20				1.02	1.31	0.0002	1.48	1.07	0.14
Caprate	1.27					1.25	0.0015			
Lactate	1.33					1.63	<0.000001			
Alpha-hydroxyisobutyrate	1.36		-1.05	0.46		1.14	0.002	1.23	1.17	0.56
Acetate	1.92				1.07	1.40	0.000004	1.00	1.34	0.02
N-acetylglutamate	2.04	0.69	1.72	<0.000001	0.84	1.79	<0.000001	1.21	1.47	0.0005
Methionine	2.14				1.66	2.58	<0.000001	1.45	2.38	0.018
Formiminoglutamate	2.17	1.31	1.25	0.019						
Succinate	2.41							1.00	-1.00	0.94
9-methyluric acid	3.16				0.34	1.23	0.00005			
Pyruvate	2.35	3.26	-1.66	0.0009	2.84	1.25	0.41	1.00	1.44	0.023
2-oxoglutarate	2.45	1.81	2.20	<0.000001	1.98	2.08	<0.000001	2.53	1.56	0.009
Citrate	2.52	2.15	2.50	<0.000001	2.44	1.07	0.12	8.51	1.05	0.67
Dimethylamine	2.72	1.40	1.29	0.0004	0.98	1.40	0.000001	1.73	1.16	0.17
Trimethylamine	2.89							1.78	-1.04	0.91
Creatine	3.04	1.31	1.21	0.00001	2.80	1.17	0.000013	5.40	1.14	0.012
N,N,N-Trimethyllysine	3.12	1.11	1.66	0.00008	0.89	1.51	0.00004	0.81	1.42	0.05
Citrulline	3.16							2.11	-1.02	0.25
Acetylcarnitine	3.19	4.29	3.54	<0.000001	4.90	3.78	<0.000001	1.79	3.00	0.023
Carnitine	3.23	9.42	5.20	<0.000001	11.80	4.68	<0.000001	8.97	2.95	0.015
Betaine aldehyde	3.24	1.14	2.21	<0.000001	0.90	2.28	<0.000001	1.34	1.76	0.032
TMAO	3.27	32.93	-1.54	0.00002	16.37	-1.10	0.17	7.22	1.31	0.2
1,3-Dimethylurate	3.30				1.45	1.62	0.022			
Methylguanidine	3.36	2.04	2.05	<0.000001	1.05	1.68	<0.000001	2.64	1.01	0.82
1,3-Dimethylurate	3.43				1.00	1.87	0.000013	2.32	1.92	0.034
m-Hydroxyphenylacetate	3.48	1.00	2.16	<0.000001	1.48	2.25	<0.000001	2.46	1.44	0.17

Choline	3.52	1.96	2.67	<0.000001	1.71	2.44	<0.000001	1.59	1.59	0.014
Phenylacetate	3.55					1.73	<0.000001	1.45	1.26	0.014
Glycine	3.57	4.82	-1.05	0.55	6.33	1.02	0.89	5.17	-1.08	0.57
Myoinositol	3.63	0.76	1.55	<0.000001						
Phenylacetylglutamine	3.67							1.87	1.53	0.0035
3-methylhistidine	3.70							8.45	1.46	0.012
Delta-hydroxylysine	3.78							1.98	1.47	0.00079
Guanidinoacetate	3.80							1.40	1.18	0.14
Indole-3-acetate	3.65	1.58	2.00	<0.000001	1.57	2.00	<0.000001	2.23	1.52	0.001
Dimethylglycine	3.72	2.95	2.49	<0.000001	2.74	2.28	<0.000001	5.32	1.36	0.009
trans-aconitate	3.74				1.58	2.29	<0.000001			
N-Phenylacetylglycine	3.75					2.07	<0.000001	2.20	1.46	0.0022
Aspartate	3.91				1.38	2.40	<0.000001			
Betaine	3.90	1.26	2.28	<0.000001						
Creatine	3.95	5.01	1.84	<0.000001	5.52	1.83	<0.000001	8.59	1.74	0.0042
Hippurate	3.97	4.45	-1.23	0.07	4.25	-1.14	0.82	25.66	-1.29	0.008
Creatinine	4.06	15.04	-1.49	<0.000001						
1-methylnicotinamide	4.48	0.82	3.83	<0.000001	0.69	3.24	<0.000001			
NADPH	4.61				0.69	2.53	<0.000001			
Glucose	4.64	1.36	2.66	<0.000001	1.21	2.69	<0.000001	2.34	1.47	0.09
L-Fucose	5.21								1.43	0.023
Allantoin	5.40								1.26	0.62
Ribose	5.24	1.04	2.22	<0.000001	0.96	2.00	<0.000001	2.25	1.44	0.014
5-Hydroxyindole-3-acetate	7.21	1.26	-1.57	0.0003	1.03	-1.20	0.2			
Phenylacetate	7.29	0.90	-1.63	0.000001	0.60	-1.29	0.015			
Phenylacetylglutamine	7.37	2.79	-1.66	0.0003	1.72	-1.13	0.29			
5-Hydroxytryptamine	7.42	1.27	-1.77	0.001	0.85	-1.22	0.43			
Hippurate	7.55	3.52	-3.18	<0.000001	3.74	-2.44	<0.000001			
Hippurate	7.64	1.71	-2.25	<0.000001	1.76	-1.74	0.000001			

Alpha-hydroxyhippurate	7.84	3.36	-3.32	<0.000001	3.16	-2.55	<0.000001			
N-Formyl-L-aspartate	8.07							0.68	1.36	0.44
Inosine	8.19							0.75	-1.98	0.000041
Guanosine triphosphate	8.13	0.48	1.82	<0.000001	0.57	1.73	0.000001	0.94	1.61	0.02
Formate	8.46				0.34	1.32	0.005	0.44	1.19	0.98

Positive fold change (FC) indicates higher relative concentration in HCC than controls while negative value indicates down-regulation

8.3.4 Additional statistical analysis

Metabolites corresponding to the resonances that contributed most strongly in discriminating between groups were subjected to further analyses. Particularly, liver disease controls were given an ordinal score of one (1), cirrhosis; a score of two (2), intermediate HCC (BCLC A-C); three (3) and advanced HCC (BCLC D); four (4).

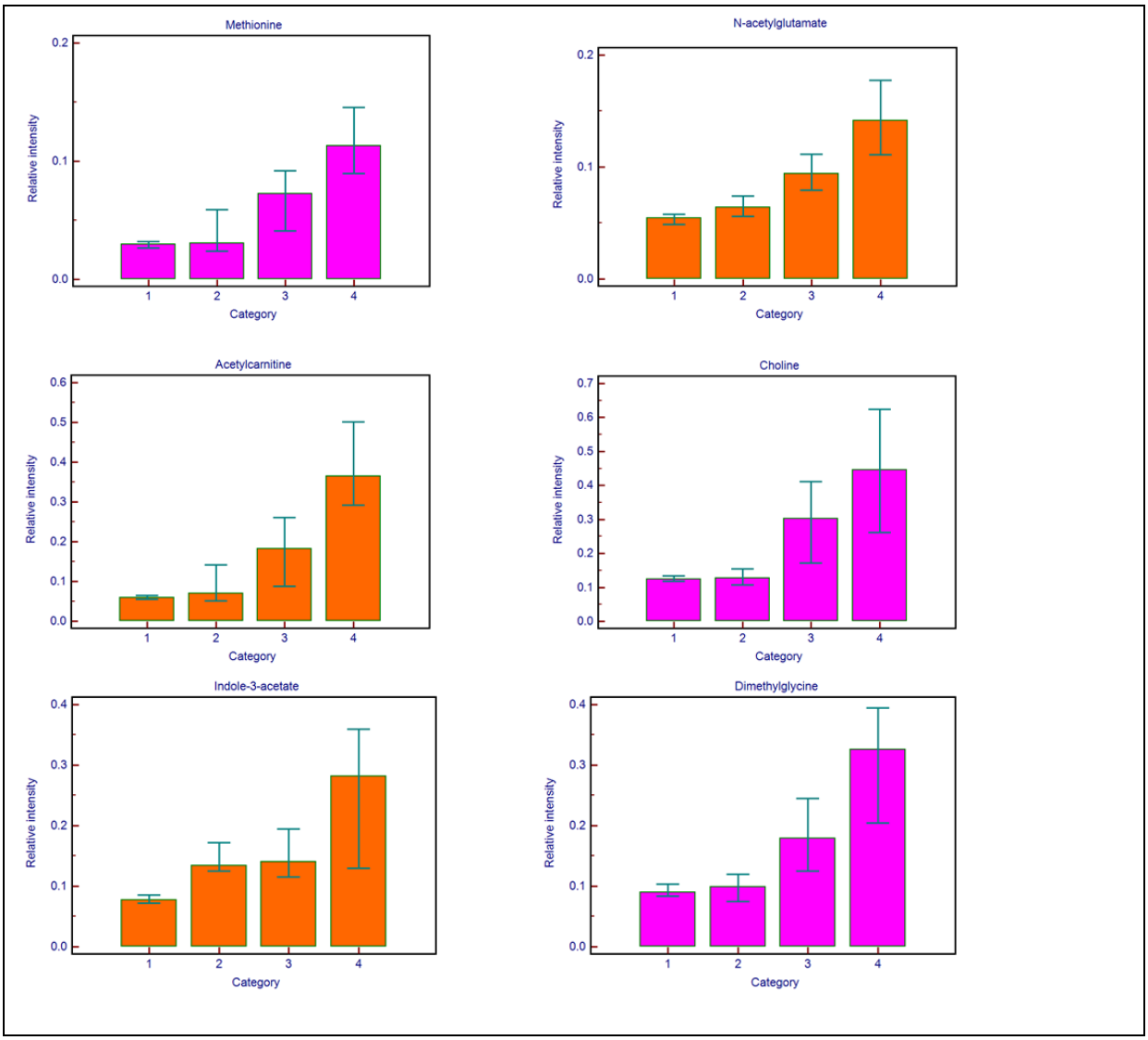

Figure 33. Trends in metabolite concentrations of some identified metabolites by study classes ($p<0.000001$ in most cases). Holm-Sidak's comparison tests found significant association between categories; 1: non-cirrhotic liver disease; 2: cirrhosis; 3: BCLC stage A-C HCC; 4: BCLC stage D HCC

Changes in median concentrations of urinary metabolites among the 4 categorised phenotypic states; chronic liver disease control, cirrhosis, BCLC stage A-C, and BCLC stage D showed significant positive trends, relative to disease states (Figure **33**).

8.3.4 Correlation of metabolites with stage of HCC

There was poor correlation with stage of HCC by serum AFP (p=0.7). However, the relative concentration of *N*-acetylglutamate, methionine, acetylcarnitine, indole-3-acetate, aspartate, dimethylglycine, and creatine were significantly positively correlated to category and clinical stage of HCC (Figure **34**).

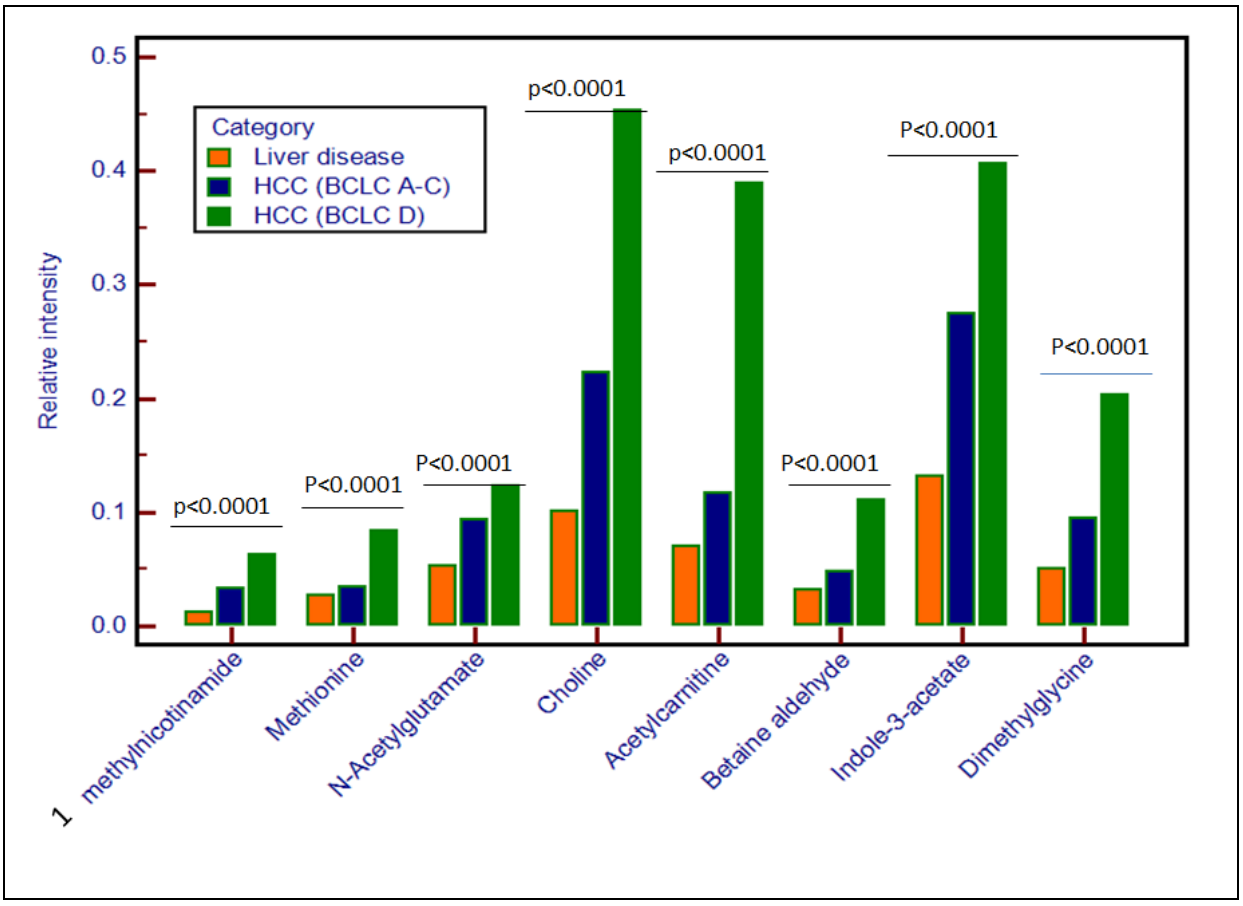

Figure 34. Correlation of relative concentration of metabolites by BCLC stage of HCC (2=BCLC stage A-C; 3=BCLC stage D)

8.4 DISCUSSION

In this multi-centre cohort, including subjects from two West African countries, it was possible to use urinary NMR spectroscopy to distinguish patients with HCC from a) healthy volunteers, b) non-cirrhotic (predominantly hepatitis B-related) liver disease, and importantly, c) those with cirrhosis. Using pattern recognition, as well as complex multivariate and univariate statistics, urinary metabolic profiles performed significantly better than serum AFP in discriminating HCC from both the disease and healthy control groups. Several metabolites with significant high fold changes were identified that discriminated between HCC and controls. Furthermore, the relative concentration of creatine, acetylcarnitine, N-acetylglutamate, methionine and dimethylglycine significantly correlated with the clinical stage of HCC.

For all three binary classes examined (HCC vs. NC, HCC vs. DC and HCC vs. Cir), models obtained by ^{1}H NMR spectroscopy prediction found a higher AUC (0.8-0.9), whereas AFP performed less well (AUC=0.7-0.8) in discrimination of HCC. Since most HCC occur in a background of liver disease, it was important that metabolites discriminating HCC from liver disease be well characterised. In general 33, 37 and 25 metabolites that had relative concentrations significantly different between HCC and the three groups of control (NC, DC and Cir, respectively) were found. However, examining the trends in metabolite concentrations, relative to disease category, acetylcarnitine, methionine, creatine, creatinine and *N*-acetylglutamate showed a significant trend in stage of HCC, compared to liver disease.

The carnitines (carnitine and acetylcarnitine), metabolites involved in the initial stage of β-oxidation of free fatty acids, were significantly elevated in the urine of subjects with HCC compared to all the non-HCC controls. The finding of relatively higher

carnitine corroborates data in the literature (Chen *et al*., 2011b;Shariff *et al*., 2010). The high excretion of these metabolites in urine may be a result of suboptimal renal function, leading to poor reabsorption, excess ingestion of carnitine-containing foods or increased biosynthesis. As the renal function, estimated by serum creatinine in the present study were comparably similar and ingestion of foods high in carnitine content, such as meat, is rather rare in West African populations (proteins are traditionally restricted from diet of patients with liver disease (Shariff *et al*., 2010), increased synthesis of carnitines in order to meet the high metabolic demands of the rapidly growing tumour in the liver may be responsible for this phenomenon. This is further supported by the fact that acetylcarnitine was proportional to the stage of HCC. Moreover, acyl co-A synthase, the initial enzyme prior to β-oxidation of fatty acid has been reported to be up-regulated in HCC tissues, relative to adjacent non-HCC tissues (Sung *et al*., 2003a;Sung *et al*., 2003b). Downstream, carnitine palmitoyltransferase-1 (CPT1) has similarly been shown to be more highly expressed in HCC tissues, compared to non-involved tissues (Kurokawa *et al*., 2011). These data therefore support the up-regulation of alternative energy pathways; in this case, β-oxidation of fatty acids in HCC.

Creatine is an amino acid that occurs in tissues and in urine, and was significantly raised in urine of HCC compared to controls in the present study. Creatine functions as part of the energy shuttle of the cell in a reaction reversibly catalysed by creatine kinase. Creatine has been reported to be elevated in the urine of patients with HCC relative to cirrhosis by our study group (Shariff *et al*., 2010), as well as after partial hepatectomy in experimental rats (Bollard *et al*., 2005). Recently, mitochondrial isoenzyme of creatine kinase activity was found to be higher in serum of cirrhosis patients with HCC than in those without HCC (Soroida *et al*., 2012). Relatively higher

urinary creatine in HCC may thus represent excess energy transfer process, a requirement of rapidly growing HCC cells.

In the human body, creatine is synthesized mainly in the liver by the use of component parts from three amino acids - arginine, glycine, and methionine. There was a tendency for glycine to be lower in the urine of patients with HCC, compared to controls although this was not significant, suggesting its high utilisation by rapidly growing HCC tissues.

Methionine is an amino acid required for normal growth and development. It is useful for protein synthesis, as well as an intermediate in transmethylation reactions, such as methyl group donation for DNA and RNA intermediates. This reaction is catalysed by methionine adenosyltransferase (MAT), the responsible for the synthesis of s-adenosylmethionine (SAMe), the principal methyl donor in the reaction. Research has demonstrated that MAT1A, the subunit of MAT expressed mostly in adult normal liver, is often silenced in human HCC (Avila *et al*., 2000;Cai *et al*., 1996;Frau *et al*., 2012;Wang *et al*., 2009a) . It is thus postulated that excess methionine as a result of reduced utilisation by tumour cells is excreted in higher concentration in HCC patients than in controls, resulting in hypomethylation.

Urinary *N*-acetylglutamate was significantly higher in HCC than in controls and correlates with tumour stage. This finding corroborates previous data which revealed higher glutamate signals in HCC tissues and serum of HCC patients than in adjacent healthy liver tissues and serum of cirrhosis patients respectively (Nahon *et al*., 2012;Yang *et al*., 2007). Up-regulation of glutaminolysis has been reported to provide an alternative energy source for tumorigenesis (Meng *et al*., 2010). Glutamine is “trapped” in malignant cells for this process (Dang, 2010). Through

glutaminolysis, more energy is made available to rapidly dividing HCC cells, as well as the generation of 2-oxoglutarate, an intermediate substrate in the tricarboxylic acid cycle.

There was a higher relative intensity of creatinine in the urine of patients with cirrhosis and healthy volunteers than in those with HCC. This finding corroborates those of Shariff and colleagues in a previous study (Shariff *et al*., 2010). More recently, Chen and colleagues have reported, using mass spectroscopy, that serum creatinine had a significantly high fold change in healthy volunteers compared to patients with HCC (Chen *et al*., 2011b). Creatinine is a breakdown product of phosphocreatine in muscle produced at a fairly constant rate and excreted in the kidneys. Reduced muscle mass, commonly observed in end-stage liver disease patients in Africa (Ladep & Taylor-Robinson, 2007;Shariff *et al*., 2010) may underlie the basis of inverse correlation of this metabolite with HCC relative to other controls. Another source of creatinine would be meat which is often traditionally excluded in the meals of patients with overt liver disease, such as cirrhosis and HCC. The malnourished status of patients with end-stage liver disease in the study cohort is suggested further by the significantly lower serum albumin in those with HCC and cirrhosis compared to normal serum albumin concentrations in those with non-cirrhotic liver disease and healthy volunteers. Therefore, the observed inverse relationship of urinary creatinine concentration in HCC relative to controls may thus represent sarcopenia of cancer cachexia, a feature most prevalent in West African patients.

Identified metabolites, highly expressed in HCC confirm the fact that cancer cells use alternative energy for their metabolic processes, as well as complement genetic and epigenetic information during HCC pathogenesis. Earlier data had reported

increased acyl Co-A synthase activity, global DNA hypomethylation, enhanced glycolysis and glutaminolysis by HCC. These data corroborate work found in non-African populations and additionally demonstrates the feasibility of collaborative networking in solving research needs of resource-limited countries.

To the best of knowledge, only a few human studies of urine proton NMR spectroscopy have so far been conducted to characterise HCC metabolites. These pilot studies involved small numbers of patients and were restricted to single populations (Shariff *et al*., 2010;Shariff *et al*., 2011). In contrast to these, HCC patients recruited in the current study were about 4 times more in number than in previous studies. HCC diagnosis was based on established international guidelines (EASL) and included 3 control groups. Additionally, the concentrations of identified metabolites were normalised relative to creatinine peaks whereas earlier data utilised data normalised to the total spectral integral. This is pertinent as renal function as well as sarcopenia would affect the excretion of metabolites. As creatinine excretion is not the most sensitive measure of renal function, it remains to be proven whether normalization to the creatinine peak is better than normalization to total spectral integral.

It is uncertain how the discriminatory metabolites found on urinary NMR will perform in patients with early HCC in the African population, although a serum-based mass spectroscopic experiment successfully discriminated between TNM stage 1 HCC and healthy volunteers in a Chinese population (Chen *et al*., 2011b). There were only two patients at early stage HCC in the present study which made it statistically impractical to assess these as a separate group. These were combined with intermediate stage HCC patients. A significant difference in the relative concentration between this intermediate stage and liver disease controls was demonstrated.

As well as answering some research questions, the present study raises more questions. It is yet to be determined whether the metabolic changes found in HCC are specific to primary liver tumours, or representative of tumorigenesis in general. Studies that would compare the metabolic profiles of secondary liver tumours to those of HCC would be relevant in this regard. Further, larger studies including profiling of other adenocarcinomas, such as colorectal metastases, ovarian and prostate cancer are required. However, the present findings provide discrimination of HCC from both cirrhotic and non-cirrhotic liver disease, using urinary metabolic profiling. The development of a urinary dipstick for use at village level in Africa for screening of at-risk populations is the ultimate goal and this study provides some evidence that a battery of discriminate metabolites may be a future possibility that would be simple to perform and may outperform blood tests, such as AFP. Further larger scale validation studies are required.

