The Plasma and Serum Metabotyping of Hepatocellular Carcinoma in a Nigerian and Egyptian Cohort using Proton Nuclear Magnetic Resonance Spectroscopy

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Background/Aims: **Previous studies have observed disturbances in the ^{1}H nuclear magnetic resonance (NMR) blood spectral profiles in malignancy. No study has metabotyped serum or plasma of hepatocellular carcinoma (HCC) patients from two diverse populations. We aimed to delineate the HCC patient metabotype from Nigeria (mostly hepatitis B virus infected) and Egypt (mostly hepatitis C virus infected) to explore lipid and energy metabolite alterations that may be independent of disease aetiology, diet and environment.** ***Methods:*** **Patients with HCC (53) and cirrhosis (26) and healthy volunteers (19) were recruited from Nigeria and Egypt. Participants provided serum or plasma samples, which were analysed using 600 MHz ^{1}H NMR spectroscopy with nuclear Overhauser enhancement spectroscopy pulse sequences. Median group spectra comparison and multivariate analysis were performed to identify regions of difference.** ***Results:*** **Significant differences between HCC patients and healthy volunteers were detected in levels of low density lipoprotein ($P = 0.002$), very low density lipoprotein ($P < 0.001$) and lactate ($P = 0.03$). *N*-acetylglycoproteins levels in HCC patients were significantly different from both healthy controls and cirrhosis patients ($P < 0.001$ and 0.001).** ***Conclusion:*** **Metabotype differences were present, pointing to disturbed lipid metabolism and a switch from glycolysis to alternative energy metabolites with malignancy, which supports the Warburg hypothesis of tumour metabolism.** (J Clin Exp Hepatol 2017;7:83–92)

Hepatocellular carcinoma (HCC) is the second commonest cause of cancer-related death and bears a poor prognosis in developing countries due to late diagnosis.[1–3] Curative treatment options, namely orthotopic liver transplantation and surgical resection, are limited to low-grade cancers that are identified early.[4] The widely accepted HCC screening using serum alpha-fetoprotein (AFP), a foetal glycoprotein, has shown evidence of improvement in mortality and morbidity.[5] Although most HCC tumours secrete AFP, the tumour marker has poor sensitivity and specificity of less than 70%.[6–8] Furthermore, serum AFP testing is unavailable in many parts of Africa, where HCC is most prevalent.

"Metabonomics" is the study of global metabolic responses to physiological, drug and disease stimuli. The most commonly used method of metabolite characterisation is proton nuclear magnetic resonance (^{1}H NMR) spectroscopy.[9] There is a paucity of data concerning the value of blood profiling using ^{1}H NMR in HCC, but previous studies have identified a number of altered metabolites, implicating changes in hepatic function, lipid metabolism and bile acid metabolism.[10–13] Heterogeneity in genotype, diet, environment, co-morbid status and liver disease aetiological factors in man, may influence the ability to translate these findings to human disease.[14]

Keywords: hepatocellular carcinoma, Nigeria, Egypt, proton nuclear magnetic resonance spectroscopy, serum metabotype

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Abbreviations: ^{1}H NMR: proton nuclear magnetic resonance; HCC: Hepatocellular carcinoma; HBV: Hepatitis B virus; HCV: Hepatitis C virus; NOESY: Nuclear Overhauser enhancement spectroscopy; LDL: Low density lipoprotein; JUTH: Jos University Teaching Hospital; US: Ultrasonography; CT: Computed Tomography; MRI: Magnetic resonance imaging; WHO: World Health Organisation; EDTA: Ethylenediaminetetraacetic acid; ALT: Alanine transaminase; ALP: Alkaline phosphatase; AFP: α-fetoprotein; IQR: Interquartile ranges; 1-D: One-dimensional; RD: Relaxation delay; t_m: Mixing time; FID: Free induction decays; PCA: Principal components analysis; PLS-DA: Partial least squared discriminant analysis; HBsAg: Hepatitis B surface antigen; ELISA: Enzyme-linked immunosorbent assay; VLDL: Very low density lipoprotein; ppm: Parts per million; PC: Principal component; PPARα: Peroxisome proliferator-activated receptor α; IDL: Intermediate density lipoprotein