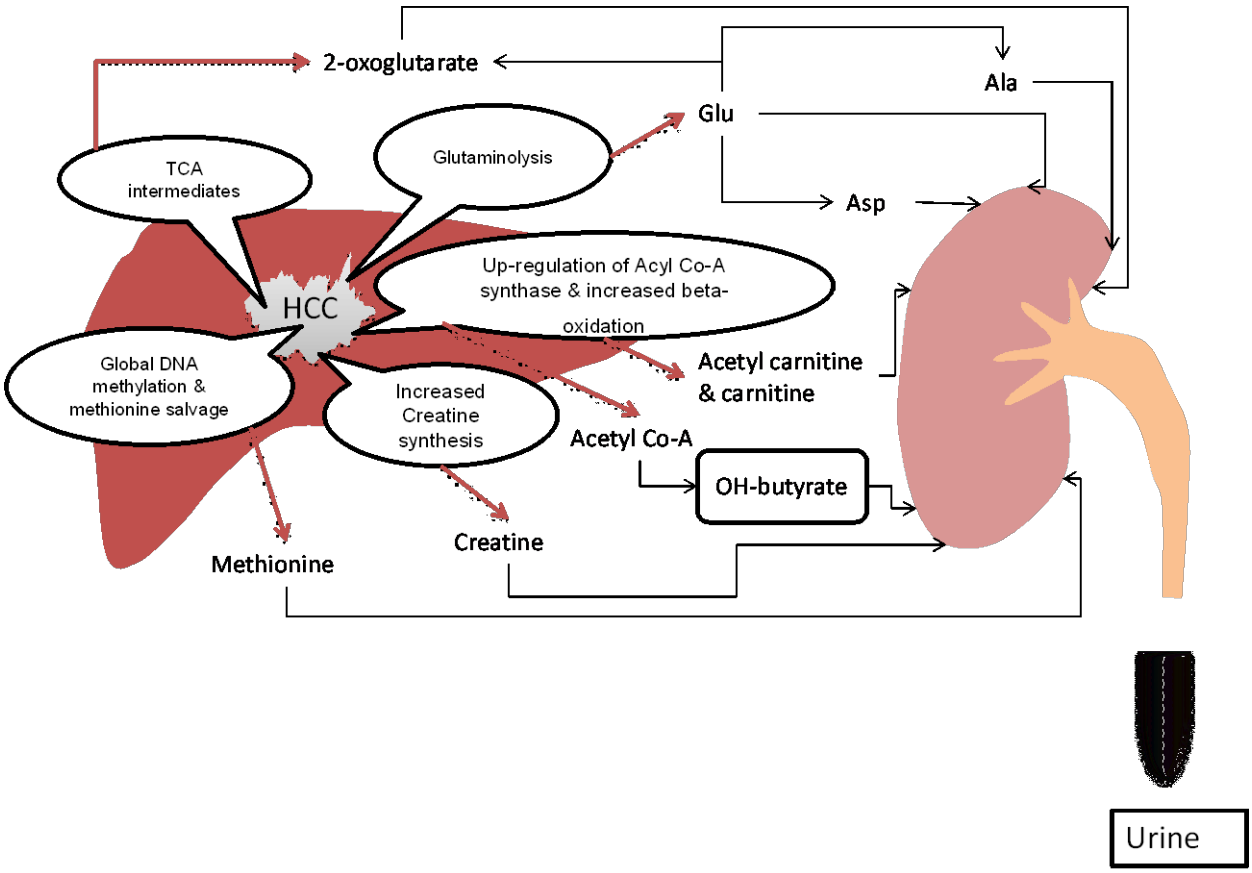

Figure 35. Schema of altered intermediary metabolism of the liver in HCC identified in the present study

The development of a diagnostic urinary dipstick would be practical for screening at-risk populations for HCC. First and foremost, there is need to widely validate the performance of the panel with or without current recommendations. It may prove valuable to combine the use of urinary metabolites with USS (where the technology allows) to improve the diagnostic yield in the evaluation of small size HCC. In resource-limited settings, liver imaging could be reserved for those who are screened "positive" by urinary metabolite profiles of HCC. Those patients in whom dynamic imaging reveals atypical findings may be identifiable by this technology and/or liver histology. Furthermore, the altered metabolic pathways identified in this present study could serve as therapeutic targets for HCC (Figure 35).

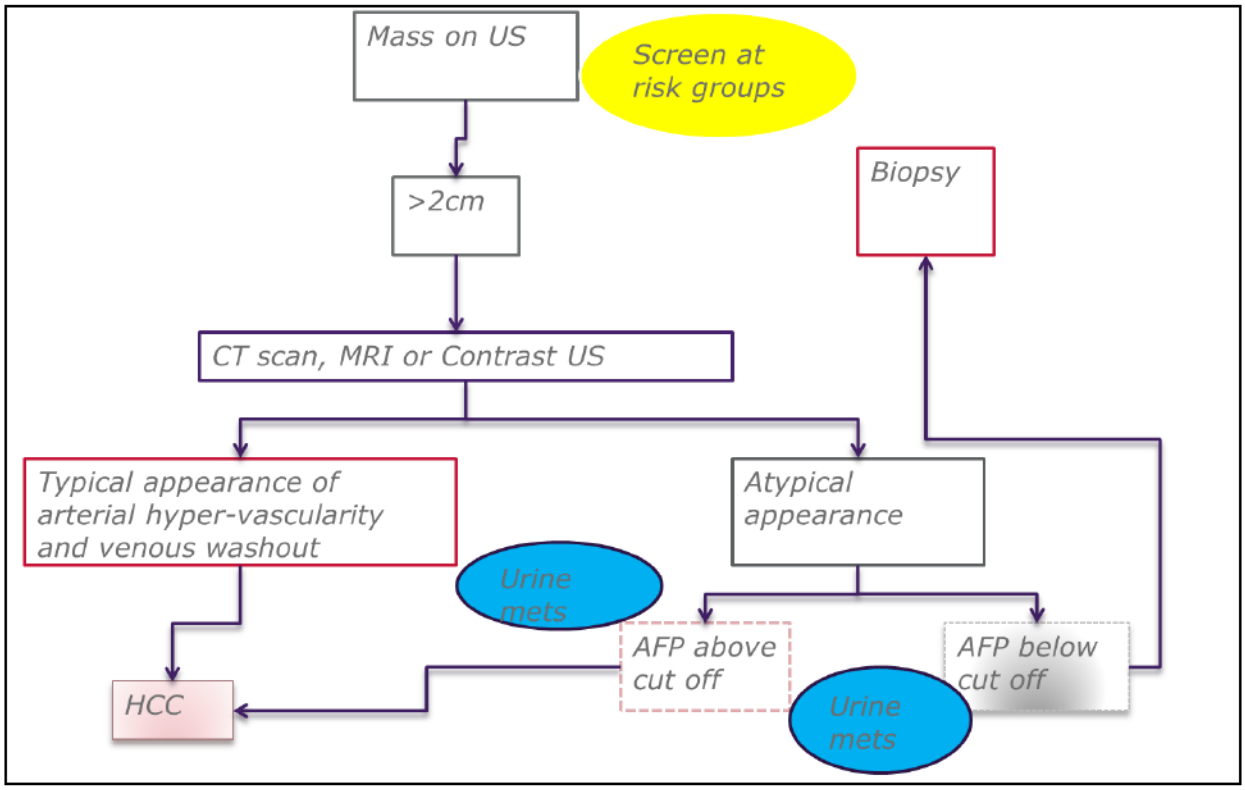

Figure 36. Possible clinical translation of urinary metabolite panel to improve current diagnostic schema of HCC

In conclusion, proton NMR spectroscopy of urine of this West African population has identified metabolites that provide clues for the implication of altered energy-related pathways in the pathogenesis and progression of HCC. Additionally, it has shown that the metabolic profiles yielded a better diagnostic performance than serum AFP. These findings confirm that a panel of urinary metabolites may prove useful for screening HCC in at-risk populations (Figure 36).

9. BIOMARKER DISCOVERY IN UK HCC PATIENTS USING PROTON NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

9.0 ABSTRACT

Background: There has been a proliferation of biomarker discovery studies for HCC using metabonomics. Most of these studies are hampered for generalizability as they were carried out on samples from subjects with predominantly single aetiological factors for and relatively advanced HCC. The current study aimed to determine the urinary metabolites of small, treatable HCCs and assess the diagnostic performance of discovered metabolites.

Methods: Urine samples were collected from patients seen at the Liver Unit of St Mary's Hospital, London. 600MHz NMR was used to acquire one-dimensional spectral data from the urine samples, using a standard 1D NMR pulse sequence with presaturation of the water peak. Identification of metabolites was ensured using multivariate statistical techniques and from library of metabolites. Relative concentrations of discriminating metabolites were compared between HCC and controls. Areas under the operating characteristic curves (AUC) of the metabolites measured in 99 patients: 17 with HCC, 48 with cirrhosis (Cir) and 34 with non-cirrhotic liver disease (DC) were computed individually and in combination (based on logistic regression), to determine their diagnostic capacity.

Results: All 17 patients with HCC were at BCLC stage A at recruitment. The laboratory parameters, including serum albumin, creatinine and ALT were comparable between cases and controls. The most common recorded aetiological factor for HCC was HCV (4 patients), followed by HBV (2), HCV/alcohol (2), and alcohol (2). Seven patients either had non-alcoholic steatohepatitis (NASH) or did

not have a definite aetiologic agent identified. Orthogonal partial least squares discriminant analysis (OPLS-DA) found a good separation between HCC and controls. Uracil and glycine were up-regulated in the urine of HCC patients compared to controls whereas creatinine and phenylacetylglutamine were down-regulated. The sensitivity and specificity of a combination of these markers were 90% and 81% respectively ($p<0.0001$) (AUC>0.8 for both HCC vs. Cir and HCC vs. DC).

Conclusions: Urinary metabolic profiling using NMR in this aetiologically diverse sample of small HCC patients has identified discriminatory biomarkers, possessing high diagnostic predictability. Further validation experiments in larger sample of patients would be worthwhile.

9.1 BACKGROUND

In the previous chapter, it was described how ^{1}H NMR was used on a total of 290 urine samples from patients that were recruited under the case-control platform of PROLIFICA to identify candidate biomarkers of HCC. The technique discriminated patients with HCC, compared to healthy and disease controls. Most importantly, the diagnostic performance of the urinary metabolites was significantly better than the current clinically-utilised serum marker, AFP. Additionally, there was a significant correlation between the relative concentration of some urinary metabolites and clinical stage of HCC.

The findings of the previous study corroborate earlier preliminary study of patients from Nigeria (Shariff *et al*., 2010) as well as in an Egyptian population (Shariff *et al*., 2011) in whom urinary ^{1}H NMR studies identified discriminatory urinary metabolites for HCC. Additionally, it complements several Chinese studies that utilised mass spectrometry for the same purpose. The metabolites identified are mostly those involved in cellular energy processes of HCC, confirming differential tumour metabolism.

As most of the HCC patients recruited in the West African study were at intermediate/advanced clinical stage at presentation, it is debatable whether NMR is able to discriminate early HCC; a group for which curative therapy would prove valuable. A recent study by Tan and colleagues using serum liquid chromatography-mass spectrometry, has shown that patients with solitary HCC lesions <2cm could be discriminated from those with chronic liver disease with a sensitivity and specificity of 87.5% and 72.3% respectively (Tan *et al*., 2012). The pursuit for more studies of differential urinary metabolites of HCC would prove valuable as urine can

easily be accessed and such a test can simply be performed in the community, clinic and/or bedside. Moreover, most of the aforementioned studies have been performed in HCC patients with single aetiologic agents (i.e. HBV in Sub Sahara Africa and China, and HCV in Egypt). A study of biomarker of HCC in England, where liver transplant services for small HCCs exist, and the possibility of encountering more widespread aetiological factors of HCC would be important.

9.1.1 Hypothesis

Urinary metabolites can discriminate small HCCs from controls (healthy, non-cirrhotic liver disease and cirrhosis).

9.1.2 Aims

- To determine the urinary metabolic profile of small HCCs using NMR spectroscopy
- To assess the diagnostic performance of discriminatory urinary metabolites of small HCCs

9.2 METHODS

9.2.1 Study design and subject recruitment

This was a laboratory-based case control study of HCC (cases) and two groups of liver disease controls, including:

i) Cirrhosis, confirmed by histology; and

ii) non-cirrhotic liver disease.

Random urine samples were collected from subjects at St Mary's Hospital Imperial NHS Trust, London between October 2008 and July 2012. All subjects gave written and informed consent in accordance with the local Research Ethics Committee

approval. HCC cases were identified during the monthly Liver multidisciplinary team meeting at St Mary's Hospital. HCC was diagnosed using EASL guidelines (two imaging techniques showing early arterial enhancement and rapid venous washout; or one characteristic imaging with serum AFP >400 ng/mL) (2012).

9.2.2 Multivariate statistical analyses

Obtained NMR spectra were normalised and aligned using an in-house MATLAB script and then subsequently transferred to SIMCA 13.0 (Umetrics, Umea, Sweden) for unsupervised, principal components analysis (PCA) followed by supervised, orthogonal partial least squares discriminant analysis (OPLS-DA) (Stenlund *et al.,* 2008). Using these techniques, outliers (mostly as a result of analytical variation) were identified; and excluded. The supervised models were subjected to "leave-one-out" validation, as previously described (Kohavi and Ron, 1995).

9.2.3 Univariate statistical analysis

The metabolites that correlated strongest with disease (case/control) groups were identified through analysis of the orthogonal partial least squares discriminant analysis (OPLS-DA) loadings plot. Identified metabolites were confirmed using STOCSY. Intensities of the metabolites were charted on heat maps of relative intensity of each sample by metabolite. Using MedCalc Statistical Software version 12.7.7 (MedCalc Software bvba, Ostend, Belgium; http://www.medcalc.org; 2013), Mann-Whitney U tests were applied to study statistical differences in the median values of identified metabolites between HCC, and controls (cirrhosis and non-cirrhotic liver disease).

9.2.4 Diagnostic performance of panels of metabolites

The ability of each model and some highly expressed metabolites to discriminate HCC from cirrhosis and non-cirrhotic liver disease controls were examined for sensitivity and specificity using area under the receiver operating characteristic curves (AUC).

9.3 RESULTS

9.3.1 Study population

Seventeen (17) patients with HCC (all cases were at BCLC stage A); 48 with cirrhosis (Cir); and 34 non-cirrhotic liver disease (DC) subjects were recruited in the study (Table 14). There was no significant difference between the age of cases versus controls. There were also no significant differences between serum albumin, ALT, bilirubin and creatinine of the subjects.

Table 14. Clinical and baseline laboratory characteristics of patients and control subjects recruited at Imperial NHS Trust, St Mary's Hospital, London

Parameter	HCC (n=17)	Cir (n=48)	DC (n=34)
Age, yrs (Median, range)	65(52-78)	63(27-86)	51(23-79)
Sex (M/F)	13/4, (76.5/23.5)	32/16, (66.7/33.3)	21/13, (61.8/38.2)
BMI	26.3	27.5	25.7
Serum albumin, g/L	33.0	36.0	40.5
Serum ALT	51.0	40.5	43.0
Serum Cr	88.0	74.5	78.0
Serum bilirubin	15.0	15.5	15.0
Aetiology of Liver Disease			
HCV	4	16	11
HBV	2	2	1
HCV/ALC	2	9	0
ALC	2	15	3
Unknown/NASH	7	2	11

9.3.2 Multivariate analysis

OPLS-DA of binary combinations, including: HCC vs. Cirrhosis and HCC vs. DC showed some distinguishable clustering of HCC versus controls (Figure **37**). The relative intensities of some of these metabolites varied between HCC and controls (Figure **37**).

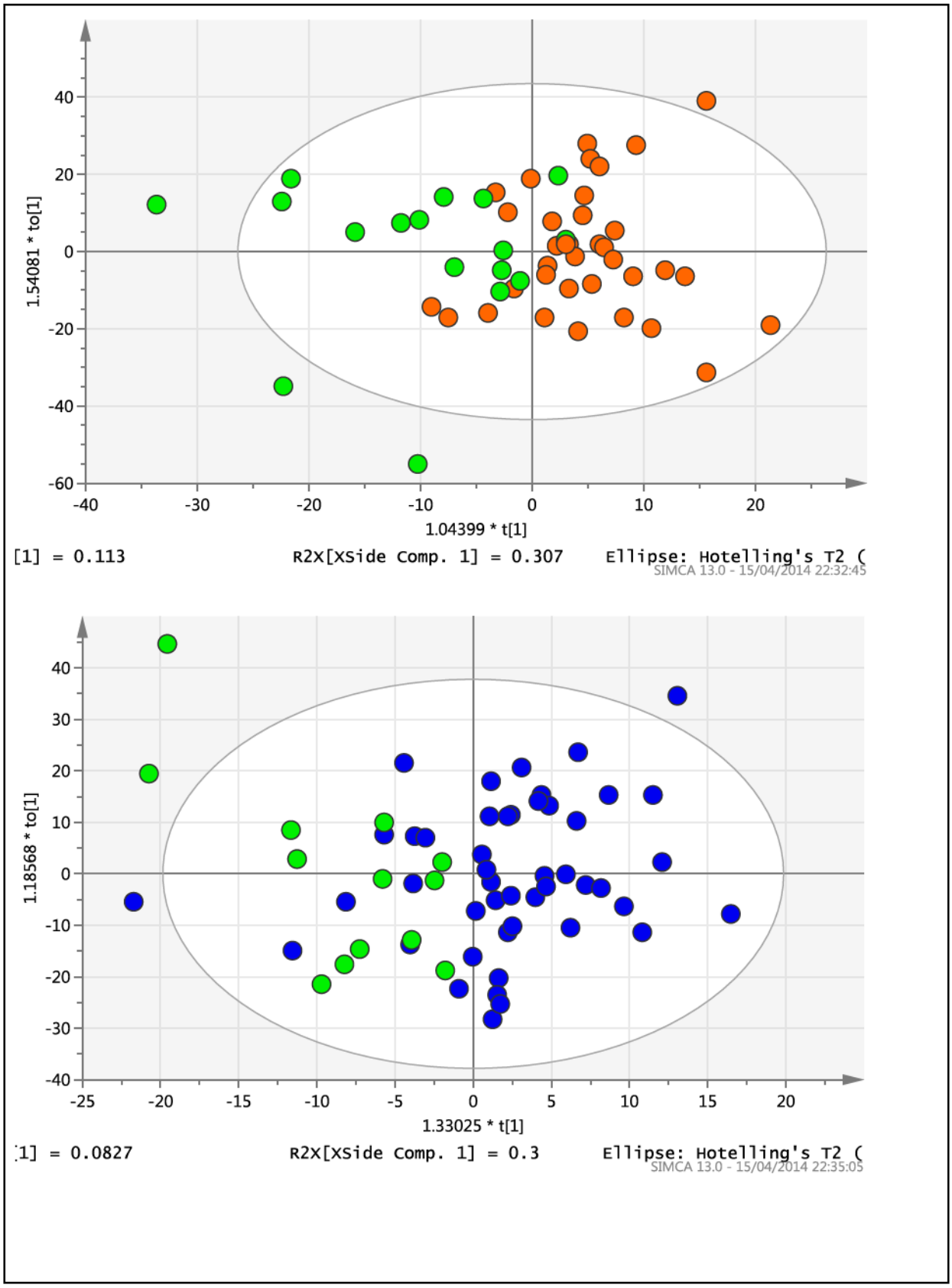

Figure 37. Orthogonal partial least squares discriminant analysis results of HCC versus controls (HCC=green circles; non-cirrhotic liver disease=red circles; Cirrhosis=blue circles) of subjects recruited in the UK.

9.3.3 Univariate analysis

There was significantly altered concentration of several urinary metabolites in HCC compared to controls. Some of these include ketone bodies (acetate), betaine aldehyde, glycine, ribose, indole-3-acetate, phenylacetylglutamine, among others (Tables 15 & 16, Figure **38**).

Table 15. Summary of urinary metabolites discriminating small HCCs from Liver disease in UK patient population

Metabolite	Chemical shift	VIP	FC	p value
Acetate	1.92	3.75	1.28	0.01
Adenine	8.20	0.70	-1.04	0.89
Beta-hydroxybutyrate	1.20	15.41	1.31	0.12
Betaine aldehyde	3.43	1.57	1.38	0.02
Citrate	2.52	3.67	1.19	0.61
Dimethylglycine	3.73	2.62	1.53	0.01
Glycine	3.57		1.38	0.03
GTP	8.13	0.52	1.68	0.02
Hydroxyindole_3_acetate	7.21	1.56	-1.33	0.11
Hydroxyphenylacetate	7.16		1.50	0.49
Indole-3-acetate	3.65	5.93	1.43	0.03
Methionine	2.16	3.44	1.30	0.33
3-methylhistidine	3.70	6.16	1.25	0.016
N,N,N-Trimethyllysine	3.12	1.44	1.08	0.49
N-Alpha-acetylornithine	4.18	1.65	1.21	0.14
Phenylacetylglutamine	7.36	2.36	1.17	0.84
Phosphorylcholine	3.22	1.36	1.37	0.09
Pyruvate	2.35	3.33	-1.53	0.2
Ribose	5.24		1.50	0.01
Syringic acid	3.84	1.60	1.53	0.02
Thiamine	5.45	2.55	1.58	0.008
TMAO	3.27	4.51	1.26	0.38

Table 16. Summary of urinary metabolites discriminating small HCCs from cirrhosis in UK patient population

Metabolite	Chemical shift	VIP	FC	p value
1,3-dimethylurate	3.30	3.84	-1.13	0.61
1-methylnicotinamide	4.48	1.01	-1.47	0.29
5-hydroxytryptamine	7.10		2.60	0.01
Acetylcarnitine	3.19	1.00	-1.05	0.96
Arginine	1.73	2.55	-1.14	0.15
Beta hydrobutyrate	1.20	3.20	-1.10	0.06
Betaine aldehyde	3.24	1.48	1.12	0.38
Caproate	0.89	2.77	-1.07	0.22
Carnitine	3.22	1.19	1.19	0.77
Choline	3.52	3.31	-1.19	0.14
Citrate	2.55	2.12	1.19	0.81
Creatinine	4.06	6.00	1.04	0.65
Dihydrothymine	1.07	1.42	-1.08	0.37
Glycine	3.57	3.20	1.21	0.11
Indole-3-acetate	3.66	7.76	-1.20	0.001
Lactate	1.33	2.34	1.19	0.07
Methionine	2.14	2.23	1.01	0.91
Methylguanidine	3.36	1.57	1.04	0.69
Myoinositol	3.63	3.30	-1.07	0.71
N,N,N-Trimethyllysine	3.12	3.19	1.03	0.98
Phenylacetylglutamine	3.67	7.60	-1.28	0.0009
Sarcosine	3.61	1.61	-1.05	0.24
TMAO	3.27	19.19	1.16	0.22
Trimethylamine	2.89	1.71	-1.40	0.01

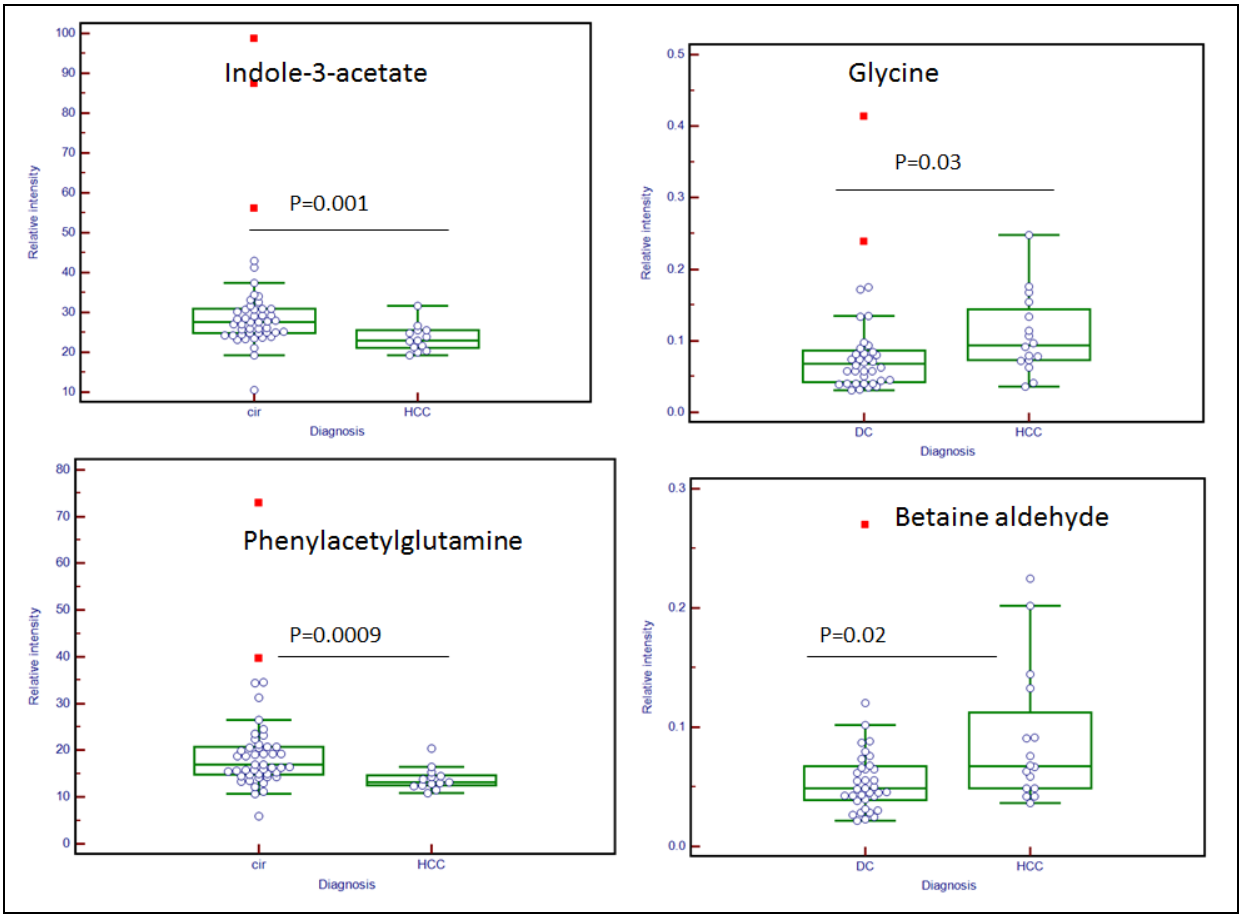

Figure 38. Some of the urinary metabolites that significantly discriminated between HCC and controls shown by relative intensities

9.3.4 Diagnostic performance of panels of metabolites

The relative intensities of those metabolites that were significantly different in concentration in urine of patients with HCC versus controls were logistically regressed in order to assess diagnostic potential of panels of urinary metabolites. The sensitivities (95% CI) of these panels to discriminate HCC from DC and Cir were 68.7% (41.3-89.0) and 84.6% (54.6 - 91.8) respectively. Similarly, the specificities of these panels were 100% (90.3-100) and 89.4% (76.9 – 96.5) for HCC vs. DC and HCC vs. Cir respectively. The corresponding AUC of these models were 0.87 and 0.93 for HCC vs. DC and HCC vs. Cir respectively ($p<0.0001$) (Figure **39**).

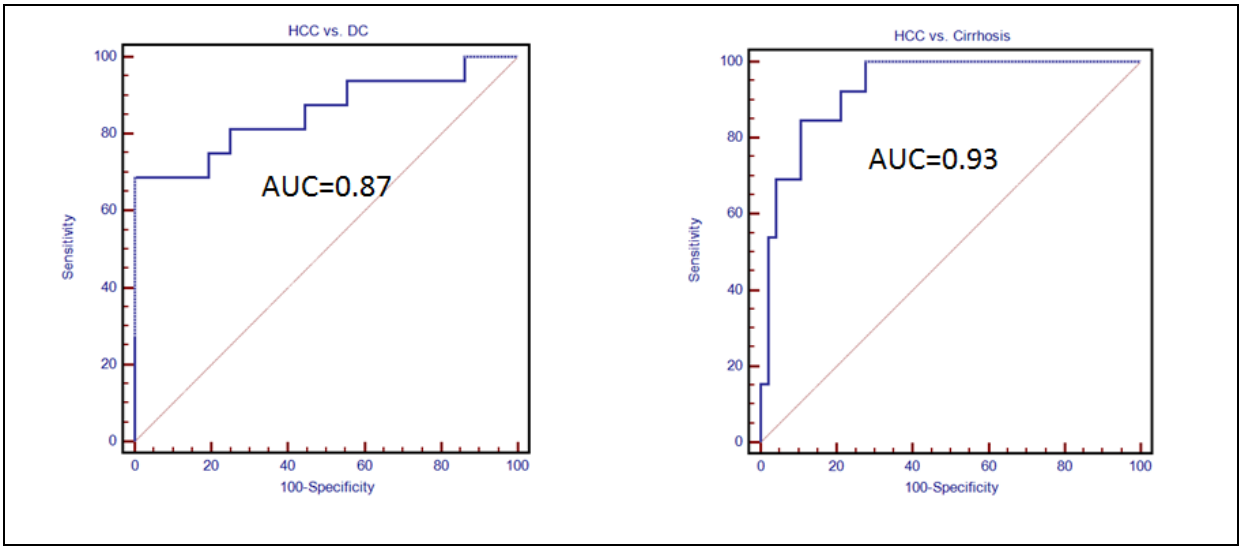

Figure 39. Area under the receiver operating characteristic curves (AUC) to assess the diagnostic performance of panels of metabolites discriminating HCC from non-cirrhotic liver disease (DC) and HCC from cirrhosis.

9.4 DISCUSSION

Translation of putative tumour markers has been hampered by the fact that most biomarker discoveries are undertaken in subjects with advanced tumours. To investigate whether patients with small HCCs could be discriminated metabolically from those with cirrhosis or non-cirrhotic liver disease, urinary NMR of patients from the UK whose HCC qualify for curative therapy were analysed. The supervised multivariate model (OPLS-DA) found a good separation of the cases (pre transplant HCCs) from controls; and metabolites responsible for the separation were identified. 5-hydroxytryptamine, betaine aldehyde, dimethylglycine, GTP, thiamine, ribose and glycine were significantly up-regulated in HCC patients while indole-3-acetate, trimethylamine and phenylacetylglutamine were significantly down-regulated. Intriguingly, the sensitivity and specificity of a panel of these urinary metabolites were about 85% and 100% respectively. Metabonomic technology, using NMR can thus discriminate HCC from liver disease controls irrespective of the clinical stage of the tumour. This thus possesses the potential for biomarker discovery that could be useful for diagnostic and pathophysiological postulations, thus has the potential to augmenting genetic and epigenetic mechanisms.

The metabolites found in this experiment are similar to some that discriminated controls from intermediate/large HCC from African patients (in the last chapter). In contrast, whereas indole-3-acetate was down-regulated and glycine up-regulated in HCC patients in the present study, these metabolites were altered in the opposite directions in the West African sample of HCC patients, as well as in a similar study involving an Egyptian population (Shariff *et al*., 2011).

Reasons for the differences are unclear, although it is to be noted that whereas the HCC patients from Egypt had advanced disease, HCC patients in the present study population included only early stage HCC patients. This divergent finding may thus represent differential metabolic activity related to tumour size of HCC patients.

Two studies have reported up-regulation of urinary glycine excretion in HCC. A study of 33 TNM stage 1 HCC patients (Chen *et al*., 2011b) and one designed to identify metabolic signatures of early HCC recurrence after surgery (Ye *et al*., 2012b), both found increased urinary glycine excretion in HCC. Involvement of glycine in methylation process of nucleic acid synthesis of malignancy may thus underlie the differential urinary glycine concentration found in the present study. This is further buttressed by the fact that GTP was significantly higher in the urine of patients with HCC than in those of controls in the present study. Urinary nucleosides have been observed to be significantly higher in HCC patients than in healthy volunteers in a study in Taiwan by Jeng and coleagues (Jeng *et al*., 2009).

Phenylacetylglutamine, a normal urinary metabolite in humans was down-regulated in HCC patients in the present study. Its lower excretion in HCC is of uncertain significance, but does not seem to be as a result of suboptimal synthetic function of the liver, as serum prothrombin time and albumin; both markers of hepatic synthetic function were not significantly different between HCC patients and controls. The fact that Glutamine N-acetyl transferase, the enzyme that catalyses the formation of phenylacetylglutamine from phenylacetyl-CoA and glutamine, has been purified from human liver mitochondrion suggests that modulation of this pathway may be important in the early stage of hepatocellular carcinogenesis. Thus the up-regulation and down-regulation of glycine and phenylacetylglutamine respectively in this present study population, relative to the West African patient population suggests

some dynamism in the tumour microenvironment during HCC carcinogenesis. There is an obvious need to verify this in future experiments, as this may prove vital as therapeutic targets in individualised therapeutic approach.

The alterations of urinary metabolites in this present study suggest pathways that could be disturbed in early stage HCC pathogenesis. Tryptophan, choline, nucleic acid and glucose metabolism were significantly prominent. 5-hydroxytryptamine is known to regulate biochemical processes and is synthesised from tryptophan. Its role in tumorigenesis has recently been suggested by researchers (Liang *et al*., 2013;Soll *et al.,* 2010). To further implicate the role of tryptophan metabolism, indole-3-acetate, a breakdown product of this amino acid was significantly altered in the urine of HCC patients in this study.

The finding of a high diagnostic performance of urinary metabolites in this study population puts to rest the contention regarding the ability of proton NMR to identify biomarkers for discrimination of early stage HCC and suggest that the clinical translation of a urinary diagnostic would be globally applicable. Important also is the fact that the HCC population was diverse, including several aetiological agents of HCC. The biomarkers thus represent hepatocarcinogenesis rather than specific to aetiological agents of HCC. It may however, prove useful to consider that the panel of markers that would suffice for diagnosing early stage HCC would incorporate some, and/or be different from the ones that would be required to diagnose medium/large HCCs.

The strength of the present study is enhanced by the fact that all HCC cases were confirmed to be early and qualified for curative therapy. Also, cirrhosis was routinely diagnosed based on histological evidence. However, these findings are limited by

the small sample size of HCC patients, not helped by the fact that several of the patients had to be excluded on account of containing drug or sugar metabolites. The fact that the internal validation outcome (using 1000 times permutations) did not yield a favourable measure, particularly for HCC versus cirrhosis suggests over-fitting of the models used in this experiment. In as much as these metabolites can be applied to separate HCC from controls by about 85% sensitivity, it may not be applicable to a new set of patients. There is thus the need to carry out this study in a larger sample size, in order to improve its power.

Another issue is the fact that the metabolites we have identified could be indicative of carcinogenesis and not specific to hepatocellular carcinoma. A further study that compares the urinary metabolic profiles of HCC against those of patients with other solid tumours such as the prostate, breast, ovary and colon, as well as metastatic tumour to the liver will best test whether or not these metabolites are specific to HCC.

In conclusion, NMR of urine of patients with early, transplantable HCC identified discriminatory metabolites with high diagnostic accuracy. Some of these metabolites share similarity to the ones identified in the west African study and confirms that they are effects of hepatocarcinogenesis and not specific to aetiology of HCC. The divergent finding of the behaviour of glycine and phenylacetylglutamine suggests sequential changes in metabolism of tumours relative to their growth sizes. These findings need to be confirmed in a larger sample population of patients.

10. BIOMARKER DISCOVERY OF HCC IN WEST AFRICAN PATIENTS USING MASS SPECTROMETRY

10.0 ABSTRACT

Background: Preliminary data of subjects recruited in the PROLIFICA project in two West African countries show that urinary metabolic profiles may discriminate HCC from controls using proton NMR spectroscopy. Ultra-Performance Liquid chromatography mass spectrometry (UPLC-MS) presents a high throughput assay that has higher ability to verify and or discover additional metabolites; and which has been used to identify metabolic profiles of HCC elsewhere. This present study hypothesised that urinary UPLC-MS will confirm HCC metabolites in this West African cohort and identify additional metabolites that will enhance discrimination of HCC from controls.

Method: Urine samples for the experiment were from subjects that formed the population of the proton NMR spectroscopy work reported in chapter 8. Metabolite profiling was performed by ultra-performance liquid chromatography quadrupole-time-of-flight mass spectrometry (UPLC Q-TOF MS) (Waters, Manchester, UK) that was equipped with an electrospray ion (ESI) source operating in either positive or negative mode. Following processing of acquired chromatograms using XCMS within R environment, further multivariate statistical methods used were principal components and partial least squares discriminant analyses (in SIMCA). Additionally, tandem mass spectrometry was employed to enhance identification of some of the compounds that discriminated HCC from controls. Identification of candidate metabolites were done by comparing the fragments obtained with standard online databases [HMDB (www.hmdb.ca) and metlin (www.metlin.scripps.edu)]. Univariate

statistical methods were applied using MedCal statistical software version 12.7.5 (MedCalc Software bvba, Ostend, Belgium; http://www.medcalc.org; 2013) in order to define the extent of alteration of identified metabolites.

Results: Urine samples from 35, 13, 35 and 32 subjects with HCC, cirrhosis, liver disease (non-cirrhosis) and healthy controls were analysed. Multivariate models demonstrated good separation, confirmed by 999 permutations tests, between binary combinations of HCC compared to the three control groups in both positive and negative ion modes. A panel of metabolites discriminated HCC from cirrhosis with sensitivity and specificity of 91% and 100% in the positive mode; and 94% and 100% in the negative mode. Good correlation between the relative concentrations of some of the adducts, versus BCLC stage of HCC was observed. Tandem MS enabled identification of 23 compounds that were discriminatory for HCC. 3'-sialyllactose, kynurenic acid, taurine, glycochenodeoxycholic acid, 9-decenoylcarnitine, among others were newly identified. The diagnostic performances of some of the metabolites were better than that of serum AFP. Furthermore, acetylcarnitine and kynurenic acid showed significant positive correlations with disease category as well as with stage of HCC.

Conclusion: Urinary UPLC-MS identified several urinary metabolites, which confirm some that were identified by proton NMR spectroscopy. Most importantly, HCC was able to be discriminated from cirrhosis, the most important group of patients for which diagnosis has been a challenge.

10.1 BACKGROUND

Around the world, the prognosis of HCC is appalling at present, mostly as a result of late diagnosis. The mortality rate of HCC is about the same as its incidence in most developing countries (Shariff *et al*., 2009). AFP, the screening tool for HCC that is still being utilised in most developing countries is known to have a low diagnostic capability (Ishida, Matsuo, & Inoue, 2010). USS of the liver, widely recommended to be the base tool for screening by most guidelines, especially in developed countries is expensive, requires stable infrastructure and technical skills that are impractical for resource-limited countries (Ladep & Taylor-Robinson, 2007). Of note, the burden of HCC is highest in these developing countries.

The search for biomarkers of HCC that will diagnostically perform better than current screening tools has become a priority with aim to curb the rising mortality from this tumour. Several investigational serum markers have not been validated to be significantly better than AFP in terms of diagnosing small tumours (Abu El, 2012). As urine presents a readily available biofluid for diagnosis that contains metabolites that are fingerprints of tissue processes as well as being more stable than blood, it has become an important direction of research endeavor in this regard. For any metabolite or combination of metabolites to be useful diagnostically, it must outperform AFP in terms of sensitivity and specificity, its concentration should mirror the burden of HCC, attenuate with response to treatment and lastly, accentuate with recurrence.

Metabonomics hold promise to discover high-performing biomarkers that could revolutionise HCC diagnosis (Wang *et al*., 2013a). Investigations of urinary biomarkers of HCC have identified substances that await validation with regards to

the conditions required for an ideal biomarker(s) (Wang *et al*., 2013b;Yin *et al*., 2009). Differences in the metabolic profiles of malignant liver tissues compared to adjacent non-malignant tissues have been described (Huang *et al*., 2013). Preliminary data have indicated that urine could be important as a source of non-invasive means of diagnosis of HCC (Shariff *et al*., 2010). However, data on the metabolic profile of HCC in patients from Africa, a region that will readily utilize this point-of-care test, are still limited. As the biological behavior of HCC in Africans may be different to patients elsewhere, the study of urinary biomarkers of HCC in this population is paramount. Moreover, as the application of this technology would be greatly embraced in this region of the world, it is essential that patients from West Africa be studied.

Preliminary data of subjects recruited in the PROLIFICA project in two West African countries found that urinary metabolic profiles could discriminate HCC from controls using NMR spectroscopy with higher diagnostic accuracy than serum AFP (chapter 8). Liquid chromatography-mass spectrometry (LC-MS) presents a high throughput assay that has higher ability to discover additional metabolites and verify ones that were discovered by NMR, and which has been used to identify metabolic profiles of HCC elsewhere (Wang et al., 2013b;Wu et al., 2009).

Hypothesis

Urinary LC-MS will confirm HCC metabolites in the PROLIFICA cohort and identify metabolites that could enhance discrimination of HCC from controls.

Aims

- To identify urinary metabolites of HCC and assess their ability to discriminate HCC from healthy, non-cirrhotic liver disease and cirrhosis
- To determine correlation of relative concentration of urinary metabolites with stage of HCC

10.2 METHODS

10.2.1 Study population

All of the samples for the present study were from subjects recruited in Nigeria and Gambia, who participated in the Prevention of Liver Fibrosis and Carcinoma in Africa (PROLIFICA) project. Essentially they were from the population that was involved in the NMR experiment (chapter 8). All subjects provided informed written consent prior to collection of samples used in the study. The study was approved by the Ethics committee of Jos University Teaching Hospital, Nigeria; Medical Research Council, Gambia and Imperial College London.

10.2.2 Study design

This is a case-control study in which HCC subjects and three classes of control were recruited, as follows:

i. mainly hepatitis B-related cirrhosis,

ii. mainly non-cirrhotic chronic liver disease, and

iii. healthy volunteers from the same African populations.

Binary comparisons of clinical, laboratory and metabolite parameters of the cases and controls were embarked upon throughout the analyses.

10.2.3 Preparation of urine samples

Urine was collected in universal containers, pipetted into Eppendorf bottles and stored at -80^{o}C prior to air-transportation on dry ice to Imperial College London for analysis. At the laboratory, the samples were centrifuged at 13,000 rpm for 10 minutes at 4^{o}C in order to remove debris. The supernatant was aliquoted into labeled wells before mass analysis.

A 2 µL aliquot of the urine was injected into 150 mm x 2.1 mm, 1.8 µm HSS T3 column (Waters, Elstree, UK) at temperature of 40^{o}C using an ultra-performance liquid chromatography system (Waters, Manchester, UK). All samples were kept at 4^{o}C during the analysis and mass spectrometry data collected with the use of a Waters Q-TOF Xevo Tandem Quadrupole (TQ) Mass Spectrometer (Waters, Manchester, UK) that was equipped with an electrospray ion source operating in either positive or negative ion mode. Data were collected from 40 to 1200 *m/z* with a scan time of 0.3 s and interscan delay of 0.02 s over a 13 min analysis time. As the analytes elude at different times, dependent on their polarity and hence solubility in the aqueous (water) or organic (acetonitrile) solvents, formic acid was added to provide a source of protons in reverse phase LC-MS and to reduce the pH of the mobile phase, suppressing the ionisation of weak organic acids and thereby improving retention.

All analyses were acquired using the LockSpray to ensure accuracy and reproducibility. 200 pg/mL leucine-encephalin (m/z 556.2771) in H_2O:CAN (50:50) was used as the lock mass with a flow rate of 30 mL^{-1}. Data were collected in the positive and negative ion centroid mode with LockSpray frequency set at 200 sec and 10 data scans on average. The mass spectrometer was operated with a capillary voltage of 3.2 kV and 2.4 kV for positive and negative ion modes

respectively, cone voltage of 35 V, nebuliser gas flow of 900 L/hr, and desolvation temperature of 120^{o}C. The instrument was set to acquire data over the mass range of m/z 50-1000 with acquisition time of 5 scans per second and an interscan delay of 100 ms, according to the validated method of Want and colleagues (Want *et al*., 2010).

10.2.4 Data processing

The acquired raw mass spectrometry (MS) data were exported in NetCDF format by MassLynx software (version 4.1; Waters, Manchester, U.K.). CDF files were extracted using XCMS software (within R statistical software) (sourced from *http://metlin.scripps.edu/xcms/installation.php;* La Jolla, Carlifornia, USA) (Smith et al., 2006). The parameters used were retention time range 0-9.5 min, mass range 50-1000 Da, mass tolerance 0.02 Da. The result of these processes led to retention time deviation correction and generation of data matrix that contain information of arbitrarily assigned peak indices (retention time; *m/z* pairs), sample names (observations), and ion intensity information (variables).

The two sets of data resulting from positive and negative ion modes were analysed separately by multivariate and univariate statistical methods. Identification of metabolites was achieved using further analysis of the MS/MS (tandem mass spectrometry) data acquired during the experiment.

10.2.5 Statistical analyses

10.2.5.1 Multivariate analysis (SIMCA)

Each data set was imported into SIMCA-P 13.0 software (Umetrics, Umeå, Sweden). For the present study, 7 round cross-validations was applied with 1/7th of the samples being excluded from the model in each round to guard against over-fitting

was embarked upon. To study how well the model clustered the groups, an unsupervised multivariate statistical technique in the form of principal components analysis was carried out. This was followed by orthogonal partial least squares discriminant analysis, a supervised multivariate technique, in order to visualize the metabolic alterations between HCC and each of the three control groups. The performance of these models was validated by permutation testing. S-plots were applied to obtain mass/charges for subsequent metabolite identification which was performed separately; using web-based resources including: Human Metabolome Database (http://www.hmdb.ca/) and Metlin database (http://metlin.scripps.edu/index.php).

10.2.5.2 Univariate analyses

Using Graphpad Prism (version 6, CA, USA) and MedCalc for windows statistical software (version 12.5, Ostend, Belgium), univariate statistical analyses in the form of Mann Whitney U tests were performed to measure the significance of representative metabolites in separating HCC patients from healthy volunteers and non-cirrhotic liver disease and cirrhosis patients. The corresponding up and down regulated intensities of how these differential masses varied between HCC and controls were displayed using heat maps (Microsoft Excel®).

Analysis of variance was used to calculate trends in the relative intensity of metabolites and 3 phenotypic states (cirrhosis, BCLC stage A-C and BCLC stage D). In order to further identify differences between cirrhosis and categorized stages, the differences between the relative intensity each of the three phenotypic groups were estimated using the Holm-Sidak multiple comparison test.

10.2.5.3 Diagnostic performance of urinary metabolites

Logistic regression analysis by backward method was used to identify the best performing masses and their predictive values fitted for calculation of area under the receiver operating characteristic curves (AUC). Calculation of sensitivity and specificity of binary combination of diagnostic panels was undertaken. Lastly, Kendall's tau was calculated in order to estimate correlation of relative intensity of some metabolites to stage of HCC. Statistically significant observations were placed at p values less than 0.05.

10.2.6 Tandem mass spectrometry

The methods so far described are limited to identification of masses which do not necessarily represent authentic metabolites. Identification of specific metabolites required accessing information of the tandem mass spectrometry (fragmentation experiment) data available in its raw form in the MassLynx software. The most significant chromatographic peaks were explored and their respective m/z vs. RT compared to online databases (http://metlin.scripps.edu/index.php). Identified discriminatory metabolites were subjected to multivariate and univariate statistical tests, comparing their diagnostic performances using area under the receiver operating characteristic curves and trends between controls and increasing HCC tumour burden (BCLC stage).

10.3 RESULTS

10.3.1 Clinical parameters

The clinical characteristics of HCC patients and other subjects are shown in Table 17. In the study cohort, fewer patients with cirrhosis than the rest of the other groups were recruited. There were more males and who were also older than the rest of

groups. Serum albumin, alanine aminotransferase and prothrombin time were abnormally different in HCC and cirrhosis groups, compared to subjects with chronic non-cirrhotic liver disease and to healthy volunteers.

Table 17. Clinical and laboratory characteristics of patients with HCC and controls whose urine samples were analysed for detection of discriminatory metabolites

Parameter	HCC	Cirrhosis	Disease control	Healthy	p value
Number	35	13	35	32	0.0084
M/F	31/4	10/3	19/16	16/15	0.0033
Age, median (years)	50	45	40	40	0.015
ALT	77	41	35	24	<0.001
Albumin	16	21	41	43	<0.001
Bilirubin	15.3	85.0	10.2	12.0	<0.001
Prothrombin time	20.0	29.0	16.0	22.0	0.1
AFP	256	78	39	3.4	0.1
Creatinine	86	76	86	72	0.07
HBV, n	15	10	33	0	

10.3.2 Data processing

Obtained spectra were processed using XCMS in R statistical software. Following normalisation, the resultant processed data can be seen in Figure **40**.

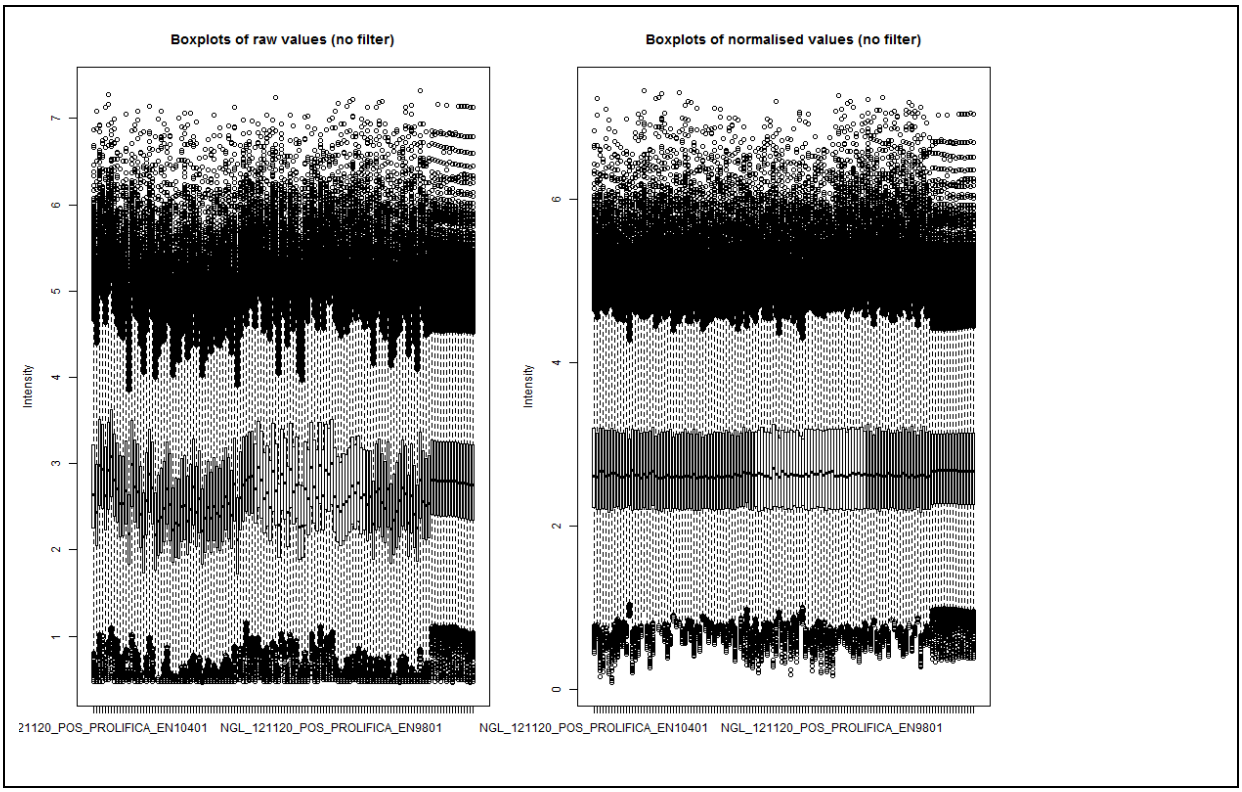

Figure 40. Boxplots showing results of normalisation of negative mode data

10.3.3 Multivariate analysis

Principal components analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA) were performed on the normalised datasets for both positive and negative ion modes. Definite trends of clustering were found for the PCA and there were excellent separations of HCC from controls on the scores plots of OPLS-DA for both modes. Notable also was the fact that all the QC samples clustered very well in both modes (grey circles). Figure **41** illustrates the scores plots of the models of study subjects by classes. In both modes, all the HCC urine samples (green circles) were correctly separated from the urine of subjects with cirrhosis (blue circles).