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One previous study, performed in a Chinese population utilised ^{1}H NMR of serum to discriminate patients with HCC (*n* = 39) from patients with cirrhosis (*n* = 36).[15] In this study, alterations were observed in levels of lipoproteins, amino acids, *N*-acetylglycoproteins, ketoacids and lipids. Unfortunately, no information was provided on age, gender or liver disease aetiology of the participants, which is particularly relevant when utilising this method to distinguish patients with cancer to those without. In 1986, Fossel and colleagues proposed using the line widths of methyl (CH_3) and methylene ($(CH_2)_n$), measured by 400 MHz ^{1}H NMR spectroscopy, as a sensitive test for cancer.[16] Levels of these metabolites were found to be significantly elevated in patients with a variety of tumours (*n* = 81). A number of validation studies performed on similar cohorts of patients using similar or higher magnetic field strengths, refuted this finding, citing age, triglyceride content and number of freeze thaw cycles as confounding variables that were likely to have contributed to Fossel's original findings.[17–20]

We have previously found discriminatory metabolites for HCC using urinary metabolic profiling with ^{1}H NMR spectroscopy in Nigerian, Egyptian and Gambian populations.[21–23] The aim of the study presented here was to investigate whether serum and plasma ^{1}H NMR profiles, collected in parallel with the published urinary studies, are different in patients with HCC compared to patients with cirrhosis and healthy volunteers in well-characterised populations from Nigeria and Egypt. These study populations were subject to widely different environmental, dietary and aetiological factors.

METHODS

Patient and Healthy Volunteer Selection

Subjects were recruited in two cohorts from Jos University Teaching Hospital (JUTH) in Nigeria and The National Liver Institute, Menoufiya University, Shebeen El Kom, Egypt. The Nigerian study protocol was approved by the research ethics committee of JUTH, Nigeria and the Egyptian protocol by Menoufiya University, Egypt. The metabolic profiling protocol was approved by the research ethics committee of Imperial College London, UK. All volunteers provided informed, signed consent.

Hepatocellular carcinoma was diagnosed by radiological measures: ultrasonography (US) and/or computed tomography (CT) in Nigeria, while in Egypt, CT or magnetic resonance imaging (MRI) was used. Cirrhosis was diagnosed on clinical findings, by the presence of portal hypertension (esophageal varices or ascites) and US or CT confirmation. Tumours were staged according to the Okuda system, which includes tumour size, the presence of ascites, bilirubin and albumin levels as its criteria.[24] This scoring method was chosen out of necessity as other, more comprehensive scoring tools, such as the Barcelona Clinic Liver Cancer staging algorithm, require World Health Organisation (WHO) performance status, presence of portal vein invasion and encephalopathy as criteria, which were not recorded for most of the Nigerian patients in this study, owing to lack of axial imaging resources.

Sample Collection

5 mL fasted blood samples were venesected into either plain serum or ethylenediaminetetraacetic acid (EDTA)-containing sterile tubes and placed immediately on ice or into a refrigerator at 4 °C. Samples were centrifuged within 1–2 h at 4 °C, 1000 rpm for 10 min. The supernatant was then transferred as 2 mL aliquots into 2 mL microvial tubes and stored at −80 °C undergoing no freeze thaw-cycles until analysis. Forty-eight of 56 Nigerian samples were collected into tubes containing EDTA as an anticoagulant. The remainder of samples were collected into plain serum tubes. All of the Egyptian samples were collected into plain serum tubes, with no additives. Previous studies have reported similar ^{1}H NMR metabolic profiles from serum and plasma, allowing the two to be compared with relative assurance.[25–27] These studies highlight the fact that clinical differences between groups were profoundly more influential than spectral differences between EDTA plasma and plain serum samples.

Blood Laboratory Tests

For the Nigerian samples, serum urea, creatinine, alanine transaminase (ALT), alkaline phosphatase (ALP), total bilirubin and albumin levels were measured using automated techniques (Abbott™ Architect Ci16200 Analyser, UK) at St Mary's Hospital, London. Serum AFP was measured using an automated Siemens™ Immulite 2500 Analyser, (Deerfield, USA). For the Egyptian samples, serum AFP, creatinine, ALT, aspartate aminotransferase (AST), bilirubin and albumin were measured at the time of collection in Egypt using a Cobas Integer 400-Autoanalyzer, (Roche, Germany). Median and interquartile ranges (IQR) were calculated for each assay and median levels were compared using unpaired Mann–Whitney tests of significance.