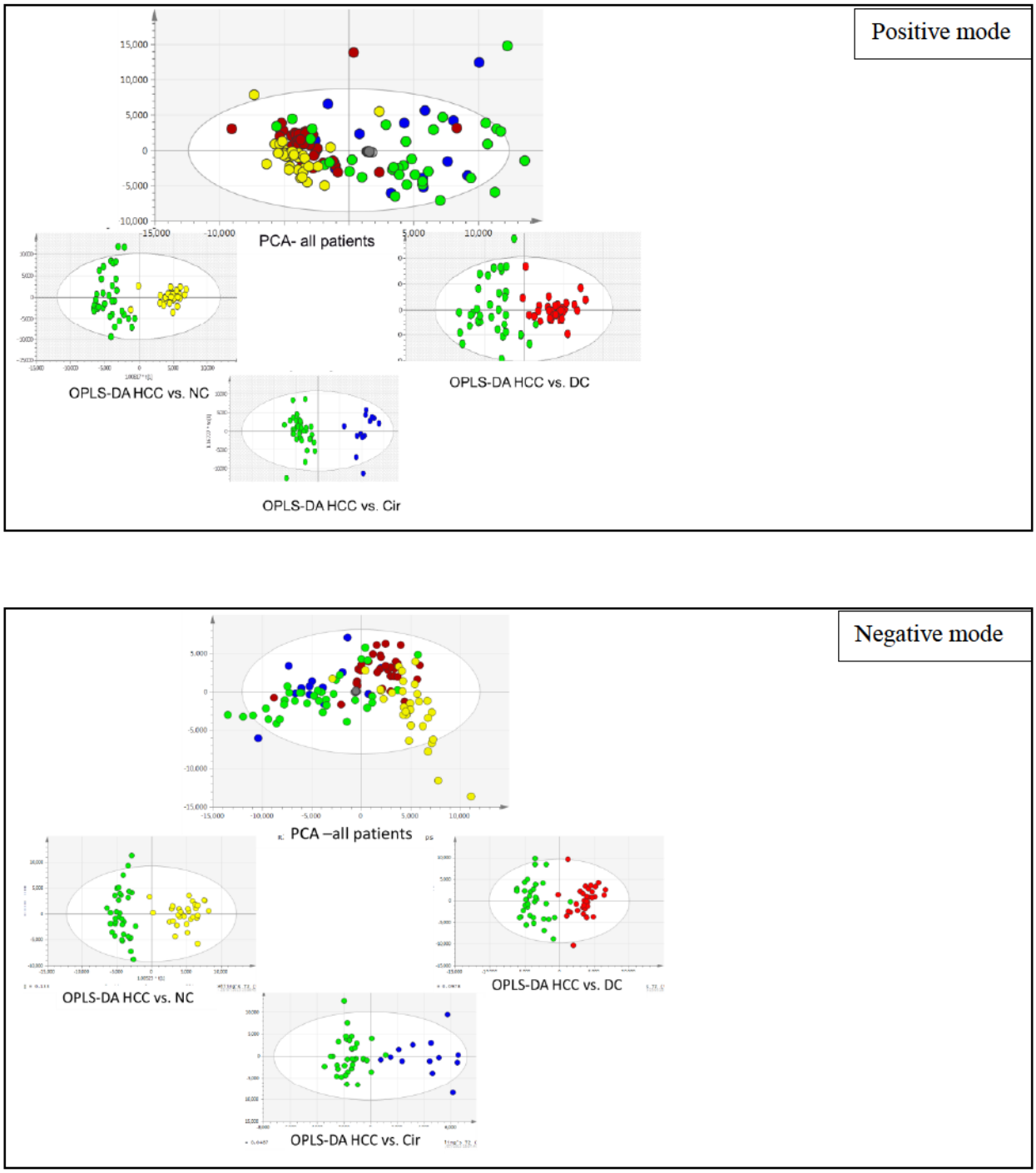

Figure 41. Multivariate analyses in SIMCA P 13.0 of Positive mode data (top) and Negative mode data (bottom) demonstrating clustering of study groups in principal components analysis (PCA). The supervised model; orthogonal partial least squares discriminant analysis (OPLS-DA) of hepatocellular carcinoma (HCC) versus healthy controls (NC), non-cirrhotic liver disease controls (DC) and cirrhosis patients (Cir) for both modes are correspondingly shown. Colour code: green circles (HCC); blue circles (cirrhosis); red circles (DC) and yellow circles (healthy volunteers).

10.3.4 Fitness of model test

The permutation tests (999 times) for HCC vs. Cir of the OPLS-DA models of both positive and negative ion modes, corresponding to their respective PCA models including the correlation coefficients between the original Y and the permutated Y versus the cumulative R2 and Q2, with regression line is shown in Figure **43**. The intercepts (which correlate to extent of over fitting) are small (Pos ion mode: R2=0.76 and Q2= -0.054; Neg ion mode: R2 = 0.77 and Q2 = -0.03) and thus suggest that the models are satisfactory (Figure **42**).

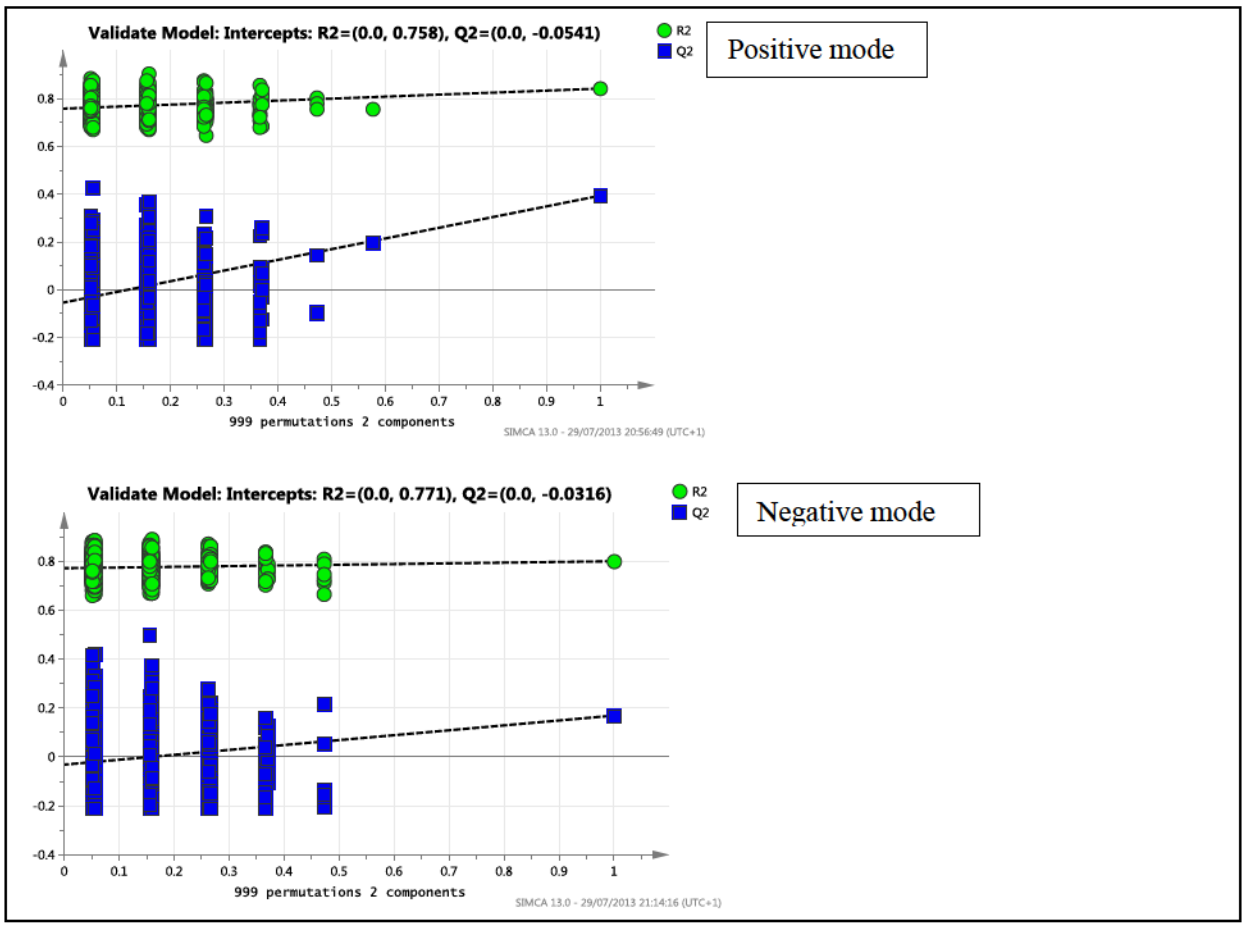

Figure 42. Summary of 1000 permutations of positive and negative ion mode data for HCC versus Cirrhosis partial least squares discriminant analysis (PLS-DA)

10.3.5 Tandem mass spectrometry analyses

In order to confirm the parent metabolites that were important in the discrimination of HCC from controls, raw chromatographic data were interrogated and those parent compounds that were further fragmented during tandem mass spectrometry were identified by comparison to fragmentation patterns of online database (metlin.scripps.edu) of standard compounds.

10.3.6 Chromatograms

Obtained metabolic chromatographic patterns of urine of subjects and controls following fragmentation experiments are shown in figure 38 below.

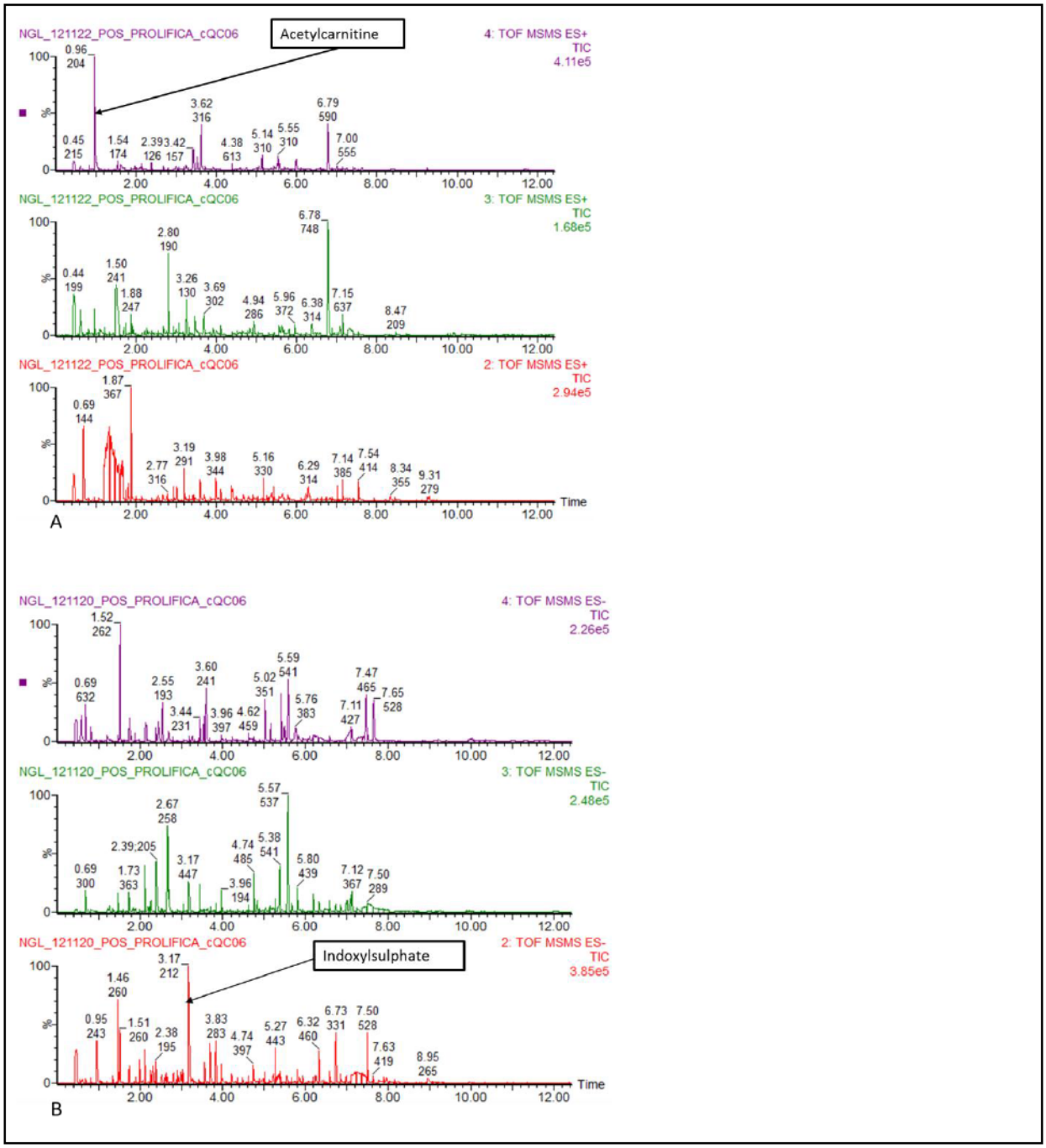

Figure 43. Chromatograms obtained following tandem mass spectrometry of urine in (A) positive ion mode and (B) negative ion mode

10.3.7 MS/MS patterns of some metabolites

Summaries of masses obtained at specific retention times were obtained in both positive and negative ion modes and results compared to mass fragmentation patterns obtained from online databases within accuracy of 30 ppm. Via this mechanism, the parent compounds were identifiable (Figure **44** and Figure **45**).

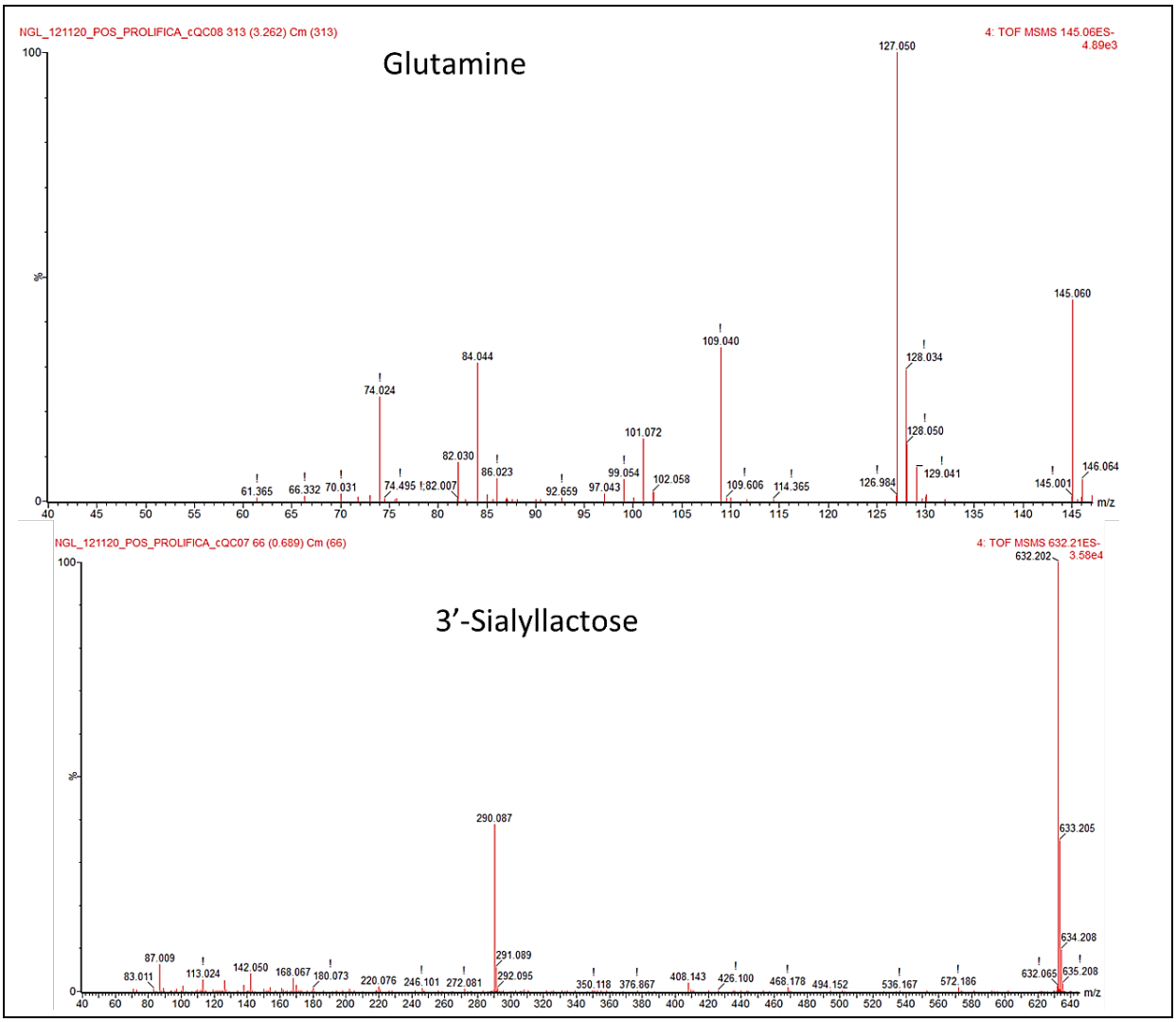

Figure 44. Tandem mass spectrometry fragmentation patterns of masses 145.060 and 632.202; identified as Glutamine and 3'-sialyllactose respectively

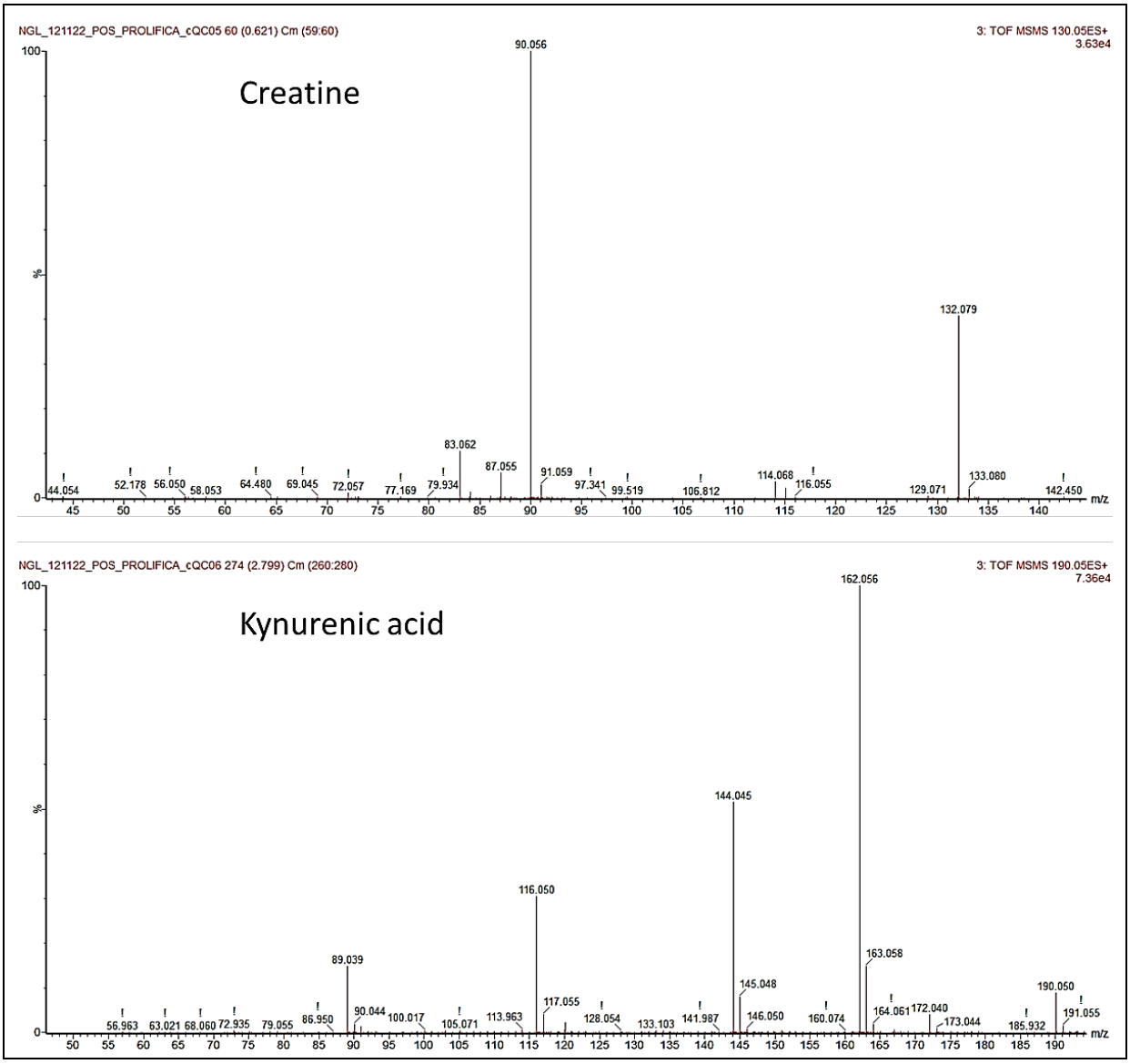

Figure 45. Tandem mass spectrometry fragmentation patterns for masses 132.078 and 190.050; identified as creatine and kynurenic acid respectively

10.3.8 Summary of metabolites

Several compounds including amino acids, nucleic acid, an oligosaccharide, bile components, hormones and gut flora metabolites were identified as perturbed between categorized groups of patients (Table 18).

Table 18. Summary of identified metabolites that were significantly expressed in positive and negative ion modes

Positive ion mode			Negative ion mode		
Mass	Retention time	Metabolite	Mass	Retention time	Metabolite
204.124	0.96	Acetylcarnitine†	212.002	3.17	Indoxylsulphate
190.050	2.80	Kynurenic acid	263.023	1.52	3-methoxy-4-hydroxyphenyl ethylene glycolsulfate
166.073	1.80	1-methylguanine	539.350	5.57	Tetrahydroxyhypoaldosterone-3-glucuronide
124.085	0.45	N-methylhistamine	195.053	2.38	1,3-dimethyluric acid
314.235	5.99	9-decenoylcarnitine	632.202	0.69	3'-Sialyllactose
132.078	0.62	Creatine†	124.007	0.54	Taurine
130.050	3.26	Glutamate†	145.060	3.26	Glutamine
150.055	1.97	Methionine†	178.051	3.26	Hippurate†
301.217	5.64	9-cis retinoic acid	*528.264	7.54	Glycochenodeoxycholic acid
229.155	1.34	Pro Leu	*283.080	3.84	P-cresol glucuronide
139.052	0.95	Urocanic acid			

†Metabolites corroborated by NMR and MS

10.3.9 Relative intensity of metabolites (HCC vs. disease controls)

Whereas glutamine, tetrahydroaldosterone-3-glucuronide and indoxylsulfate were significantly lower in the urine of patients with HCC, there were several metabolites that were up-regulated in HCC (Figure **46**). 9-decenoylcarnitine, glycochenodeoxycholic acid, 3'-Sialyllactose, methionine, taurine, acetylcarnitine and kynurenic acid were higher in HCC relative to liver disease controls.