Sample Preparation

Samples were prepared according to standard validated protocols.[28] Samples were thawed at room temperature and 200 μL were transferred into 1.5 mL Eppendorf (Cambridge, UK) tubes to which 400 μL NaCl/D_2O (90%/10%) were added. External reference standards, such as 3-trimethylsilyl-(2,2,3,3-$^2H_4$)-1-propionate (TSP), were not added, as in blood they may bind to protein, resulting in a final NMR signal that is reduced and has a very broad linewidth.

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The mixture underwent centrifugation for 5 min at 13,000 rpm and 550 μL of supernatant were transferred to Norell 5 mm 507-HP-7 NMR tubes (Norell, Landisville, New Jersey, USA) ready for ^{1}H NMR analysis. Samples were analysed on the same day as preparation.

^{1}H NMR Spectroscopy

All samples were run in a random, non-grouped order. All samples were run at the Department of Biomolecular Medicine, Imperial College London on two Bruker Ultrashield Plus™ 600 NMR systems operating at 600.29–600.44 MHz ^{1}H frequency (Bruker Biospin, Rheinstetten, Germany).[29] The systems were tuned, matched and frequency-locked on to ^{1}H as the nucleus of interest. A representative sample was utilised to set shim gradients to ensure a homogenous magnetic field across the sample, a 90° pulse length and the water suppression offset parameters. These settings were saved and utilised for the whole sample set. Spectra were acquired using nuclear Overhauser enhancement spectroscopy (NOESY) 1-D pulse sequence with water presaturation, during the relaxation delay (RD) and mixing time (t_m) using the following pulse programme: -RD-90°_t-90°-t_m-90°-acquire; where RD = 2.0 s and t_m = 0.1 s. For each sample, 128 free induction decays (FIDs) were collected into 32,000 data points with a spectral width of 20 parts per million (ppm). A line broadening function of 0.3–1.0 Hz was applied prior to Fourier transformation. Spectra were manually phased, baseline corrected and referenced to the α-glucose doublet at 5.23 ppm in TOPSPIN v2.0 (Bruker Biospin, Rheinstetten, Germany). Spectral peaks were assigned with reference to the literature.[30–32]

Data Pre-processing

Spectra were exported to MATLAB R2010 (MathWorks, Natick, Massachusetts, USA) and the water region from 4.5 to 6 ppm was excluded. As the concentration of EDTA varied between the serum and plasma samples, regions were excluded where it resonated, to avoid modelling differences between EDTA concentrations between samples. In a recent analysis of the effect of EDTA on metabolic profiling information recovery, it was reported that the resonances EDTA obscures commonly resonate elsewhere in the spectrum with few exceptions. Furthermore, the effect of EDTA on other molecules, in terms of spectral resonance or peak shift was found to be negligible.[33] Data were normalised to median fold-change and median spectra for all groups were generated to allow visual comparison of spectra and allow the selection of regions that were divergent for use in multivariate and univariate analyses.

Univariate Analysis

Data were exported to GraphPad Prism (La Jolla, California, USA) for univariate analysis in the form of Mann–Whitney *U*-tests comparison of medians between groups, assuming non-parametric distribution of data. *P*-values of <0.05 were considered significant.

Multivariate Analysis

Median spectra of each group (HCC, cirrhosis and healthy volunteer) were compared in a combined analysis of Nigerian and Egyptian data. Regions that were visually divergent were selected for multivariate analysis. These areas are recorded in Table 1. The integral areas of these regions were recorded in a data matrix and exported to SIMCA (Umetrics, Umea, Sweden). Data were mean-centred and principle components analysis (PCA) was performed first to model overall variation and identify outliers. Only mean-centred data were used for further analysis. After outliers were identified and excluded, partial least squared discriminant analysis (PLS-DA) was performed to identify the discriminant strength of the metabolite based model and to generate a loadings plot from which metabolites could be identified which most greatly contributed to differences between the groups. In SIMCA-P v12, PLS-DA models were generated through seven-fold cross

Table 1 Spectral Regions Selected for Multi- and Univariate Analyses.