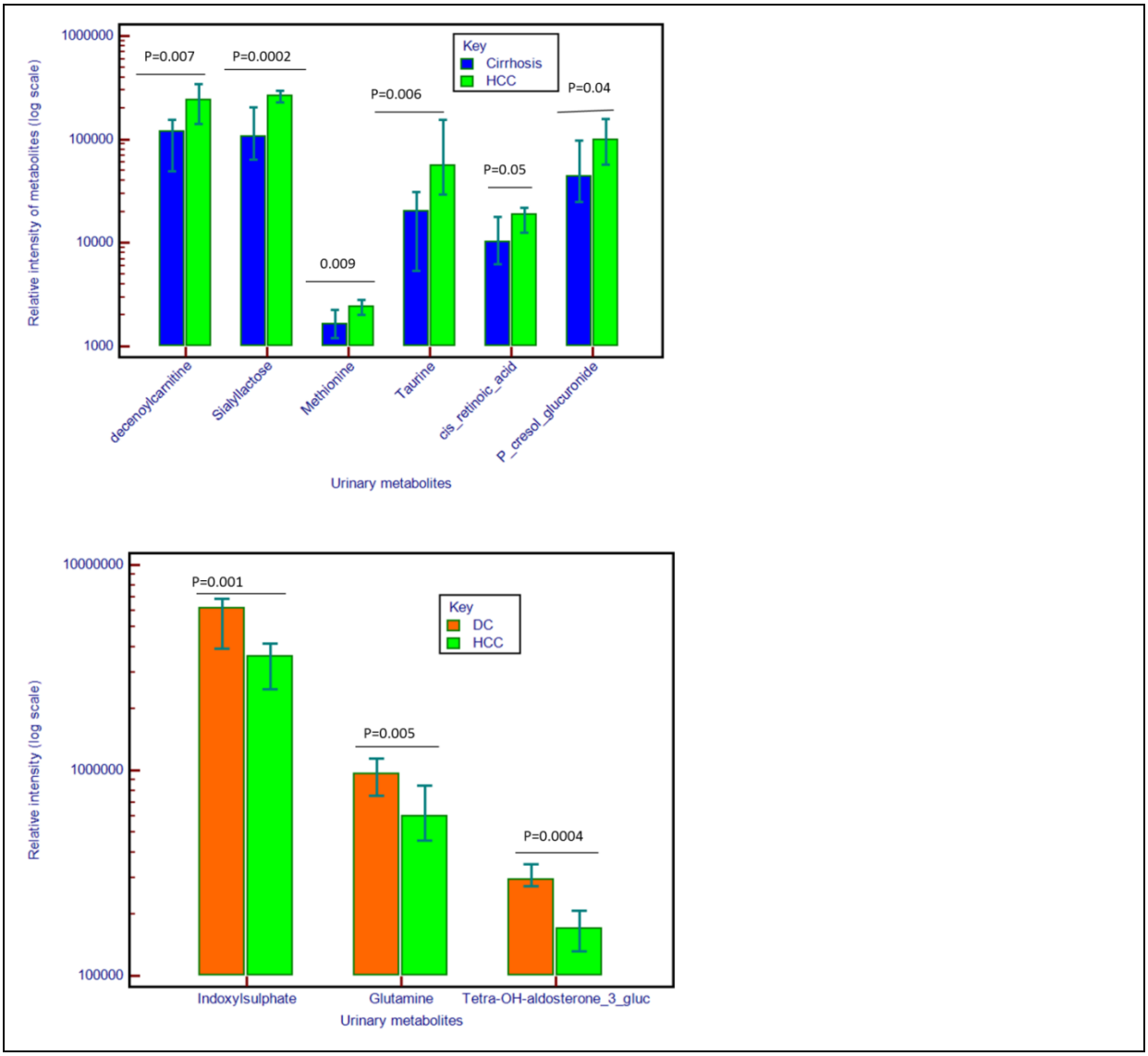

Figure 46. Bar charts showing urinary metabolites that were significantly up-regulated (above) and down-regulated (below) in urine of HCC patients (green bars) relative to controls (blue bars-cirrhosis and pink bars-liver disease). Error bars indicate 95% confidence interval

10.3.10.6 Trends of intensity by disease category

The relative intensity of acetylcarnitine and kynurenic acid showed a significant positive trend to the disease category as well as stage of HCC (Figure **47** and Figure **48**). No correlations were observed between intermediate and late stage HCC for the other metabolites.

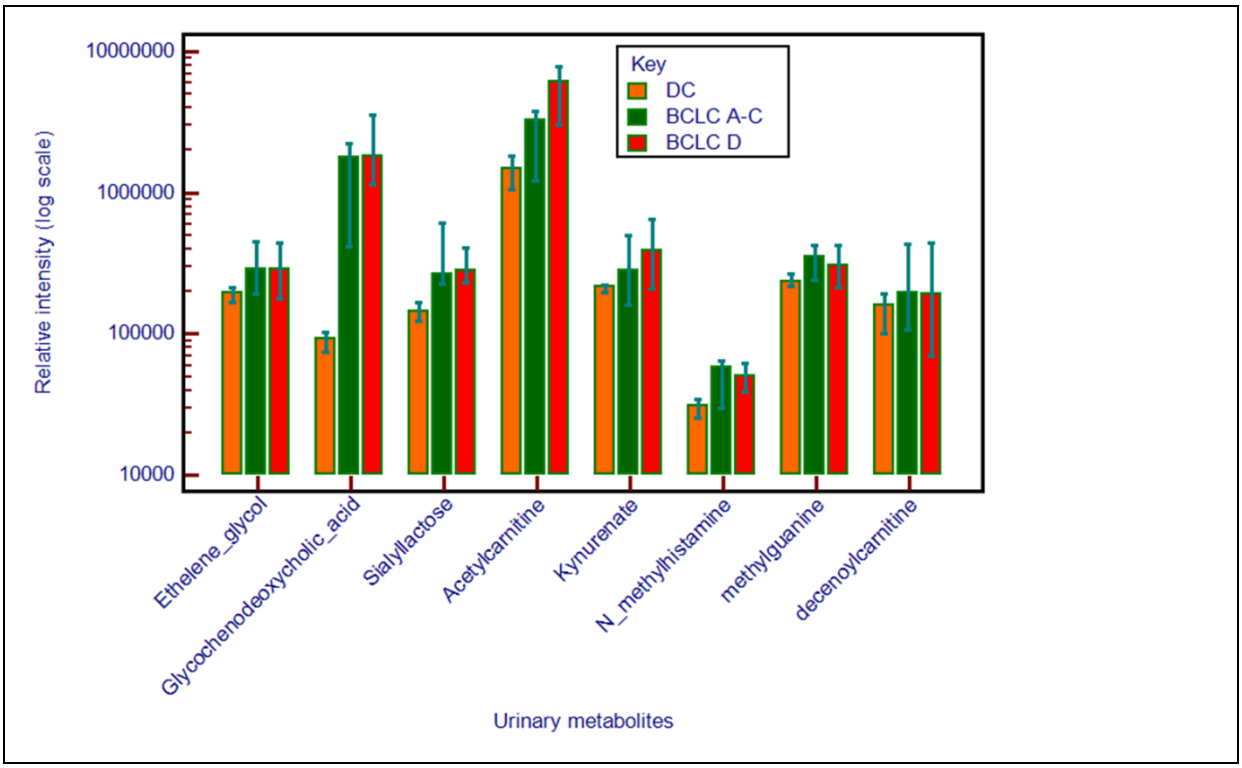

Figure 47. Clustered bar charts showing patterns of increase in the relative intensity of urinary metabolites against disease category and stage of HCC

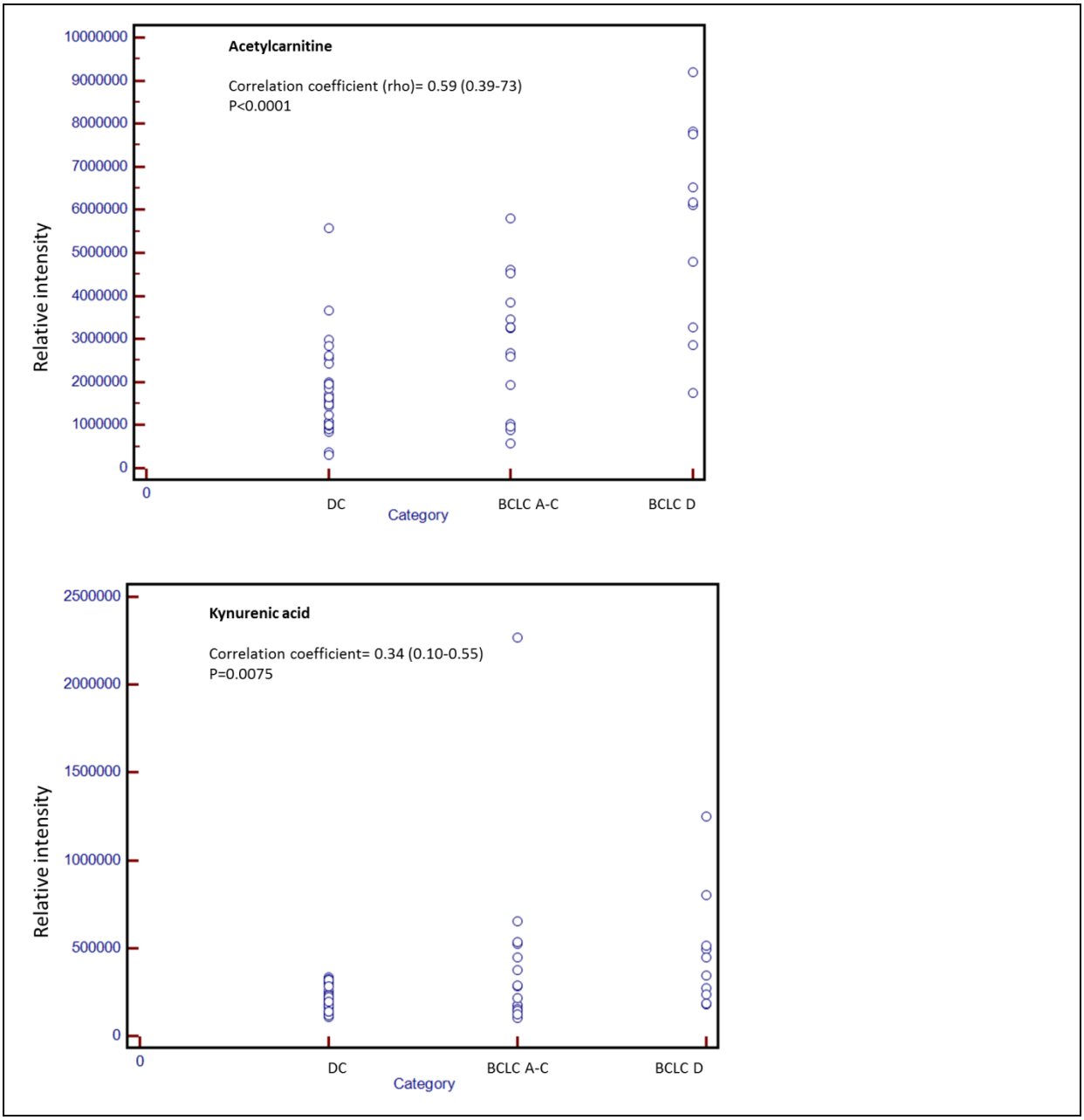

Figure 48. Graphs of Spearman's rank correlation coefficients for intensity of acetylcarnitine and kynurenic acid relative to disease category and stage of HCC

10.3.10 Diagnostic models of urinary metabolites

Urinary glycochenodeoxycholic acid, 3'-Sialyllactose, taurine, methionine, 9-decenoylcarnitine and acetylcarnitine, as single metabolites each possessed higher sensitivity and specificity than serum AFP in the discrimination of HCC from liver disease controls (Table 19). Two panels; comprising 3 urinary metabolites derived from logistic regression (as significant in the models) found a further higher ability of urinary metabolites to diagnose HCC compared to serum AFP (Table 19). Panel II confirms the best diagnostic schedule of metabolites to be a combination of 3'-Sialyllactose, 9-decenoylcarnitine and methionine.

Table 19. Diagnostic performance of serum AFP and urinary metabolites (single and multiple combinations) identified by tandem mass spectrometry

Diagnostic plan	Candidate metabolites	AUC	Sensitivity (%)	Specificity (%)	+ LR	- LR
AFP (serum)						
	HCC vs. Cir	0.64	50.0	66.7	1.5	0.8
	HCC vs. DC	0.67	53.8	68.7	1.7	0.7
Urinary metabolites						
	Glycochenodeoxycholic acid	0.90	85.7	91.4	10.0	0.2
	3'-Sialyllactose	0.86	80.0	76.9	3.5	0.3
	Taurine	0.76	65.7	84.6	4.3	0.4
	Methionine	0.75	65.7	76.9	2.9	0.5
	9-decenoylcarnitine	0.75	60.0	92.3	7.8	0.4
	Acetylcarnitine	0.73	62.9	85.7	4.4	0.4
Combination of metabolites (urine)						
Panel I						
	3'-Sialyllactose 9-decenoylcarnitine 3-methoxy-4-hydroxyphenyl ethylene glycolsulfate	0.92	94.3	88.6	8.3	0.1
Panel II						
	3'-Sialyllactose 9-decenoylcarnitine Methionine	0.96	91.4	100	>11.9	0.1

10.4 DISCUSSION

To be able to curb the menace of HCC, the discovery of more sensitive tools for surveillance and screening of this tumour is a priority. Using liquid chromatography mass spectrometry and complex multivariate/ univariate statistical analyses, several urinary metabolites possessing high discriminatory power for HCC were discovered in the present work. Most importantly, HCC was able to be discriminated from cirrhosis, the most challenging group of patients for which diagnosis would be invaluable. Metabolites, some of which complemented those that were identified by NMR spectroscopy were discovered via this high throughput technique. Besides those, additional diagnostically relevant compounds were found to be up-regulated in HCC population of subjects involved in this study.

The finding of differential relative concentration of some of the metabolites between HCC and controls corroborates published data of patients from Chinese, Egyptian and Nigerian populations. These findings corroborate Warburg's hypothesis and suggest that single or a panel of urinary metabolites could be developed to enhance early diagnosis of HCC. The finding of a high (91%) sensitivity and (100%) specificity of a panel, comprising 3'-Sialyllactose, 9-decenoylcarnitine and methionine confirms the penultimate hypothesis.

The burden of HCC tumour mass, described by the clinical stage of disease was proportional to the relative intensity of acetylcarnitine in this present work, confirming preliminary finding (chapter 8). Another metabolite that was detected in this work to correlate with tumour size was kynurenic acid, a derivative of tryptophan; which has yet to be reported in patients with HCC. However, a study of 19 experimental rats has suggested that the metabolism of tryptophan may be altered in aflatoxin B1-

induced hepatoma animals (Lemonnier *et al*., 1975). The finding of kynurenic acid in this present study population may support the altered tryptophan metabolism of HCC in this aflatoxin-prone population (Gouas *et al*., 2012). Of interest is the finding of down-regulation of indoxylsuphate, another tryptophan metabolite, in urine of HCC patients in this study population. Further studies are warranted in order to define the role of tryptophan metabolism in the pathogenesis of HCC.

3'-Sialyllactose was observed to be significantly up-regulated in HCC patients relative to disease controls in this study population. This sialylated oligosaccharide, secreted in human breast milk has not featured significantly as an important urinary metabolite of HCC. Zhang and colleagues recently discovered its up-regulation in the urine of patients with pre-operative epithelial ovarian cancer relative to healthy controls (Zhang *et al*., 2013). In patients with malignant melanoma, serum sialic acid concentration was discovered to positively correlate with tumour stage (Silver *et al*., 1979). Also, a single murine study, 3 decades ago discovered that sialic acid correlated with metastatic ability of tumours (Yogeeswaran and Salk, 1981). 3'-sialyllactose may be thus be important in the modulation of cell-cell interaction of tumours leading to decreased containment of malignant tumours, such as HCC. The up-regulation of 3'-sialyllactose in HCC of this study population may thus partly explain the relatively higher fatality rate and short diagnosis-to-death period observed in West African patients.

Glycochenodeoxycholic acid, one of the major human bile salts was relatively higher in the urine of HCC patients compared to controls in this study population. Alteration of bile acids had been demonstrated in serum (Ressom *et al*., 2012;Wang *et al*., 2013b) and urine (Chen *et al*., 2011) of HCC patients relative to controls and found to be perturbed. The present finding thus corroborates those of earlier researchers

and suggests important roles for bile acids in the modulation of carcinogenetic process of HCC. The role of glychochenodeoxycholic acid (GCDA) in HCC is poorly understood and may be important in the modulation of hepatocarcinogenesis. Liao and colleagues have suggested that GCDA may be important as a carcinogen and or a survival agonist in the development of HCC (Liao *et al*., 2011).

This work further confirms the possibility of the development of a urinary diagnostic of HCC, most probably via the use of panels, containing a number of metabolites; in this case, a combination of three metabolites. Exploration of complementing serum AFP by these urinary metabolites did not provide any significant increase in the diagnostic accuracy, suggesting that the panel correctly classified those that were diagnosable by AFP. Noteworthy in these findings is the enhanced illumination on the possible mechanistic development of HCC these data have provided. The down-regulation and up-regulation of certain metabolites confirm differential metabolism of HCC and could be downstream effects of genetic and epigenetic changes. This could herald discovery of pathways that may enhance drug development for HCC.

The strength of the present work lies in the fact that it is the first urinary metabolite profiling of HCC by mass spectrometry of HCC patients in a sub-Saharan African population, a region least studied in this regard. Second, the identification of HCC cases was embarked upon via validated international guidelines (EASL) and controls were well characterized by radiological and histological means. Third analysed urine samples underwent very strict laboratory conditions of storage, transportation and preparation. The samples were well preserved and transported on ice and only thawed prior to preparation for mass spectrometry. Moreover, rigorous statistical applications ruled out over-fitting of the models and thus provided validity to the

results obtained. This was confirmed by clustering of the quality control (QC) samples in both modes and low intercept of Q2 following 999 permutations.

The findings are however limited by the fact that most of these metabolites were identified based on adducts that satisfy the classification and biological plausibility; although robust metabolite libraries were utilised (www.hmdb.ca and www.metlin.scripps.edu). To achieve a high degree of confirmation of metabolite identity, tandem mass spectrometry was utilized and some of the metabolites were identified (23 compounds). The highest level of confidence in the metabolite identity would be based on spiking experiments of reference laboratory standards. Future work is planned to verify that the metabolites are correctly identified. Furthermore, validation of these findings in a new set of patients that have been recruited on the PROLIFICA project would be a significant way forward.

11. VALIDATION OF HCC BIOMARKERS IN WEST AFRICAN PATIENTS USING PROTON MAGNETIC RESONANCE SPECTROSCOPY

11.0 ABSTRACT

Background: In order to achieve early diagnosis and hence curb the current trend of HCC, there has continued to be a search for biomarkers. Candidate urinary biomarkers are being identified but await verification and or validation in at-risk populations. The present study being reported aimed to validate urinary biomarkers of HCC in a separate West African cohort to the original study population.

Methods: Urine samples were collected from a separate group of patients and controls at two sites in West Africa on the case-control platform of PROLIFICA. 600MHz NMR was used to acquire one-dimensional spectral data from the urine samples, using a standard 1D NMR pulse sequence with presaturation of the water peak. Areas under the operating characteristic curves (AUC) of the relative concentration of metabolites identified, measured in 463 subjects: 141 with HCC, 56 with cirrhosis (Cir), 178 with non-cirrhotic liver disease (DC) and 88 healthy volunteers (NC), were compared to serum AFP. Following post-acquisition processing of obtained spectra in MATLAB, multivariate statistical techniques in the form of PCA and OPLS-DA were applied using SIMCA. Further statistical handling (univariate and rank correlation) was carried out using MedCalc Statistical Software version 12.7.7 (MedCalc Software bvba, Ostend, Belgium; http://www.medcalc.org; 2013) with statistical significance defined as $p<0.05$.

Results: There was found distinct urinary metabolic profiles of patients with HCC, compared to Cir, DC and NC with sensitivity/specificity of 83.6%/63.5%, 83.6%/96.0% and 87.1%/75.0% respectively. Several metabolites were significantly

increased ($p<0.0001$) in urine of HCC patients compared to non-HCC liver disease controls (DC and Cir), including: *N*-acetylglutamate, betaine, methionine, acetylcarnitine, carnitine, 2-oxoglutarate, indole-3-acetate, creatine and several others. Urinary metabolite profiles performed significantly better than AFP in discriminating HCC from other non-malignant liver conditions (AUC: HCC vs. DC; metabolite profile [0.9] vs. AFP [0.8], $p=0.0003$; HCC vs. Cir; metabolites [0.8] vs. AFP [0.7], $p=0.034$). There was observed a positive correlation of *N*-acetylglutamate, acetylcarnitine, methionine, betaine aldehyde, ribitol and creatine with BCLC stage of HCC.

Conclusions: Urinary NMR of this separate validation cohort of the West African population has confirmed metabolites that implicate altered energy-related pathways in the pathogenesis and progression of HCC. Additionally, the urinary metabolic profiles performed better than serum AFP in the discrimination of HCC from non-malignant liver diseases, thus validating the preliminary results.

11.1 BACKGROUND

A couple of studies have suggested that metabonomics of urine can potentially be used to characterise underlying biochemical mechanisms and hopefully enhance early diagnosis of HCC (Chen *et al*., 2011b;Shariff *et al*., 2010;Shariff *et al*., 2011). Preliminary work reported in chapter 8, involving about 300 patients have confirmed that HCC patients could be discriminated from chronic liver disease patients with higher sensitivity and specificity than serum AFP, using metabolite panels identified by NMR spectroscopy. The identified metabolites involved in the process are mostly those indicative of altered energy metabolism, particularly of beta-oxidation, urea cycle, TCA cycle and gut floral activities. Parallel experiment (chapter 10) on a subset of urine samples from the cohort reported in chapter 8, using ultra high Performance liquid chromatography mass spectrometry confirm the possibility of achieving diagnosis of HCC using urinary biomarkers more accurately than serum AFP.

The ideal biomarker that would overcome the hurdles against clinical utility in HCC should be elevated in concentration in the presence of higher burden of disease and attenuate with tumour ablation or response to treatment. It was possible to demonstrate the former phenomenon only in this West African population, as most patients recruited in this project presented late and with tumours that were not amenable to curative therapy. It is noted that there has been a dearth of data regarding true validation of urinary metabolites of HCC. Most published data utilised leave-one-out validation as well as internal validation; which are both limited by the fact that they only statistically manipulate the same cohort on which their training set data are based. The best model for such a validation would be in a different sample that share similar clinical characteristics. Additionally, more rapid advances have

been made in the prediction, detection and understanding of disease using metabonomics, presenting more opportunities to characterise metabolites with high precision. The on-going recruitment of HCC patients on the case control study of PROLIFICA also enabled a robust platform to study an entirely new set of patients with similar clinical and laboratory characteristics.

Hypothesis

Urinary metabolite profiles can discriminate HCC from controls better than serum AFP and thus validate preliminary experiments.

Aims

- To validate discovery of urinary metabolites of HCC in a separate validation cohort of West African patients, from the original study population
- To determine the accuracy of urinary metabolites and compare with serum AFP in the diagnosis of HCC
- To interrogate the correlation of relative concentration of urinary metabolites with clinical stage of HCC

11.2 METHODS

11.2.1 Study design/subject selection

The design and conduct of this validation experiment were as outlined earlier (chapter 8). Samples were from subjects recruited in Nigeria and Gambia, who were formally consented to participate in the Prevention of Liver Fibrosis and Carcinoma in Africa (PROLIFICA) project. PROLIFICA is a European Union Framework 7-funded project (PROLIFICA: www.prolifica.eu). The case-control platform involved identifying cases and controls, including: mainly hepatitis B-related cirrhosis, non-cirrhotic chronic hepatitis B-related liver disease, and non-cancer subjects that were negative to HBV and HCV screening. All the patients in the present cohort were newly recruited during August 2012 to July 2013 and did not participate in the initial NMR experiment (chapter 8).

Urine samples were collected for metabonomic studies according to earlier outlined protocol. The metabonomic analyses were undertaken using urinary proton (^{1}H) nuclear magnetic resonance spectroscopy (NMR) at Imperial College London. Samples collected from patients, diagnosed with HCC according to the European Association for the Study of the Liver (EASL) guidelines were stored at -80^{o}C until air-transported on dry ice to Imperial College London. Ethics committees of JUTH, Nigeria and MRC, Gambia; as well as Imperial College London granted approval for the study.

All controls were confirmed not to have tumours on ultrasound scan and negative to HBsAg, anti-HCV and HIV screening. Staging of HCC was performed according to the Barcelona Clinic Liver Cancer (BCLC) classification.