	Region (ppm)	Molecule Moeity	
1	0.8–0.85	Low density lipoprotein	CH_3
2	0.85–0.88	Very low density lipoprotein	CH_3
3	1.21–1.24	Low density lipoprotein	$-(CH_2)_n-$
4	1.25–1.30	Very low density lipoprotein	$-(CH_2)_n-$
5	1.31–1.32	Lactate	CH_3
6	2.02–2.05	*N*-Acetylglycoproteins	$NHCOCH_3$
7	2.22–2.23	Acetoacetate	CH_3
8	4.098–4.108	Lactate	CH
9	8.445–8.45	Formate	CH

validation. In this method, every 7th sample was excluded (1st, 7th, 14th, 21st and so on), a model generated from the remaining samples and the excluded "training set" predicted back into the model. This was repeated for all the samples (grouping the 2nd, 9th, 16th and 3rd, 10th, 17th and so on) until all the samples were excluded once. The results were averaged to produce a model which was externally cross-validated. Spectral peaks which contributed most to PLS-DA models, and those visually different on median spectra comparison, were selected for peak integration. All data were mean centred prior to multivariate analysis. Country-specific and male-only analyses were performed to ensure that findings were due to metabolite characteristics secondary to HCC and not due to population or gender disparities between groups.

RESULTS

Subject Selection and Demographics

A total of 98 volunteers were recruited for study, 56 from Nigeria and 42 from Egypt. Subjects were recruited in three cohorts, 53 patients with ultrasound or computed tomography proven HCC (29 Nigerian + 24 Egyptian, median age: 50, 70% male); 26 patients with clinically-confirmed cirrhosis with features of portal hypertension, but no HCC (12 Nigerian + 14 Egyptian, median age: 48.5, 69% male); and 19 healthy subjects with no history of liver disease (15 Nigerian + 4 Egyptian, median age: 40, 42% male). All patients, except one, in the Nigerian HCC and cirrhosis groups were hepatitis B surface antigen (HBsAg) positive. The single non-HBV patient with cirrhosis was also anti-hepatitis C virus (HCV) antibody negative and was therefore classified as having idiopathic liver disease. In the Egyptian cohort, all the patients with cirrhosis and 23/24 patients with HCC had chronic HCV. The single HCC patient without HCV had idiopathic liver disease. All healthy volunteers were HBsAg and anti-HCV antibody negative with no history of liver disease.

There was no significant difference between the ages of all three groups, although patients in the healthy volunteer group had a median age of 40 years, compared to that of 50

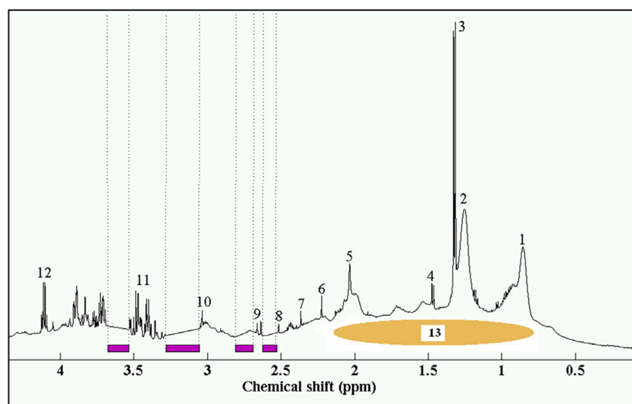

Figure 1 Representative plasma spectrum with EDTA exclusion. **Key:** (1) and (2) LDL/VLDL; (3) lactate (CH_3); (4) alanine; (5) N-acetylglycoproteins; (6) acetoacetate; (8) and (9) citrate; (10) creatinine; (11) glucose resonances; (12) lactate (CH); (13) albumin and albumin-bound fatty acids (Nicholson et al., 1995).[31] Purple bars indicate areas of EDTA resonance exclusion.

years for patients with HCC ($p = 0.09$). There were fewer males in the healthy volunteer group (42% versus 70% in the HCC group, $p = 0.052$). The biochemical analyses of the patients are outlined in Table 2. Median serum AFP levels were significantly higher (1198 IU mL^{-1}) in patients with HCC, compared to those with cirrhosis and to healthy volunteers (5.61 and 1.44, $p < 0.001$). Of note, if an AFP cut-off value of 400 IU mL^{-1} was used for HCC diagnosis, 19 tumours would have not been diagnosed (64% sensitivity). Creatinine levels were comparable across groups, but serum ALT, bilirubin and albumin were deranged in the HCC and cirrhosis groups in comparison to healthy controls. HCC was staged according to the Okuda criteria, which showed 8 patients were Stage 1, 25 patients were Stage 2 and 16 patients were Stage 3. Four Nigerian patients were unable to be staged accurately, owing to a lack of clinical data.

^{1}H NMR Spectroscopy

A representative NOESY plasma spectrum is displayed in Figure 1 with indication of which regions were excluded

Table 2 Biochemical Analysis of All Patients.

Test (range)	HCC	Cirrhosis	Healthy controls	*P*-values (Mann–Whitney)
Serum samples (*n*)	**53**	**26**	**19**	-
AFP (IU mL^{-1})	1198	5.61$^+$	1.44$^+$	[a and b]<0.001*
Creatinine (mmol L^{-1})	63.0	82.5	70.0	[a]0.39 and [b]0.04*
ALT (IU L^{-1})	52.5$^+$	32.5	22.0	[a]<0.001* and [b]0.04*
Bilirubin (μmol L^{-1})	29.0$^+$	36.8	6.9	[a]<0.001* and [b]0.43
Albumin (g L^{-1})	26.6$^+$	23.8	45.7	[a]<0.001* and [b]0.16

Key: $^+$Some data missing. Mann–Whitney non-parametric comparisons of HCC versus healthy ([a]) and versus cirrhosis ([b]).

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Table 3 Metabolite Differences Between Groups.

Metabolite	Moiety	Chemical shift (ppm)	HCC vs. Healthy	HCC vs. Cirrhosis	Pathway
LDL	CH_3	0.8–0.85	↓*	↓	Lipid production/use
LDL	-$(CH_2)_n$-	1.21–1.24	↓	↓	
VLDL	CH_3	0.85–0.88	↑*	↑	
VLDL	-$(CH_2)_n$-	1.25–1.30	↑*	↑	
Lactate	CH_3	1.31–1.32	↑*	↓	Inflammation
Lactate	CH	4.098–4.108	↑*	↓	
N-Acetyl-glycoproteins	$NHCOCH_3$	2.02–2.05	↑*	↑*	
Acetoacetate	CH_3	2.22–2.23	↑	↑*	Lipid metabolism
Formate	CH	8.445–8.45	↑	↑	1-carbon pathway

Key: ↑↓Indicates increased or decreased in patients with HCC. **P*-value <0.05.

due to EDTA resonances. The area of exclusion is, therefore, relatively small in comparison to the whole spectrum. The resolution between the overlapping peaks of low density lipoprotein (LDL) at 0.8 ppm and 1.21 ppm and very low density lipoprotein (VLDL) at 0.85 ppm and 1.25 ppm was poor, although could discernibly be distinguished.

Univariate Statistical Analysis

Univariate analyses, using the spectral integral values of one peak, which corresponds to one metabolite, were performed (Figure 5 and Table 3). The most prominent spectral peaks, arising from LDL and VLDL molecules, showed significant difference between the groups. LDL levels were reduced in patients with HCC, compared to both healthy volunteers ($p = 0.28$ and 0.002) and cirrhosis ($p = 0.12$ and 0.05). VLDL levels were raised in patients with HCC, compared to healthy volunteers ($p = 0.004$ and <0.001), but not when compared to patients with cirrhosis ($p = 0.77$ and 0.62). Lactate levels, both at 1.31 ppm (doublet) and 4.11 ppm (quadruplet), were significantly raised in patients with HCC, compared to healthy controls ($p = <0.001$ and 0.03), but not when compared to patients with cirrhosis ($p = 0.06$ and 0.12). *N*-Acetylglycoproteins levels were significantly raised in patients with HCC compared to both healthy volunteers and patients with cirrhosis ($p < 0.001$ and 0.001), while acetoacetate was non-significantly raised ($p = 0.52$ and 0.06). Finally, formate levels, although visually appearing altered between group median spectra, displayed no significant differences between the groups.