11.2.2 Sample collection and preparation

Approximately 5mL of non-fasted urine samples were collected into universal containers after obtaining informed written consent and administration of dietary questionnaires. Prior to spectral acquisition, samples were thawed at room temperature. Sample preparation and acquisition methods were performed according to published methods (Beckonert *et al*., 2007). 400 µL urine sample was mixed with 200 µL of phosphate buffer solution (0.2 M Na_2HPO_4/0.04 M NaH_2PO_4, pH = 7.4 plus 0.1% sodium azide, 1 mM 3-trimethylsilyl-1-[2,2,3,3,-$^2H_4$] propionate (TSP)) to stabilize the urinary pH. The samples were allowed to stand for 10 min prior to centrifugation at 13000 rpm for 10 min in order to remove insoluble material. 500 µL of the supernatants from each urine sample was aliquoted into 5 mm NMR tubes (Wilmad LabGlass^{TM}, New Jersey, USA) for proton nuclear magnetic resonance (NMR) studies.

11.2.3 ^{1}H NMR spectral acquisition and processing

^{1}H NMR spectra were acquired with a 600 MHz spectrometer (Bruker Avance, Massachussets, USA) operating at 600.13 MHz for ^{1}H at 300 K. It was equipped with a 5 mm broad-band inverse configuration probe. Samples were randomly analysed in automation with a B-ACS 60 sample changer system. Urine samples were analysed using water suppressed 1D NMR spectrum using the NOESYPRESAT pulse sequence (256 transients). Irradiation of the solvent (water) resonance was applied during presaturation delay (2.0 s) for all spectra and for the water suppressed 1D NMR spectra also during the mixing time (0.1 s). The pulse sequence parameters including the 90° pulse (~ 10 µs), pulse frequency (~ 4.8 ppm), receiver gain (~ 200), and pulse powers were optimised for each sample set run. The spectral width was 20 ppm for all spectra. The NMR data were processed

with an exponential line broadening of 1.0 Hz prior to Fourier transformation, which were collected with approximately 32 k real data points. Data [-1.0 to 10.0 ppm] were imported into MATLAB 7.0 software (MathWorks, Natick, MA), where they were automatically phased, baseline corrected and referenced to the TSP peak (0.00 ppm), using scripts written in-house. To reduce analytical variation between samples the residual water signal (4.70 – 5.00 ppm) was truncated from the data set. Normalization to total area was performed and metabolites identified according to the earlier protocol.

11.2.4 Multivariate statistical analyses

The acquired spectra were divided into small regions of 0.02±0.01ppm, representing specific metabolites. These regions were aligned (controls mild shifts in the spectra) and normalised (controls differential dilution of samples) using MATLAB. A matrix of samples against variables (ppm bins) was thus generated and transferred to SIMCA 13.0 (statistical software) for unsupervised principal components analysis (PCA) and supervised orthogonal partial least squares discriminant analysis (OPLS-DA). Using these techniques, some outliers identified to be due to experimental errors or huge metabolic peaks that would adversely affect analysis were removed from the models. The models were subjected to “leave-one-out” validation, a technique in which each sample in turn was automatically excluded from the analysis, a model created from the remainder of the samples and the class membership of the excluded sample predicted. This technique was applied to every 7^{th} sample until each sample was excluded once. To confirm the performance of the model, 999 permutations of comparison models were embarked upon.

11.2.5 Univariate statistical analysis

The metabolites that contributed most to the OPLS-DA separation were identified through their high performance on the variable importance in projection (VIP) and use of S-line plots in SIMCA 13.0. Identified metabolites were confirmed using an in-house statistical total correlation spectroscopy (STOCSY) tool. Intensities of the metabolites were expressed as concentration relative to creatinine. This procedure of correcting for creatinine intensity was aimed at controlling for muscle mass and differential kidney function of the subjects. Using MedCalc software, Mann-Whitney U test of significance was applied to study the differences in the median values of identified metabolites between HCC (cases) and the three classes of controls.

The ability of each model to discriminate HCC from cirrhosis, non-cirrhotic liver disease and healthy control subjects were examined for sensitivity and specificity using area under the receiver operating characteristic (AUC) curves. The AUC curves were built to compare the diagnostic performance of these metabolite panels with serum alpha fetoprotein; each for HCC versus cirrhosis, HCC versus non-cirrhotic liver disease and HCC versus healthy volunteers.

Finally, Spearman's correlation coefficients of the most influential metabolites by clinical stage of HCC were determined.

11.3 RESULTS

11.3.1 Clinical parameters

A total of 463 urine samples, 141 of which were from patients confirmed to have HCC, 56 with cirrhosis, 178 with non-cirrhosis liver disease (mostly HBV infected) and 88 from normal healthy volunteers were analysed. As shown in Table 20, most of the patients were at intermediate and advanced stage of HCC.

Table 20. Clinical and laboratory information of HCC patients and controls (cirrhosis, chronic liver disease and healthy volunteers)

	HCC subjects (n=141) Nig(n=85); Gam(n=56)	**Cirrhosis subjects (n=56)** Nig(n=32); Gam(n=24)	**Non-cirrhosis liver disease (n=178)** Nig(n=40); Gam(n=138)	**Healthy controls (n=88)** Nig(n=35); Gam(n=53)
Age; years	45(21-95)	36(15-69)	38(17-75)	46(18-81)
Sex; M/F, n(%)	104/31(77%/23%)	43/13(77%/23%)	92/85(52%/48%)	45/43(51%/49%)
BCLC stage				
A-C	60(72.3)	/	/	/
D	22(26.7)	/	/	/
Serum AFP, ng/mL	381.8(1.4-100000)	58.0(2.0-789)	5.8(0.5-558)	4.7(2.2-571)
Albumin, g/L	28.0(4.2-17.4)	25.0(11-41)	41.0(9-50)	40.0(12-71)
ALT, IU/L	46.5(4-823)	59.0(16-1074)	21.0(4-1339)	22.0(8-386)
Creatinine, μm/L	90(21-423)	74(36-297)	72(30-173)	85(15-896)
Bilirubin, μm/L	10.2(5.1-295.8)	15.3(8.2-680)	10.2(5-316)	10.2(1-102)
Aetiology of liver disease				
HBV	63(53%)	54(98%)	171(98%)	0(0%)
HBV/HCV	3(3%)	0(0%)	0(0%)	0(0%)
HCV	14(12%)	1(2%)	2(1%)	0(0%)
Negative/ Unknown	39 (33%)	0(0%)	2(1%)	84(100%)

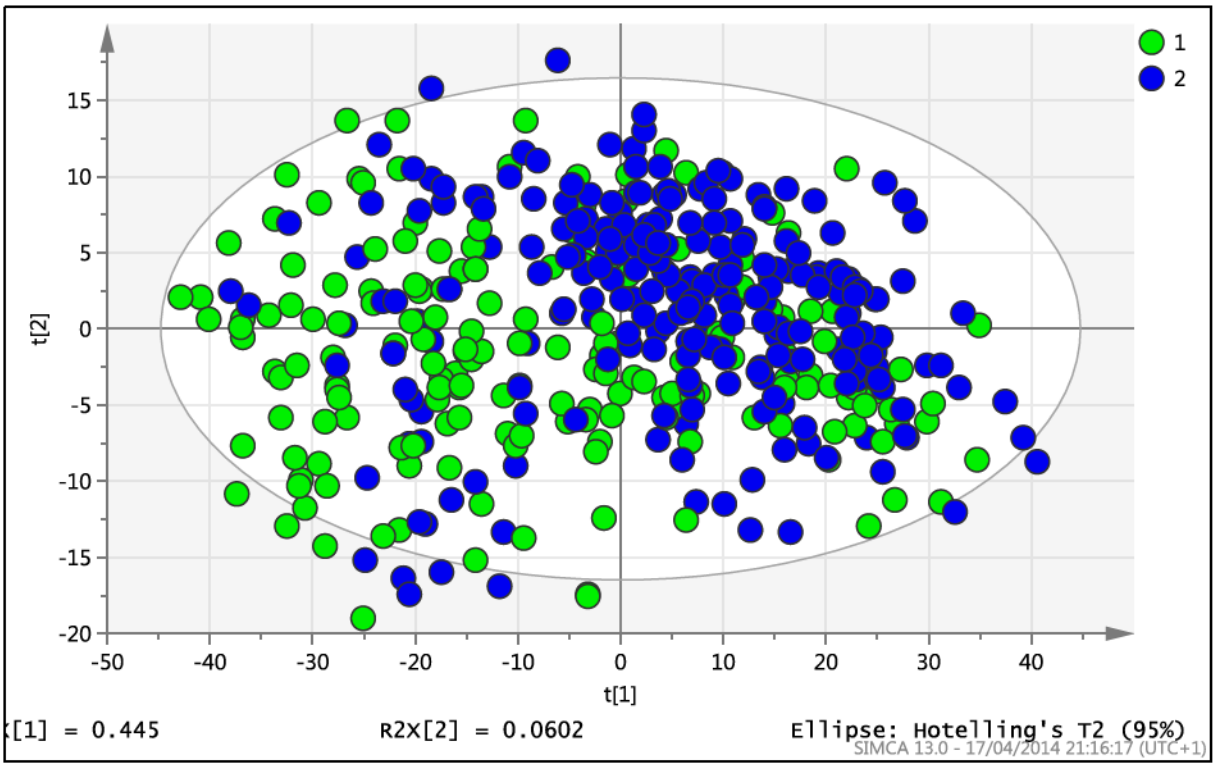

Figure 49. PCA scores plot based on urine metabolic profiles of subjects from Nigeria (green circles) and Gambia (blue circles) demonstrating suboptimal clustering

11.3.2 Multivariate analysis results

PCA scores plot by country of origin of subjects did not show any significant clustering (**Figure 49**). Scores plot of PCA demonstrated clustering of cases (HCC) and the three groups of controls (Figure **50**). The supervised OPLS-DA models each for HCC compared to healthy volunteers, HCC compared to non-cirrhotic liver disease and HCC compared to cirrhosis, showed good separation of cases from controls. The prediction of the performance of each of the models was assessed to be satisfactory using 999 permutations.

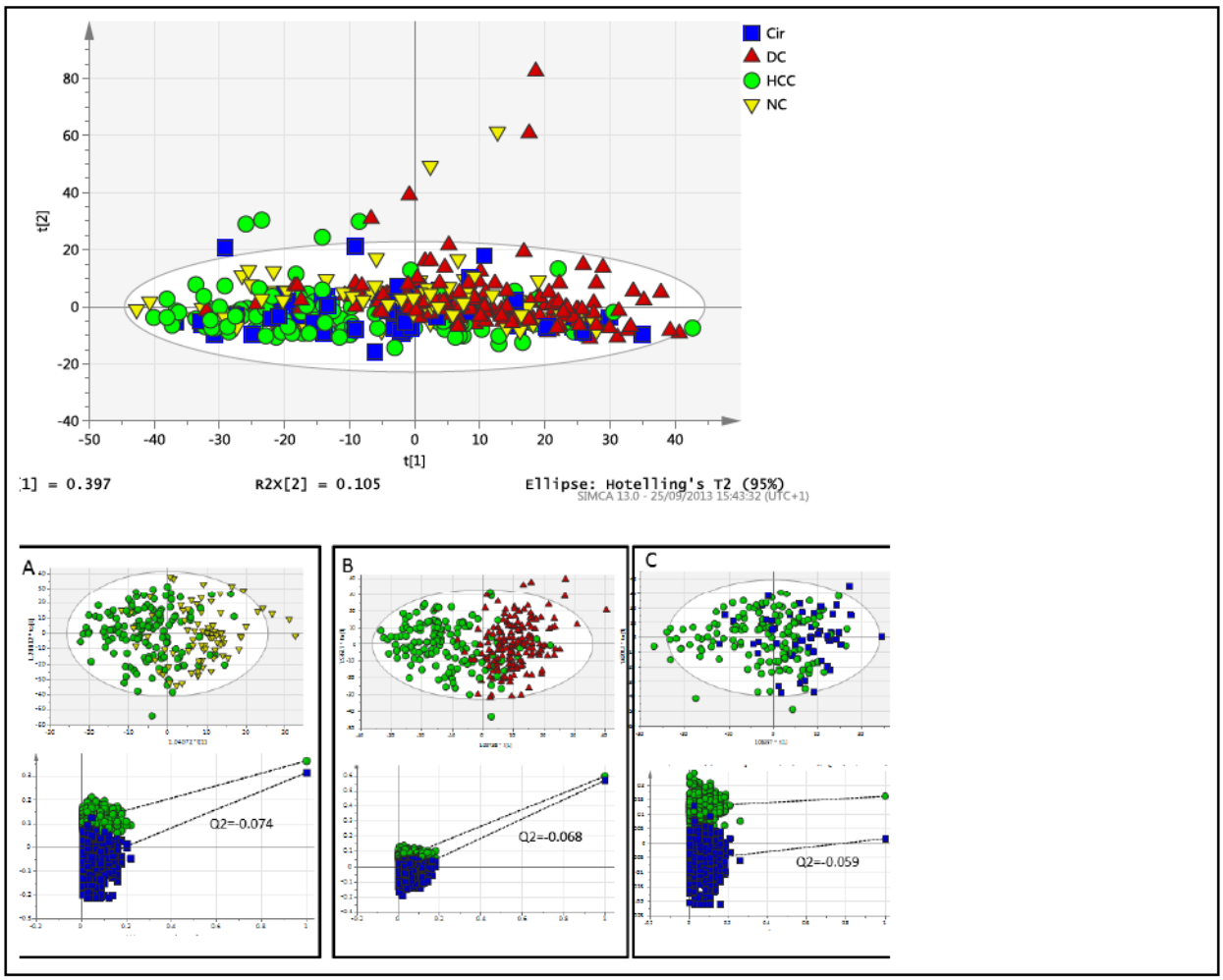

Figure 50. Scores plot of PCA model based on urine samples from HCC patients (green circles), cirrhosis patients (blue squares), liver disease (red triangles) and healthy volunteers (yellow inverted triangles). Below the PCA plot are scores plot of OPLS-DA prediction and corresponding statistical validation of PLS-DA models by permutation analyses (999) of (A) HCC vs. Healthy, (B) HCC vs. DC; and (C) HCC vs. Cir. HCC: hepatocellular carcinoma; DC: non-cirrhotic liver disease; Cir: cirrhosis

11.3.3 Diagnostic performance of urinary metabolites

Based on the models generated by the metabonomics of urinary metabolites, there was found to be a superior diagnostic accuracy of these models relative to serum AFP (Table 21). In particular, there was observed significantly better diagnostic performances of urinary models than serum AFP for HCC vs. Cir and HCC vs. DC.

Table 21. Comparison of diagnostic accuracy of models based on urinary metabolic profiles and serum AFP

Class comparison		AUC (95% CI)	SE	AUC difference	SE	p value
HCC vs. NC[α]	Model	0.90(0.85-0.96)	0.03	0.08	0.05	0.12
	AFP	0.82(0.73-0.92)	0.05			
HCC vs. DC[β]	Model	0.96(0.94-0.99)	0.01	0.08	0.03	0.003
	AFP	0.88(0.84-0.93)	0.02			
HCC vs. Cir[δ]	Model	0.81(0.73-0.89)	0.04	0.12	0.06	0.034
	AFP	0.68(0.59-0.78)	0.05			

[α]132 patients (112 HCC and 20 NC); [β]208 patients (112 HCC and 96 DC); [δ]150 patients (112 HCC and 38 Cir); comparisons were limited to those subjects that had urinary metabolic profiling performed and in whom serum alpha fetoprotein were determined.

11.3.4 Univariate analysis results

Major discriminatory metabolites that were identified from the processed normalized and aligned spectra were subjected to further univariate analyses. The metabolites were identified by a comprehensive method of checking online libraries such as the human metabolome database (www.hmdb.ca) and Chenomx nmr suite 7.7 (www.chenomx.com). Additionally, reference standards were run under the same conditions as the experimental samples and their respective spectra confirmed to be identical to the ones identified (Figure **51**).

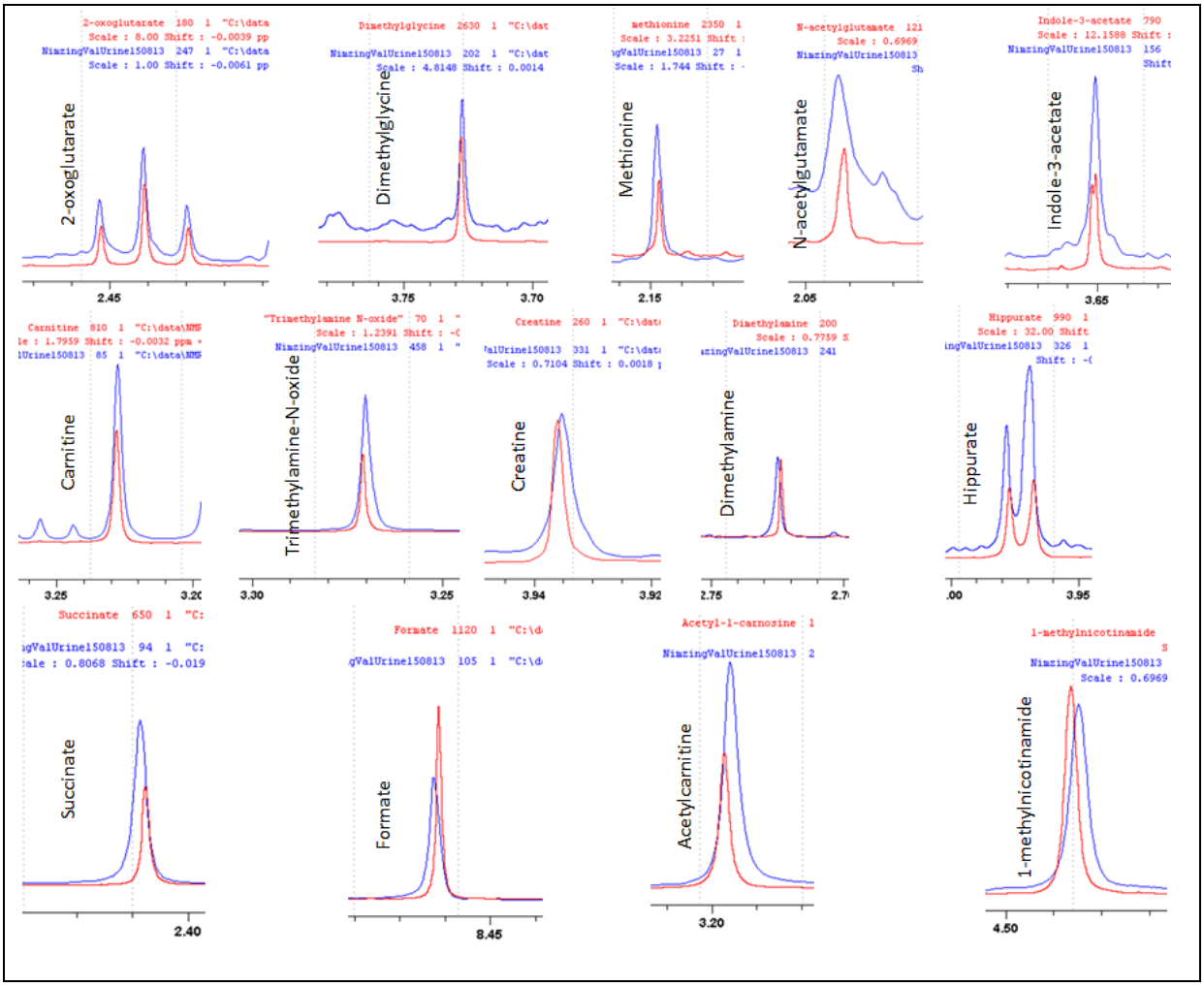

Figure 51. NMR spectra of reference standards (red) compared against urinary NMR spectra of (blue) patients

Alterations (median fold changes) in the relative concentration of urinary metabolites were observed when HCC was compared with disease and healthy controls (Table 22). The metabolites identified were mostly those already identified in the initial cohort (chapter 8) besides additional ones including: amino acids, carbohydrates, and nucleotides, co-factors of beta-oxidation, as well as those from the gut flora.

Table 22. Summary of altered urinary metabolites between HCC vs. Healthy, HCC vs. liver disease and HCC vs. cirrhosis patients

Metabolite	Chemical shift(ppm)	HCC vs. Healthy			HCC vs. Liver Disease			HCC vs. Cirrhosis		
		VIP	FC	p value	VIP	FC	p value	VIP	FC	p value
Lactate	1.33	1.43	1.17	0.0028						
Alpha-hydroxyisobutyrate	1.36		1.07	0.6		-3.78	0.000005			
Acetate	1.92				1.42	1.19	0.0006	5.08	1.10	0.037
N-acetylglutamate	2.04	1.59	1.57	<0.000001	1.06	1.91	<0.000001	1.13	1.43	0.0008
Methionine	2.14	4.46	1.55	<0.000001	2.18	1.85	<0.000001	3.30	1.39	0.009
Pyruvate	2.35	5.28	-1.29	0.0018	3.83	-1.38	0.0017			
Succinate	2.41	1.55	1.16	0.043						
2-oxoglutarate	2.44	1.33	1.49	<0.000001	1.00	1.60	<0.000001			
Citrate	2.66	1.82	1.08	0.22	3.59	1.02	0.64	4.96	-1.20	0.37
Dimethylamine	2.72	2.06	1.35	0.000002						
Methylguanidine	2.83	1.38	1.35	<0.000001						
Homocarnosine	2.93							2.18	-1.09	0.68
N-Alpha-acetyllysine	3.01							1.15	1.22	0.093
Creatine	3.04							11.33	1.15	0.035
Acetylcarnitine	3.20	11.15	2.05	<0.000001	6.61	2.36	<0.000001	10.99	1.97	0.0007
Carnitine	3.22	15.46	2.07	<0.000001	8.74	2.80	<0.000001	8.01	1.63	0.015
Betaine aldehyde	3.24	1.43	1.50	<0.000001	1.00	1.75	<0.000001	0.70	1.34	0.017
TMAO	3.27	19.81	-1.18	0.0076	10.80	-1.05	0.12	9.48	1.02	0.58
Methylguanidine	3.36							1.18	1.05	0.41
Choline	3.50	1.21	1.70	<0.000001	1.15	2.18	<0.000001			
Glycine	3.56	5.98	-1.03	0.46	9.30	-1.28	0.0003	10.40	-1.09	0.21
Indole 3-acetate	3.65	5.12	1.80	<0.000001	3.79	2.02	<0.000001			
Phenylacetylglutamine	3.67	1.81	1.11	0.14						
1-Methyl-L-histidine	3.69	1.38	1.45	0.000006						
Dimethylglycine	3.72	1.14	1.49	<0.000001	1.61	1.86	<0.000001			
N-Phenylacetylglycine	3.75				1.40	2.02	<0.000001			

Ribitol	3.83							3.83	1.54	0.0001
Pseudouridine	3.85				1.19	1.96	<0.000001			
Betaine	3.89					2.08	<0.000001			
Glycolate	3.94	3.91	1.36	0.0019						
Creatine	3.95	6.25	1.29	0.0016	3.18	1.35	<0.000001	5.44	1.19	0.14
Hippurate	3.97	8.68	-1.67	<0.000001	9.31	-1.61	<0.000001			
O-hydroxyhippurate	3.99	3.08	1.37	<0.000001	3.61	1.61	<0.000001			
1-Methylnicotinamide	4.48	1.30	1.99	<0.000001	1.00	2.88	<0.000001			
N-formyl-L-Aspartate	4.51							1.29	-1.05	0.91
Glyoxylic acid	5.09							1.31	1.24	0.072
L-Fucose	5.21	0.61	1.32	0.0018	0.60	1.67	<0.000001			
N-Acetyl-5-hydroxytryptamine	7.09							1.37	-1.62	0.019
5-Hydroxytryptophan	7.14								1.34	0.11
5-Hydroxyindole-3-acetate	7.21	1.67	-1.82	<0.000001	1.19	-1.36	0.0016			
Betaphenylpyruvate	7.28	1.54	-1.81	<0.000001	1.06	-1.54	4E-06			
5-Methoxytryptamine	7.32							1.38	1.18	0.028
Phenylacetylglutamine	7.36				1.69	-1.33	0.021			
5-Hydroxytryptamine	7.42	1.67	-1.91	0.00004	1.00	-1.63	0.003			
Phenylacetylglutamine	7.44							1.81	1.52	0.0037
Hippurate	7.55	6.97	-3.37	<0.000001	5.86	-3.62	<0.000001		1.08	0.69
Hippurate	7.64	3.60	-2.64	<0.000001	2.64	-1.71	<0.000001			
Alpha-hydroxyhippurate	7.84	6.36	-3.32	<0.000001	5.69	-3.78	<0.000001			
Formate	8.46								1.12	0.75

11.3.5 Concentration of metabolites relative to stage of HCC

Owing to the fact that most HCC patients would have background cirrhosis, the urinary metabolites that discriminated HCC from cirrhosis were further evaluated. Methionine, betaine aldehyde, carnitine, N-acetylglutamate, ribitol and creatine showed good correlation of increasing concentration relative to stage of HCC (Table 23). To study further the specific metabolites that may be contributing to the pathological processes of HCC disease, bar charts were used to illustrate the relative concentrations of identified metabolites between tumour stages relative to cirrrhosis. Of note, acetylcarnitine, N-acetylglutamate, ribitol and creatine, which are mainly metabolites involved in altered metabolism revealed increasing trends (Figure **52**).