Multivariate Statistical Analysis

Principal components analysis and PLS-DA were performed on the data matrix, consisting of those spectral regions that appeared most divergent between patient and control groups. Nine regions were identified, which are tabulated (Table 1). Principal components analysis of all groups is shown in Figure 2A. Supervised PLS-DA was undertaken and is displayed for HCC and healthy volunteer and HCC and cirrhosis groups in Figure 2B and C. The fit of the models was good ($R^2 = 0.87$ and 0.7). However,

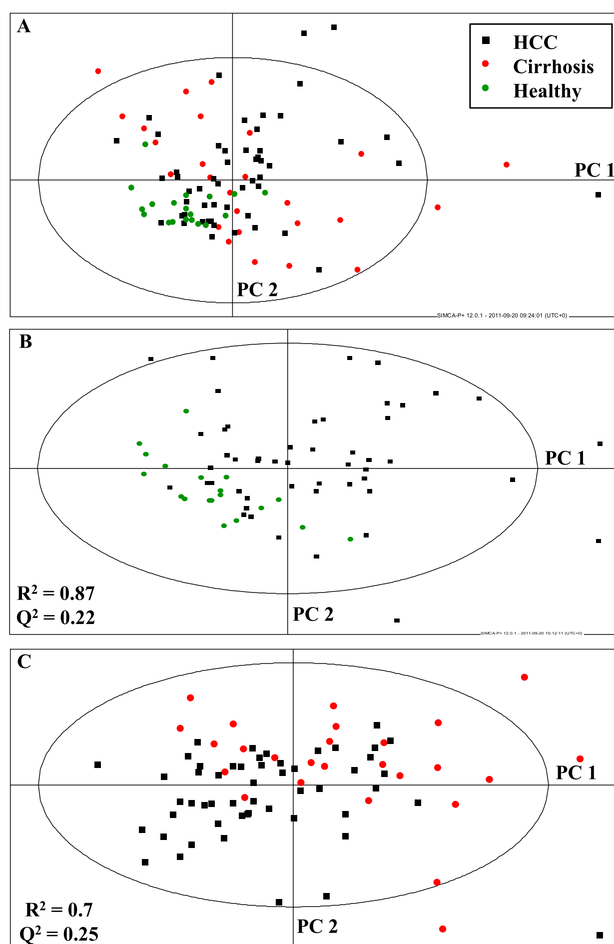

Figure 2 Multivariate analyses of combined Nigerian and Egyptian samples. (A) PCA scatter plot of all groups; (B) PLS-DA scatter plot of HCC and healthy volunteer samples; (C) PLS-DA scatter plot of HCC and cirrhosis samples.