Table 23. Summary of correlation of urinary metabolite concentration by HCC stage

Metabolite	**Coefficient (rho)**	**95%CI**	**p value**
Acetylcarnitine	0.38	0.18-0.55	0.0005
Carnitine	0.39	0.19-0.56	0.0003
Methionine	0.42	0.23-0.59	0.0001
N-acetylglutamate	0.28	0.07-0.47	0.011
Betaine aldehyde	0.43	0.23-0.59	0.0001
Ribitol	0.37	0.16-0.54	0.0007
Creatine	0.33	0.12-0.51	0.003

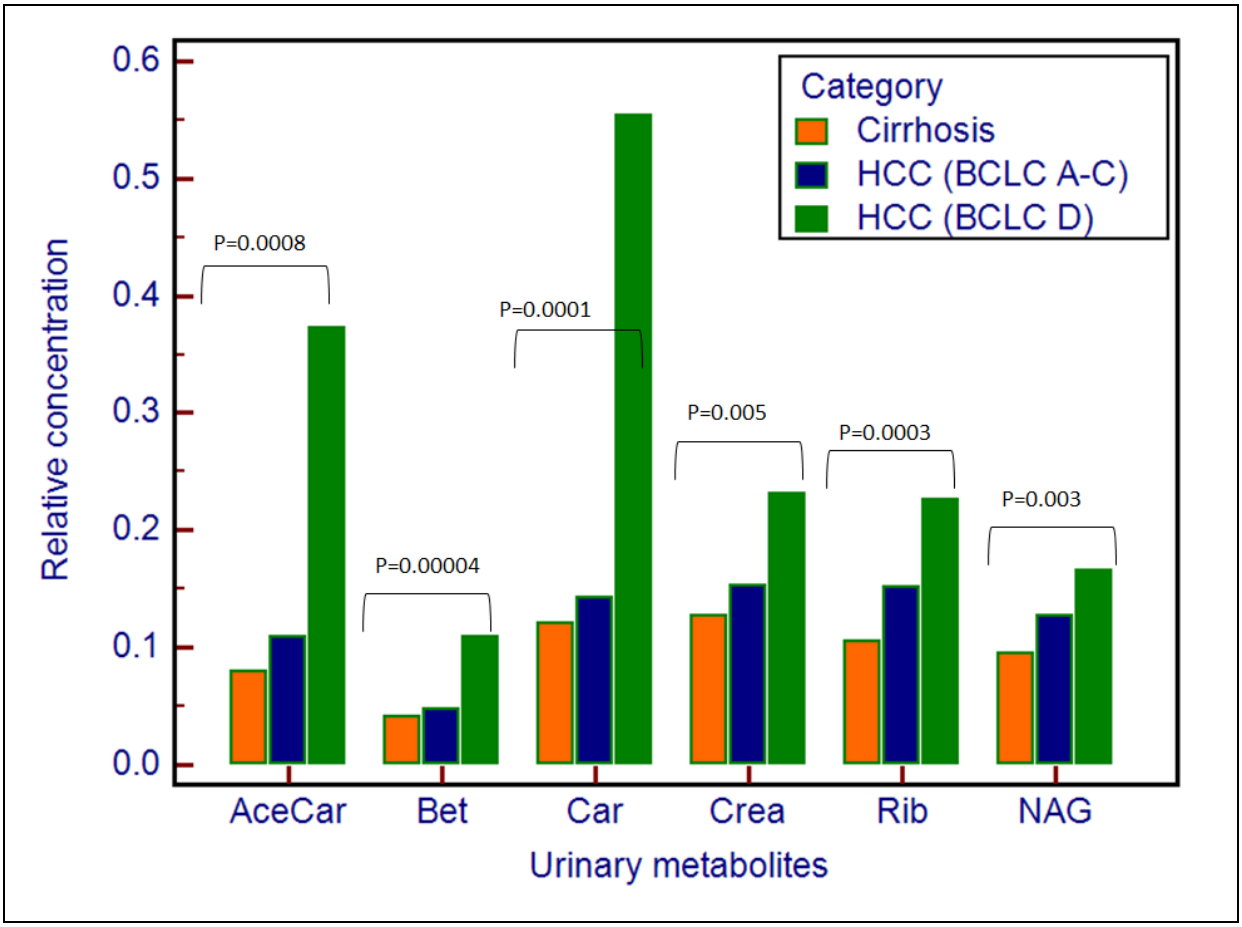

Figure 52. Bar charts showing progressive increase in relative concentration of urinary metabolites among patients at different stages of HCC relative to cirrhosis; AceCar: acetylcarnitine; Bet: betaine aldehyde; Car: carnitine; Crea: creatine; Rib: ribitol; NAG: n-acetylglutamate

11.4 DISCUSSION

Lack of verification and/or true validation of candidate biomarkers have been the bottle-neck of translation from bench to clinical utility of severally reported markers in scientific literature. As of 2007, about 1,261 proteins had been cited in the literature as being altered in human cancers, yet only about 3% are being used in some clinical capacity (Polanski and Anderson, 2007). There has been a lot of interest in defining urinary biomarkers of cancer in recent times. A review of urinary bladder cancer studies identified some studies that have been conducted; and it noted that these markers have lower specificity than invasive cystoscopy for screening of bladder cancer (Xylinas *et al*., 2013). Data are also available for urinary markers of ovarian (Macuks *et al*., 2012) and gastric cancer (Dong *et al*., 2013), but findings are yet to be validated.

Increasing attempts at defining relevant biomarkers of HCC in urine have yielded interesting results, yet these have been hampered by small sample sizes (Shariff *et al*., 2010) and lack of external validation of preliminary findings (Chen *et al*., 2011b;Shariff *et al*., 2011). The data presented in this report studied a large number of samples in which there were 141 HCC subjects, and confirmed urinary metabolites that were discovered in a separate cohort of patients from West Africa, possessing similar clinical and laboratory characteristics as in the preliminary cohort. Moreover, the profiles of the urinary metabolite models diagnostically outperformed serum AFP, similar to the findings of the initial experiment. It is to be noted that whereas most diagnostic performances of biomarkers identified in previous studies were described between cancer patients and healthy controls, this work compared the diagnostic accuracy of identified markers between HCC and at-risk populations

(cirrhosis and non-cirrhosis liver disease), besides comparison with healthy volunteers.

Almost all the urinary metabolites that were described in the preliminary work were confirmed to have similar alterations in the present work. Additional metabolites found in this validation cohort include 5-hydroxytryptamine, betaphenylpyruvate, pseudouridine, guanosine, homocarnosine and ribitol. Together with the ones found in the previous work, these metabolites indicate fingerprints of significantly altered pathways including glycolysis, tricarboxylic acid cycle, pentose phosphate pathway, DNA methylation, choline, gut flora and beta-oxidation. A schema of how these alterations may have evolved is charted as shown in Figure **53**.

The present work also confirms alterations of urinary metabolites with stage of HCC; suggesting their role for prognostication of this tumour. Until at time of writing up these data, there has been little attempt to illustrate the possible complex pathway alterations that may be important in HCC. The human metabolome database and the Kyoto Encyclopaedia of Genes and Genomes (KEGG) database were used to map modifications in the metabolic pathways. Fatty acid metabolism (acetylcarnitine and carnitine), energy provision for the tumour (acetate, butyrate and creatine), choline metabolism (dimethylglycine and TMAO), glutaminolysis (glutamate, alanine), tricarboxylic acid cycle (2-oxoglutarate and succinate), DNA methylation (formate, methionine, betaine, glycine) and gut metabolism (tryptophan, tryptamine and indole-3-acetate) pathways were particularly altered.

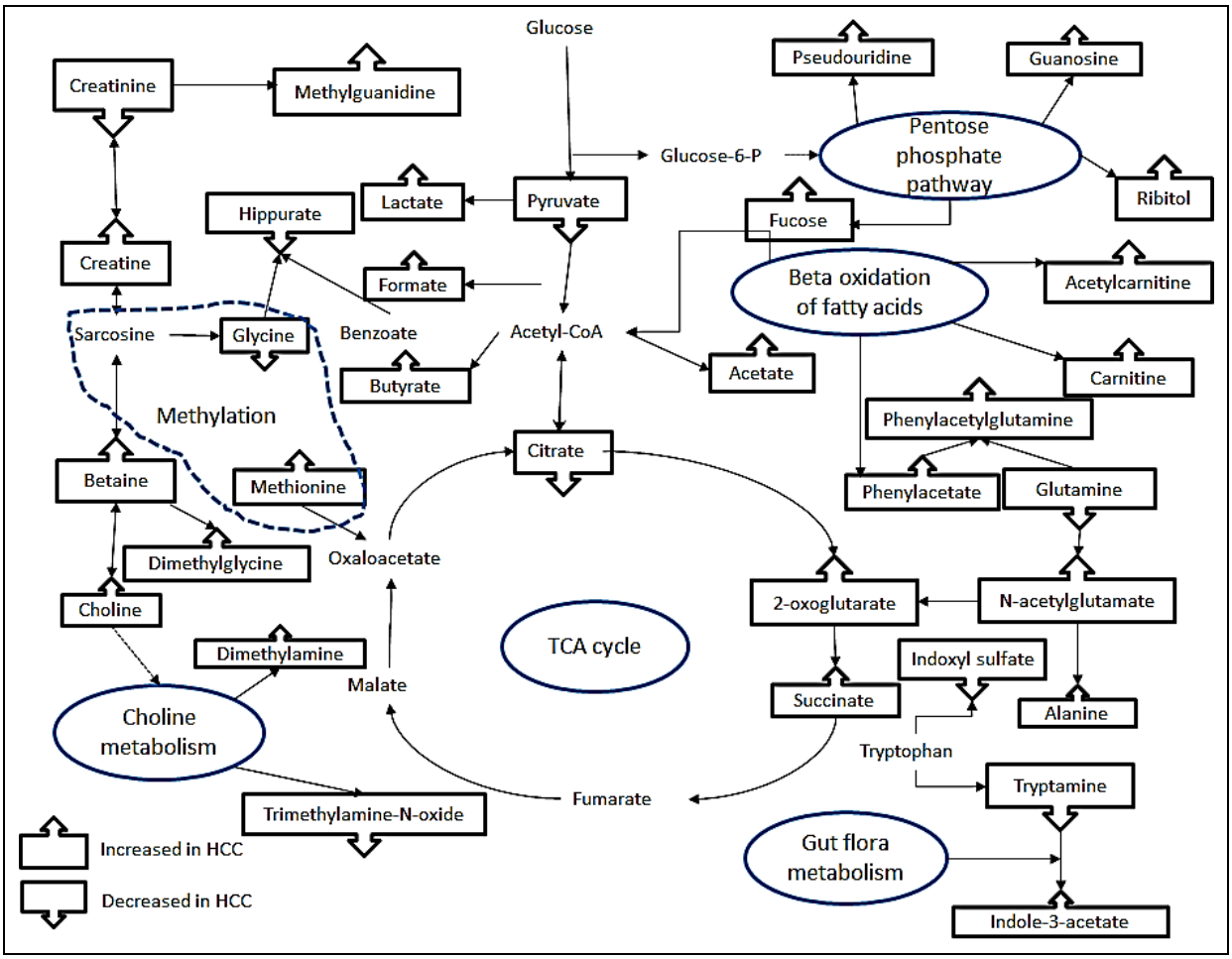

Figure 53. Presumed altered metabolic pathways in HCC identified from discriminating urinary metabolites. Items within blue borders highlight pathways; whereas direction of arrow indicate up or down-regulation

There appears to be a heightened free fatty acid metabolism in patients with HCC. In both preliminary and validation experiments, acetylcarnitine and carnitine, co-factors in the initial phase of beta-oxidation were up-regulated in urine of patients with HCC. In the carnitine shuttle, transformed fatty acid, Acyl CoA is transferred to the hydroxyl group of carnitine by carnitine acyltransferase located on the outer membrane of the mitochondrion. Acylcarnitine is then shuttled inside by carnitine-acylcarnitine translocase. Carnitine acyltransferase II, located on the inner membrane of the mitochondrion, converts acylcarnitine to acyl CoA and carnitine. The liberated carnitine is returned to the cytosol. Further evidence of heightened beta-oxidation is

shown by relatively higher acetate, butyrate and phenylacetylglutamine in the urine of HCC patients than in controls. The excess production of these metabolites in HCC patients confirms that ketone bodies are possible requirements of HCC and may fuel the growth and progression of HCC. This phenomenon had been validated by researchers in a recent study (Martinez-Outschoorn *et al*., 2012) in which ketone bodies were identified as drivers of tumour growth and metastasis.

Glycine was consistently down-regulated in urine of HCC patients. This finding corroborates data from Shariff and colleagues in a study of HCC patients from an Egyptian population (Shariff *et al*., 2011), as well as preliminary data (chapter 8). The involvement of glycine in aberrant DNA methylation, a hallmark of carcinogenesis may explain this finding. Indeed, methionine, betaine aldehyde and formate; all three metabolites significant in DNA methylation were up-regulated. However, up-regulation of 1-methylnicotinamide in this HCC cohort suggests overexpression of nicotinamide-N-methyltransferase (NNMT) and thus impairment of DNA methylation. This phenomenon has been confirmed by a metabolomic study (Ulanovskaya *et al*., 2013) and may give rise to hypomethylated histones and other cancer-related proteins. Hypomethylation has been well-described as a cause of chromosomal instability in particularly, non-HCV associated HCC tissues (Nishida *et al*., 2013) and in those patient groups exposed to aflatoxin B1 (Zhang *et al*., 2012). Methylation studies of tissues will be helpful to uncover the occurrence of this phenomenon, especially as HBV formed the most prevalent associated aetiological agent of HCC in this aflatoxin-prone study population.

Dimethylglycine (DMG), choline and TMAO, metabolites involved in choline intermediary metabolism were altered in the urine of HCC patients. Whereas DMG

and choline were up-regulated, there was a down-regulation of TMAO. These alterations corroborate the findings of published data in which choline metabolism was identified to play a significant role in malignant transformation of tissues (Glunde *et al*., 2011). The role of choline metabolism has been confirmed in tissues from patients, not only with liver cancer (Bell and Bhakoo, 1998), but also brain, breast, lung, ovarian and prostatic cancers (Glunde, Bhujwalla, & Ronen, 2011). Excess choline metabolites in urine of HCC patients thus indicate downstream changes in which genetic or epigenetic changes in HCC tissues would have led to what is observed in the present study, and could serve as a possible target of therapy. Current data support the hypothesis that altered metabolism of cancer results from active reprogramming by growth factor regulators such as oncogenes and tumour suppressors (Ward & Thompson, 2012), and which drives the process of cancer development. There is thus a significant paradigm shift from the former hypothesis that altered metabolism in cancer are a consequence of carcinogenesis (Costello and Franklin, 2012).

While creatine and methylguanidine were up-regulated in HCC, creatinine was significantly lower in HCC patients and maintained a significant negative correlation in both preliminary and validation sets of patients. Creatinine is converted to creatine reversibly by the enzyme, creatine kinase; and non-enzymatically to methylguanidine. Whereas creatine, chiefly produced in the liver from arginine, methionine and glycine, is known for its role in the energy shuttle, methylguanidine has been shown to inhibit inducible nitric oxide synthase (iNOS) and tumour necrosis factor alpha (TNF-α) release (Autore *et al*., 1999) both *in vitro* and *in vivo*. Not much is in the literature to implicate the role of methylguanidine in tumour development.

However, the immune deregulations associated with methylguanidine on TNF-α synthesis and release, may be important in promoting carcinogenesis via reduced immune surveillance.

N-acetylglutamate is up-regulated in HCC as well as correlates significantly with tumour stage. Glutamate is a non-essential amino acid and would have been produced through the action of glutaminase enzyme on glutamine. The elevated level of this amino acid in HCC patients may thus suggest enhanced glutaminolysis in HCC subjects relative to controls in this study. Glutamate has been identified as an important metabolite that contributes to triggering of cell growth and inhibition of autophagy (Duran *et al*., 2012) and thus equip tumour cells to evade apoptosis. Additionally, cancer cells solely rely on glutamine as a carbon source for the TCA cycle (DeBerardinis *et al*., 2007). The finding of up-regulation of 2-oxoglutarate and succinate in the present work confirms this phenomenon. Also, the finding of down-regulation of glutamine (chapter 10) in HCC suggests increased utilisation by the HCC tissues. This finding has implications for anti-metabolite drug developments for HCC, and which is being pursued for other cancers currently (Zhao *et al*., 2013).

The metabolites that discriminated HCC from controls in this work have thus confirmed the preliminary data and highlight several pathways that are altered in tumour metabolism. The fact that most of these metabolite alterations have previously been identified in tumours other than HCC suggest that the metabolites alterations identified may not possess specificity to HCC. Be that as it may, within the at-risk population of liver disease, most especially, HBV population of Africa, a combination of the metabolites could be helpful in screening patients for HCC. Immediate application of this approach will however, be hampered by a number of

limitations. First, most of the HCC patients recruited in the study population had intermediate or advanced stage of disease. It would be more beneficial to diagnose HCC at stages during which cure is achievable. However, as the health-care seeking behaviour of the current study population is such that most patients present when illnesses are advanced, on-going work by the PROLIFICA programme has been able to sensitise the populace and may lead to recruitment of early stage patients. Indeed the metabolic profiling result of the UK HCC population (chapter 9) is a proof of the principle that detection of early stage disease using urinary metabolites is practicable.

Second, the influence of diet, drugs and exercise on urinary metabolite excretion could significantly affect the interpretation of the current data. In order to control for this, detailed dietary, exercise and drug questionnaires were administered to consenting patients and volunteers before samples were obtained. Recall of information such as historical facts may otherwise be distorted in patients that were recruited. Some of the patients had hepatic encephalopathy, a condition that may be associated with amnesia. This was obviated by confirming historical facts from carers of the patients.

Also, advanced HCC is often complicated by cancer cachexia with associated sarcopenia. Urinary creatinine concentration has been recognised as a biomarker of sarcopenia (Pahor *et al*., 2009). Although urinary creatinine excretion is directly proportional to total body muscle mass, its determination is a Herculean task, often requiring strict dietary restrictions. This was controlled for by normalising the obtained relative concentrations of the metabolites to creatinine spectral peak.

Further verification and discovery of additional urinary metabolites by mass spectrometry and identification of a combination of metabolites vital to diagnosis of small HCCs would be the most rationale direction of future research. Most of the metabolites discovered in the experiment have been identified in several other tumours remote to the liver. Comparison of metabolite differences between HCC and secondary tumours to the liver, as well as primary cancers other than HCC deserves additional work. All the urinary metabonomic studies reported in this dissertation involved a global approach. Targeted metabonomic studies will seek to identify, quantify and determine the diagnostic performance of single or a combination of key metabolites. Several hypotheses of altered pathways that have yet to be established will form another direction of future study. For example, the involvement of gut flora metabolism in the evolution of HCC deserves further studies. The concept of gene-environment interaction in the aetiology of diseases has recently been suggested by Heinzmann and colleagues (Heinzmann *et al*., 2012). Several tryptophan metabolites, including: indole-3-acetate, indoxyl sulphate, kynurenic acid and 5-hydroxytryptamine were identified in the present work. Studies linking up the mechanistic development of HCC, involving genetic approaches would pave way to individualised anti-HCC drug options.

12. SUMMARY AND CONCLUSIONS

12.0 Background

PLC is the 5th most commonly diagnosed cancer and 3rd cause of cancer-related deaths globally. Although less common than in the developing countries, an exponential increase in the mortality from PLC in England and Wales was reported towards the end of the last century. Lifestyle and temporal changes have warranted close monitoring of the trends in the mortality, incidence, modes of diagnosis and ethnic distribution of this fatal cancer. This was, therefore, one of the objectives of my thesis.

The absence of good quality cancer database in high incident countries such as Africa limited the appraisal of the current epidemiology of PLC in this region of the world. However, the availability of resources involved in HIV research allowed me to perform some detailed examination of liver-related morbidities in a Nigerian HIV cohort. Thus, it was possible to determine the rates and impact of PLC risk factors (HBV and HCV) in the HIV population of that cohort.

The high case fatality associated with HCC engendered biomarker discovery studies using samples from two West African countries (Nigeria and Gambia), which I undertook as a spin-off of the PROLIFICA study. The poor diagnostic accuracy of serum AFP and the need to further simplify identification of HCC in resource-limited regions, as well as proliferation of new techniques (metabonomics) created opportunities for urine to be explored for delineation of HCC biomarkers.

12.1 Research questions

There are insufficient data on the most important factors responsible for rising rates of PLC and subtypes. Until my study, the question of whether changes in diagnosis, ICD classification and immigration are impacting on the increasing rates of PLC, particularly in England and Wales had not been investigated.

The incidence of HCC has been reported to have risen from 0.2 to 2.8 cases per 1000 person-years between 2000 and 2009 in HIV-infected patients in Spain (Merchante *et al*., 2013). Hitherto, studies of liver-related morbidities in HIV populations in African communities have been limited by small sample sizes and relatively shorter duration of follow up compared to data from Western countries. My study in Africa aimed to obviate the shortcomings in the published literature.

Previous pilot studies utilising urinary metabonomics have identified several metabolites that are perturbed in HCC patients, yet these remain to be validated in large cohorts; and particularly African patient groups, something which I have now addressed in this thesis. I have assessed how these metabolites perform diagnostically and have provided pointers as to how these compare in relation to tumour size. I have postulated how these techniques could provide in-roads into point-of-care diagnostic and drug development efforts for HCC, as well as complementing pathogenesis information from genetic and epigenetic research efforts.

12.2.1 Epidemiological study (PLC)

I have reported for the first time, how errors in ICD coding of tumours and changes in the mode of diagnosis of PLC could be contributing to the changing trends in the incidence of PLC subtypes in England and Wales. Estimation of survival, using MIR calculations provided information on the outlook of PLC in the immediate term. Studying the ethnic distribution of PLC, data were obtained from national registries, which include all incident cases and are thus much more representative, compared to previous data. Although a significant proportion of the registered cases were found not to have information on basis of diagnosis and ethnic background, the findings highlight information that would be relevant for follow up studies.

12.2.2 Epidemiology study (Liver-related morbidity in HIV)

In the present study, I characterised the HIV-infected patients, based on hepatitis status, comparing them with a HIV mono-infected cohort and have shown that HBV, in particular, was associated with attenuated CD4 cell increase following long-term ART. Additionally, HBsAg serological status was identified as a significant factor in the mortality of HIV-infected patients that were on ART, an effect that was annulled in those co-infected patients (HBV/HIV), who were on an ART regimen, in which Tenofovir was a component.

12.2.3 HCC biomarker discovery

It is to be noted that whereas most diagnostic performances of biomarkers identified in previous studies were described between cancer patients and healthy controls, this work additionally compared the diagnostic accuracy of identified markers between HCC and at-risk populations (cirrhosis and non-cirrhosis liver disease). As

renal function and sarcopenia could alter the nature of excreted metabolites in the urine, normalisation to creatinine peaks was embarked upon prior to univariate analyses.

Almost all the urinary metabolites that were described in the preliminary work were confirmed to have similar alterations in the validation set in which 141 HCC subjects were studied. The complex interactions of pathways that were discovered to be altered in the present study were mapped using the Kyoto Encyclopaedia of Genes and Genomes (KEGG) database. Fatty acid metabolism, ketone bodies, choline metabolism, glutaminolysis, tricarboxylic acid cycle, DNA methylation and gut metabolism pathway alterations were identified. UPLC-MS and tandem mass spectrometry coupled with multivariate analyses identified single and panels of metabolites that diagnostically outperformed serum AFP in the discrimination of HCC in the studied population.

12.3 Applications

12.3.1 Changes in epidemiology of PLC and subtypes

The findings of increasing incidence and mortality rates of PLC, and particularly, the IHBD subtype, despite ICD coding errors, provide a number of important pointers for future healthcare provision. The predilection of Afro-Caribbean ethnicity, with the projected overall worsening outcomes of PLC, carries several implications. First, efforts need to be intensified regarding control of risk factors that may be contributing to the rising incidence of PLC, as well as the development of surveillance tools aimed at early diagnosis, particularly targeted in migrant communities, in order to provide curative treatments. Second, cancer registries need to be unambiguous in their data collation, regarding the registration of diseases, particularly with reference

to ethnicity. Whenever there is an epidemiological debate, such as in the case of whether hilar CC should be referenced to C22.1 or C22.4, there should be immediate multi-disciplinary team intervention to address this. The fact that ethnic information registration in the England and Wales cancer registry was available in only a 3rd of the cases but increasing suggest that in the future, it must be possible to describe the distribution of PLC by ethnicity, more accurately. The latter will provide useful information that shall inform clinicians to provide targeted surveillance of at-risk population groups.

12.3.2 Impact of HBV in HIV-infected patients in an African cohort

With such a high and rising rate of hepatitis infection in HIV-infected patient cohort in a representative African population, there is obvious need to intensify efforts at ensuring that the recommended hepatitis screening pre-ART is adhered to by implementing partners. Tenofovir, a nucleotide analogue, with therapeutic effect on both HIV and HBV, has become available in the past few years and is recommended for use by expert panels, such as AASLD and EASL. The present study fortifies the significance of those recommendations, particularly in resource-limited settings. It would be sensible to recommend that HIV/HBV co-infected patients embarking on treatment are commenced on a Tenofovir-containing regimen, unless otherwise contraindicated. Nevertheless, HIV patients who are co-infected with hepatitis require close monitoring and follow up, and this may flag-up additional cases that may require earlier interventions with respect to the reduction of liver-related morbidities.

12.3.3 HCC urinary biomarker development

As an extension of my work, future diagnostic biomarker and drug developments are possible applications of my urinary metabonomics research. For example, a diagnostic screening and surveillance panel could improve case ascertainment of HCC in patients with cirrhosis. This is particularly so in African patients where AFP is still being relied upon for diagnosis. The present data highlighted the superior performance of urinary metabolite batteries (using both NMR and UPLC-MS) over serum AFP. A urinary diagnostic panel would be readily available and applicable to large populations and thus amenable to community surveys. Future work on larger cohorts around the world is needed to validate my findings in Africa and the UK.

Also, the findings from this work have further highlighted pathogenic alterations in HCC. Whether these pathways represent a milieu required for, or are consequent upon carcinogenesis are subjects deserving further studies. They, however, complement genetic and epigenetic data and could be valuable in drug development for HCC.

12.4 Future directions

12.4.1 Changes in epidemiology of PLC

Differential patterns of the increase in the incidence and mortality rates of HCC and IHBD, in which females were more affected than males with regard to IHBD in England and Wales deserve further study. It is uncertain whether oral contraceptive pills have any role to play aetiopathologically. Indeed, existing data suggest otherwise (Lam *et al*., 2005), although better designed studies will yield more meaningful results. Second, the use of ethnic information to implicate immigration in the current trends may be misleading and should form the subject for further studies.

Studies involving stratification of immigration patterns, such as first and second generation immigrant status will provide more meaningful results than ethnicity status, as environmental factors are also important in disease processes. The risk factors in the pathogenesis of IHBD, particularly, in England and Wales will constitute a worthwhile study area in the future; as effective control measures will rest on such findings. The impact of guidelines, adherence to surveillance schedules and tumour stage at diagnosis on the survival of patients with PLC will need further studies. A search for better performing screening and diagnostic tools should be a priority study subject, as the poor survival forecasted by the current trends suggests that available tools have suboptimal diagnostic accuracy.

12.4.2 Liver-related morbidities in HIV in African patients

Prior to availability of free ARVs, most HIV-infected patients in sub Saharan African countries died of AIDS. Most AIDS–related deaths have been on the decline (Weber *et al*., 2006), but the incidence of HCC among HIV-infected patients has been reported to be increasing. However, reports of the relatively higher incidence of HCC in HIV patients, compared to non-HIV patients have been absent from this population, although, they are reported to be so in developed countries of the world. Well-designed cross-sectional studies in which HIV/HBV patients form the cases, while HBV patient groups constitute the controls will help to answer the issues of increasing incidence of HCC in the ART era. Characterisation of HCC cases was noted to be poor in the present data and any prospective study should yield higher quality data if suspected cases of HCC are investigated according to international guidelines (EASL/AASLD).

12.4.3 HCC biomarker discovery

The ultimate goal of the current endeavour is to develop a point-of-care test that would be accessible, affordable and simple, such as a urinary dip-stick for use in the field. To be able to translate my research findings into such a reality,, there needs to be external validation of altered metabolites, in particular, in small HCCs. Also, corroboration of the pattern of alteration in blood, as well as liver tissues, would provide unequivocal evidence of the implicated metabolites. Patients recruited on to the PROLIFICA studies are being followed up in this regard. Those with cirrhosis and those who may develop HCC will have their initial urine samples compared to the samples that will be collected at the time that HCC diagnosis would be confirmed.

Most of the metabolites discovered in my work have been described in tumours other than HCC and these may therefore represent carcinogenesis, rather than specificity to HCC. Studies comparing metabolic profiles of non-HCC tumours (such as tumours of the ovary, prostate, breast, colon and metastasis to the liver) with HCC would be valuable in this regard. The pathways such as glutaminolysis, gut flora metabolism, beta-oxidation, DNA methylation and others, which were inferred to be altered from the present findings, will need further elucidation, as they may hold key to further understand pathogenesis of HCC.

13. PUBLICATIONS AND PATENT

Publications, book chapters and filed patent by me that led to, and or arising from the work being presented in this thesis; as published before December 2013 are listed below:

13.1 Original articles

Shariff, M.I.F., **Ladep, N.G**., Cox, I.J., Williams, H.R.T., Okeke, E., Malu, A., Thillainayagam, A.V., Crossey, M.M., Khan, S.A., & Taylor-Robinson, S.D. 2010. Characterization of urinary biomarkers of hepatocellular carcinoma using magnetic resonance spectroscopy in a Nigerian population. *J. Proteome. Res.,* 9, (2) 1096-1103.

Khan, S.A., Emmadossadaty, S., **Ladep, N.G**., Thomas, H.C., Elliot, P., Taylor-Robinson, S.D., & Toledano, M.B. 2012. Rising trends in cholangiocarcinoma: Is the ICD classification system misleading us? *J. Hepatol.* 56 (4), 848-854.

Ladep, N.G., Agaba, P.A., Agbaji, O., Muazu, A., Ugoagwu, P., Imade, G., Cooke, G., McCormack, S., Taylor-Robinson, S.D., Idoko, J., & Kanki, P. 2013. Rates and impact of hepatitis on human immunodeficiency virus infection in a large African cohort. *WJG*. 19, 1602-1610.

Ladep, N.G., Agbaji, O., Agaba, P.A., Muazu, A., Ugoagwu, P., Imade, G., Cooke, G., Vivas, L., McCormack, S., Taylor-Robinson, S.D., Idoko, J., & Kanki, P. 2013. Hepatitis B co-infection is associated with poorer survival of HIV-infected patients on highly active antiretroviral therapy in West Africa. *J AIDS Clinic Res*. S3: 006. Doi:10.4172/2155-6113.S3-006.

Ladep, N.G., Khan, S.A., Crossey, M.E., Thillainayagam, A.V., Taylor-Robinson, S.D. & Toledano, M.B. 2013. Incidence and Mortality of Primary Liver Cancer in England and Wales: Changing Patterns and Ethnic Variations. *WJG* (accepted for publication).

13.2 Published abstracts

Ladep, N., Shariff, M., Crossey, M., Okeke, E., Malu, A., Williams, H., Khan, S., Cox, J., & Taylor-Robinson, S.D. Urinary metabonomic study of hepatocellular carcinoma in a Nigerian population using proton magnetic resonance spectroscopy. *J. Hepatol.* 2009; 50, S299.

Ladep, N.G., Khan, S.A., Thillainayagam, A.V., Taylor-Robinson, S.D., & Toledano, M.B. Mortality rates of primary liver cancer are continuing to increase globally. *J. Hepatol.* 2012; 56, S287.

Shariff, M.I.F., Lewis, M.R., Want, E.J., Keun, H.C., Mohamed, F., Jalan, R., **Ladep, N.G**., Crossey, M.M., Khan, S.A., Holmes, E., & Taylor-Robinson, S.D. Blood lipidomic profiling of hepatocellular carcinoma in human and animal studies identifies lysophosphatidylcholine (24,0,0), a discriminatory biomarker. *Gut.* 2012; 61, A405-A405.

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Ladep, N.G., Dona, A., McPhail, M.J.W., Shariff, M.I., Crossey, M.M., Wijeyesekera, A., Okeke, E.N., Banwat, E., Lemoine, M., Njie, R., Garside, D., Khan, S.A., Thillainayagam, A.V., Thursz, M., Holmes, E.C., & Taylor-Robinson, S.D. Urinary

metabolic profile discriminates hepatocellular carcinoma better than serum Alpha fetoprotein in West Africans. J. Hepatol. 2013; 58, S49.

13.3 Book chapter

Ladep, N.G. Why is the tumour different in Africa? Clinical Dilemmas in Primary Liver Cancer. Wiley Publishing, December 2011.

13.4 Filed patent

Ladep, N.G., Dona, A., Holmes, E., & Taylor-Robinson, S.D. 2012. Urinary markers of Hepatocellular Carcinoma. 1307256.6

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Appendix A: Number of deaths from primary liver cancer (PLC) by subcategories (HCC, IHBD and Liver NOS) in England and Wales, 1968-2008

Year	PLC			HCC			IHBD			LIVER NOS		
	M	F	TOTAL	M	F	TOTAL	M	F	TOTAL	M	F	TOTAL
1968	571	396	967	292	180	472	21	17	38	258	199	457
1969	603	458	1061	282	180	462	20	30	50	301	248	549
1970	587	451	1038	276	147	423	35	22	57	276	282	558
1971	551	443	994	271	158	429	23	16	39	257	269	526
1972	599	424	1023	310	163	473	22	23	45	267	238	505
1973	637	478	1115	313	184	497	27	25	52	297	269	566
1974	611	449	1060	311	196	507	21	34	55	279	219	498
1975	589	418	1007	312	181	493	40	30	70	237	207	444
1976	629	433	1062	319	156	475	36	19	55	274	258	532
1977	625	453	1078	309	180	489	53	38	91	263	235	498
1978	606	398	1004	332	151	483	48	47	95	226	200	426
1979	583	336	919	434	208	642	54	66	120	95	62	157
1980	522	376	898	380	227	607	78	74	152	64	75	139
1981	628	419	1047	430	220	650	78	89	167	120	110	230
1982	597	463	1060	341	230	571	91	101	192	165	132	297
1983	594	396	990	409	231	640	127	123	250	58	42	100
1984	713	466	1179	479	231	710	148	148	296	86	87	173
1985	680	499	1179	425	220	645	145	171	316	110	108	218
1986	750	514	1264	456	218	674	183	206	389	111	90	201
1987	726	526	1252	437	229	666	185	206	391	104	91	195
1988	837	583	1420	467	207	674	227	242	469	143	134	277
1989	802	576	1378	465	220	685	239	248	487	107	108	215
1990	818	570	1388	458	207	665	231	252	483	129	111	240
1991	879	645	1524	501	242	743	269	301	570	109	102	211
1992	919	684	1603	528	223	751	285	372	657	106	89	195
1993	970	735	1705	348	142	490	329	355	684	293	238	531
1994	983	781	1764	374	132	506	335	405	740	274	244	518
1995	999	761	1760	366	142	508	347	402	749	286	217	503
1996	1052	770	1822	416	164	580	349	387	736	287	219	506
1997	1133	848	1981	459	156	615	414	473	887	260	219	479
1998	1118	803	1921	462	158	620	402	463	865	254	182	436
1999	1134	818	1952	491	205	696	415	451	866	228	162	390
2000	1209	849	2058	559	159	718	432	504	936	218	186	404

2001	1160	868	2028	465	164	629	456	542	998	239	162	401
2002	1223	917	2140	555	193	748	444	549	993	224	175	399
2003	1342	853	2195	622	165	787	489	543	1032	231	145	376
2004	1356	941	2297	671	197	868	509	609	1118	176	135	311
2005	1461	994	2455	716	186	902	556	680	1236	189	128	317
2006	1619	1041	2660	785	208	993	623	710	1333	210	122	332
2007	1615	1100	2715	802	240	1042	610	758	1368	200	101	301
2008	1731	1178	2909	856	238	1094	673	809	1482	200	130	330

Appendix B: Number of cases of primary liver cancer (PLC) by subcategories (HCC, IHBD and Liver NOS) in England and Wales, 1979-2008

Year	PLC			HCC			IHBD			LIVER NOS		
	M	F	TOTAL	M	F	TOTAL	M	F	TOTAL	M	F	TOTAL
1979	608	350	958	519	281	800	32	38	70	57	33	90
1980	580	415	995	471	296	767	42	42	84	67	77	144
1981	639	407	1046	509	304	813	57	61	118	73	42	115
1982	607	408	1015	476	315	791	62	53	115	69	40	109
1983	627	414	1041	524	310	834	70	68	138	33	36	69
1984	621	433	1054	524	319	843	57	76	133	40	38	78
1985	692	467	1159	592	367	959	60	59	119	40	41	81
1986	669	421	1090	571	333	904	50	60	110	48	28	76
1987	740	512	1252	634	378	1012	68	95	163	38	39	77
1988	778	501	1279	627	366	993	89	92	181	62	43	105
1989	739	485	1224	594	341	935	78	93	171	67	51	118
1990	778	471	1249	611	323	934	115	111	226	52	37	89
1991	818	553	1371	674	365	1039	100	127	227	44	61	105
1992	920	574	1494	731	371	1102	142	165	307	47	38	85
1993	913	591	1504	711	376	1087	150	179	329	52	36	88
1994	949	640	1589	719	395	1114	168	189	357	62	56	118
1995	1003	685	1688	536	280	816	290	294	584	177	111	288
1996	1127	806	1933	605	321	926	305	357	662	217	128	345
1997	1182	864	2046	668	323	991	355	413	768	159	128	287
1998	1250	864	2114	722	342	1064	362	413	775	166	109	275
1999	1281	824	2105	719	315	1034	367	385	752	195	124	319
2000	1408	925	2333	803	306	1109	420	504	924	185	115	300
2001	1347	965	2312	774	371	1145	437	473	910	136	121	257
2002	1469	968	2437	877	355	1232	441	506	947	151	107	258
2003	1465	883	2348	848	315	1163	464	474	938	153	94	247
2004	1502	996	2498	869	334	1203	489	573	1062	144	89	233
2005	1757	1101	2858	1030	368	1398	559	592	1151	131	107	238
2006	1854	1107	2961	1069	309	1378	608	676	1284	157	94	251
2007	1916	1176	3092	1140	366	1506	557	691	1248	170	93	263
2008	1996	1165	3161	1083	306	1389	639	672	1311	196	138	334

Appendix C: Cases of Hepatocellular carcinoma (HCC) in whom ethnicity was registered in England and Wales, 1993-2008 (percentages in parentheses)

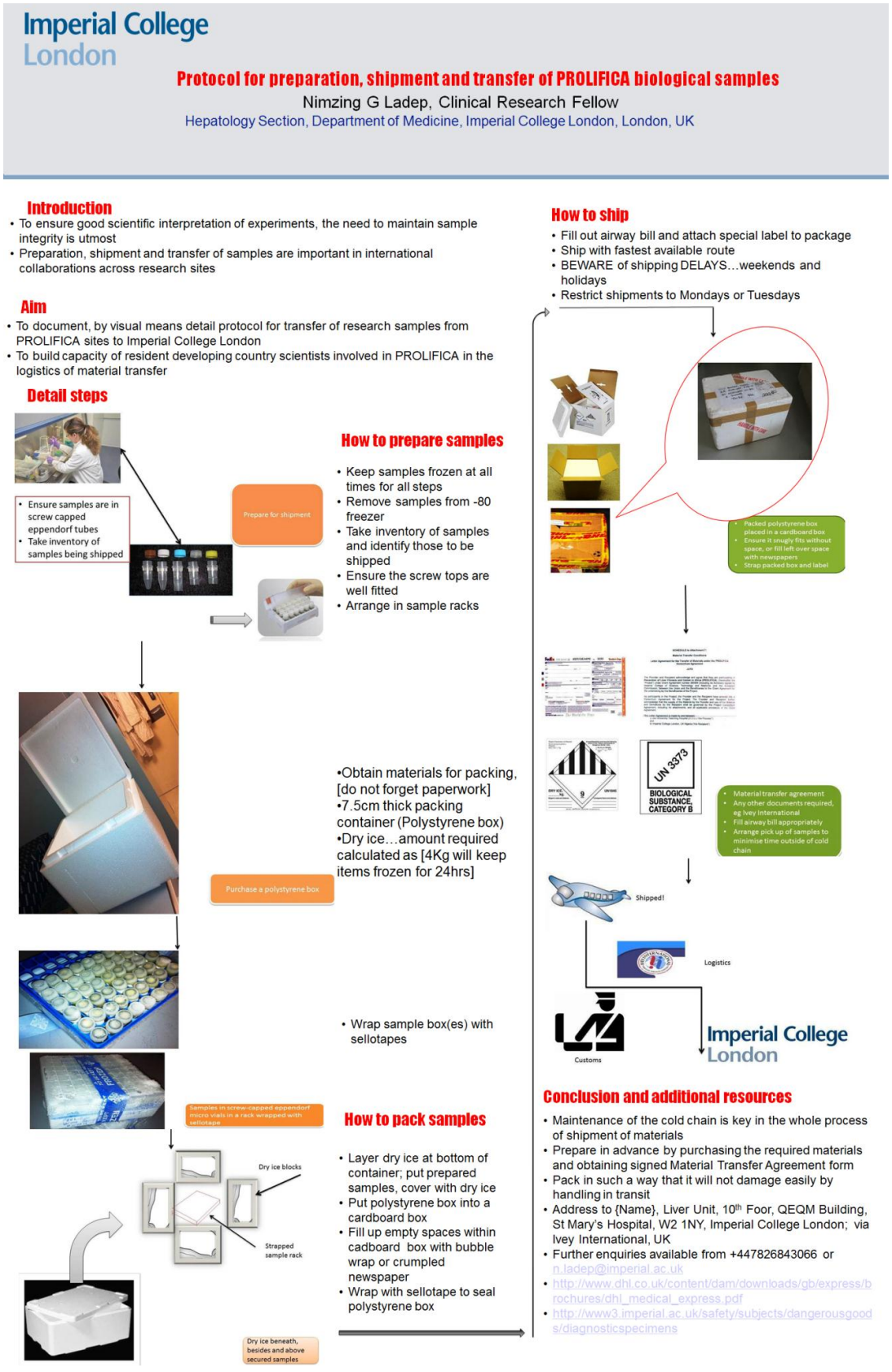
Year	Hepatocellular carcinoma						
	All HCC	Whites	Afro- Caribbeans	Black Africans	South Asians	Chinese	Others
1993	362(34.2)	355(98.1)	1(0.3)	0(0.0)	6(1.7)	0(0.0)	0(0.0)
1994	355(32.5)	346(97.5)	1(0.3)	1(0.3)	4(1.1)	0(0.0)	3(0.8)
1995	243(30.9)	229(94.2)	2(0.8)	2(0.8)	3(1.2)	4(1.6)	3(1.2)
1996	409(44.6)	390(95.4)	1(0.2)	2(0.5)	6(1.5)	2(0.5)	8(2.0)
1997	430(46.7)	410(95.3)	1(0.2)	4(0.9)	8(1.9)	5(1.2)	2(0.5)
1998	493(50.3)	462(93.7)	3(0.6)	7(1.5)	9(1.8)	5(1.0)	7(1.4)
1999	445(46.0)	412(92.6)	4(0.9)	2(0.4)	12(2.7)	5(1.1)	10(2.2)
2000	440(41.5)	396(90.0)	12(2.7)	8(1.8)	12(2.7)	3(0.7)	9(2.0)
2001	218(19.7)	167(76.6)	20(9.2)	5(2.3)	10(4.6)	6(2.8)	10(4.6)
2002	265(22.0)	220(83.0)	15(5.7)	7(2.6)	11(4.2)	7(2.6)	5(1.9)
2003	249(21.4)	196(78.7)	14(5.6)	7(2.8)	18(7.2)	3(1.2)	11(4.4)
2004	342(28.2)	275(80.4)	17(5.0)	16(4.7)	13(3.8)	6(1.8)	15(4.4)
2005	466(33.3)	388(83.3)	31(6.7)	14(3.0)	17(3.6)	6(1.3)	10(2.1)
2006	543(39.4)	421(77.5)	62(11.4)	10(1.8)	28(5.2)	10(1.8)	12(2.2)
2007	637(42.3)	492(77.2)	64(10.0)	13(2.0)	31(4.9)	15(2.4)	22(3.5)
2008	579(41.7)	493(85.1)	16(2.8)	12(2.1)	22(3.8)	10(1.7)	26(4.5)

Appendix D: Cases of Intrahepatic Bile Duct carcinoma (IHBD) in whom ethnicity information was available in England and Wales, 1993-2008 (percentages in parentheses)

Year	Intrahepatic bile duct tumours						
	All IHBD	Whites	Afro-Caribbeans	Black Africans	South Asians	Chinese	Others
1993	156(47.7)	155(99.4)	0(0.0)	0(0.0)	1(0.6)	0(0.0)	0(0.0)
1994	166(45.9)	166(100.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)
1995	289(48.2)	287(99.3)	0(0.0)	0(0.0)	2(0.7)	0(0.0)	0(0.0)
1996	378(56.6)	375(99.2)	2(0.5)	0(0.0)	0(0.0)	1(0.3)	0(0.0)
1997	447(58.1)	441(98.7)	0(0.0)	0(0.0)	3(0.7)	2(0.4)	1(0.2)
1998	463(59.7)	458(98.9)	2(0.4)	0(0.0)	3(0.6)	0(0.0)	0(0.0)
1999	412(54.6)	401(97.3)	6(1.5)	0(0.0)	2(0.5)	2(0.5)	1(0.2)
2000	444(48.1)	428(96.4)	12(2.7)	0(0.0)	3(0.7)	1(0.2)	0(0.0)
2001	174(18.9)	152(87.4)	13(7.5)	3(1.7)	1(0.6)	1(0.6)	4(2.3)
2002	211(21.8)	188(89.1)	8(3.8)	1(0.5)	7(3.3)	2(0.9)	5(2.4)
2003	205(20.7)	189(92.2)	6(2.9)	3(1.5)	5(2.4)	0(0.0)	2(1.0)
2004	323(29.2)	296(91.6)	13(4.0)	2(0.6)	7(2.2)	0(0.0)	5(1.5)
2005	370(32.1)	331(89.5)	23(6.2)	1(0.3)	8(2.2)	2(0.5)	5(1.4)
2006	487(37.9)	426(87.7)	29(6.0)	3(0.6)	16(3.3)	0(0.0)	13(2.7)
2007	526(42.1)	463(88.0)	43(8.2)	1(0.2)	9(1.7)	3(0.6)	7(1.3)
2008	536(40.9)	492(91.8)	25(4.7)	0(0.0)	11(2.1)	1(0.2)	7(1.3)

Appendix E: Cases of Liver tumours, not otherwise specified (NOS) in whom ethnicity information was available in England and Wales, 1993-2008 (percentages in parentheses)

Year	Liver NOS						
	All NOS	Whites	Afro- Caribbeans	Black Africans	South Asians	Chinese	Others
1993	45(52.3)	44(97.8)	1(2.2)	0(0.0)	0(0.0)	0(0.0)	0(0.0)
1994	49(43.4)	49(100.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)
1995	64(28.4)	63(98.4)	0(0.0)	0(0.0)	1(1.6)	0(0.0)	0(0.0)
1996	85(32.1)	83(97.6)	0(0.0)	1(1.2)	0(0.0)	1(1.2)	0(0.0)
1997	87(31.6)	87(100.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)
1998	86(33.1)	84(97.7)	0(0.0)	0(0.0)	1(1.2)	0(0.0)	1(1.2)
1999	128(41.0)	126(98.4)	0(0.0)	0(0.0)	0(0.0)	1(0.8)	1(0.8)
2000	81(28.2)	77(95.1)	0(0.0)	0(0.0)	2(2.5)	1(1.2)	1(1.2)
2001	43(18.0)	38(88.4)	2(4.7)	0(0.0)	0(0.0)	0(0.0)	3(7.0)
2002	45(17.8)	45(100.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)
2003	50(20.6)	48(96.0)	0(0.0)	0(0.0)	2(4.0)	0(0.0)	0(0.0)
2004	68(29.8)	68(100.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)
2005	56(23.5)	52(92.9)	1(1.8)	2(3.6)	0(0.0)	0(0.0)	1(1.8)
2006	114(45.4)	108(94.7)	4(3.5)	0(0.0)	2(1.8)	0(0.0)	0(0.0)
2007	96(36.5)	88(91.7)	3(3.1)	0(0.0)	1(1.0)	2(2.1)	2(2.1)
2008	129(38.6)	125(96.9)	1(0.8)	0(0.0)	1(0.8)	0(0.0)	2(1.6)



Appendix F. Visual protocol for collection, preparation, packaging and transportation of samples from Africa used in the analyses