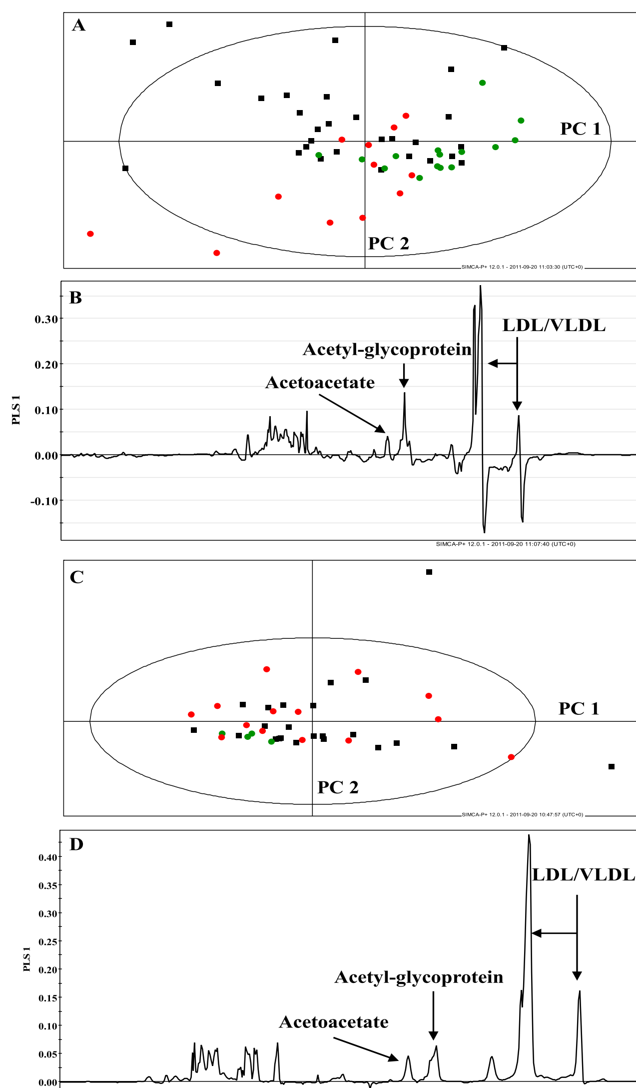

Figure 3 Multivariate analysis plots of Nigerian and Egyptian data. (A) and (B) PCA and PLS-DA loadings plot of Nigerian data; (C) and (D) PCA and PLS-DA loadings plot of Egyptian data.

the goodness of prediction or Q^2 levels was low: 0.22 and 0.25. Figure 3A-D displays the separate multivariate analyses for the Nigerian and Egyptian cohorts. These analyses confirm that the combined analyses reflect the country-specific results, with metabolites such as LDL, VLDL, *N*-acetylglycoproteins and acetoacetate as contributing most to discrimination between patients and healthy volunteer groups. Finally, male-only analyses were performed using both Nigerian and Egyptian data. This is represented in a PCA plot in Figure 4. The data displayed similar clustering to combined plots and the metabolites contributing most to discrimination between group remained very similar, confirming that gender disparities between disease and healthy volunteer groups were not confounding multivariate results (Figure 5).

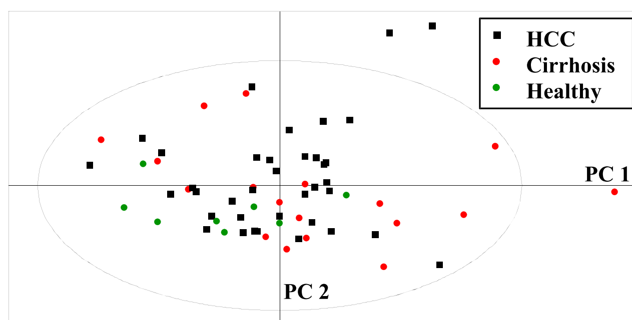

Figure 4 Principal components analysis of male volunteer samples.

DISCUSSION

This is the first study to characterise the metabolic changes in serum and plasma due to HCC in two completely diverse populations with different genetics, diet and underlying disease aetiology. Multivariate analysis displayed reasonable separation of disease and healthy groups, while comparison of median group spectra, combined with univariate analyses identified several metabolites elevated or reduced in the blood of patients with HCC. Furthermore, combined analyses, of subjects from Nigerian and Egypt, revealed similar results to country-specific analyses. Given that the majority of patients from Nigeria were HBV-infected and those from Egypt were HCV-infected, this would suggest that blood metabolite profiles in the presence of HCC are dependent on the tumour effects, rather than aetiology of liver disease.[30]

There have been several previous studies that utilised serum ^{1}H NMR for HCC identification.[12-15,34] Assi and colleagues utilised a large ^{1}H NMR study to associate lifestyle exposure with metabolomic signals of HCC in a European cohort of the European Prospective Investigation into Cancer and Nutrition (EPIC) study.[14] The study highlighted the presence of a complex interaction of dietary and lifestyle factors leading to metabolic changes that may contribute to HCC. A study by Liu and colleagues identified potential biomarkers by comparing 43 HCC patients with 42 cirrhosis patients and 18 healthy volunteers. There were significant elevations in beta-hydroxybutyrate, glycerol and oxaloacetate in the HCC group, and fatty acid elevation in the cirrhosis group, including isobutyrate, linoelaidic acid and linoleic acid, compared with the healthy volunteers.[34] Nahon and colleagues compared the serum data of patients with compensated biopsy-proven alcoholic cirrhosis, of whom 93 had cirrhosis without HCC, 28 had small HCC and 33 had large HCC determined by the Milan criteria.[12] The study showed significant increase in glutamate, acetate and *N*-acetyl glycoproteins in large HCC compared to the cirrhotic group without HCC. The significance of the results is debatable due to the various metabolic effect of chronic

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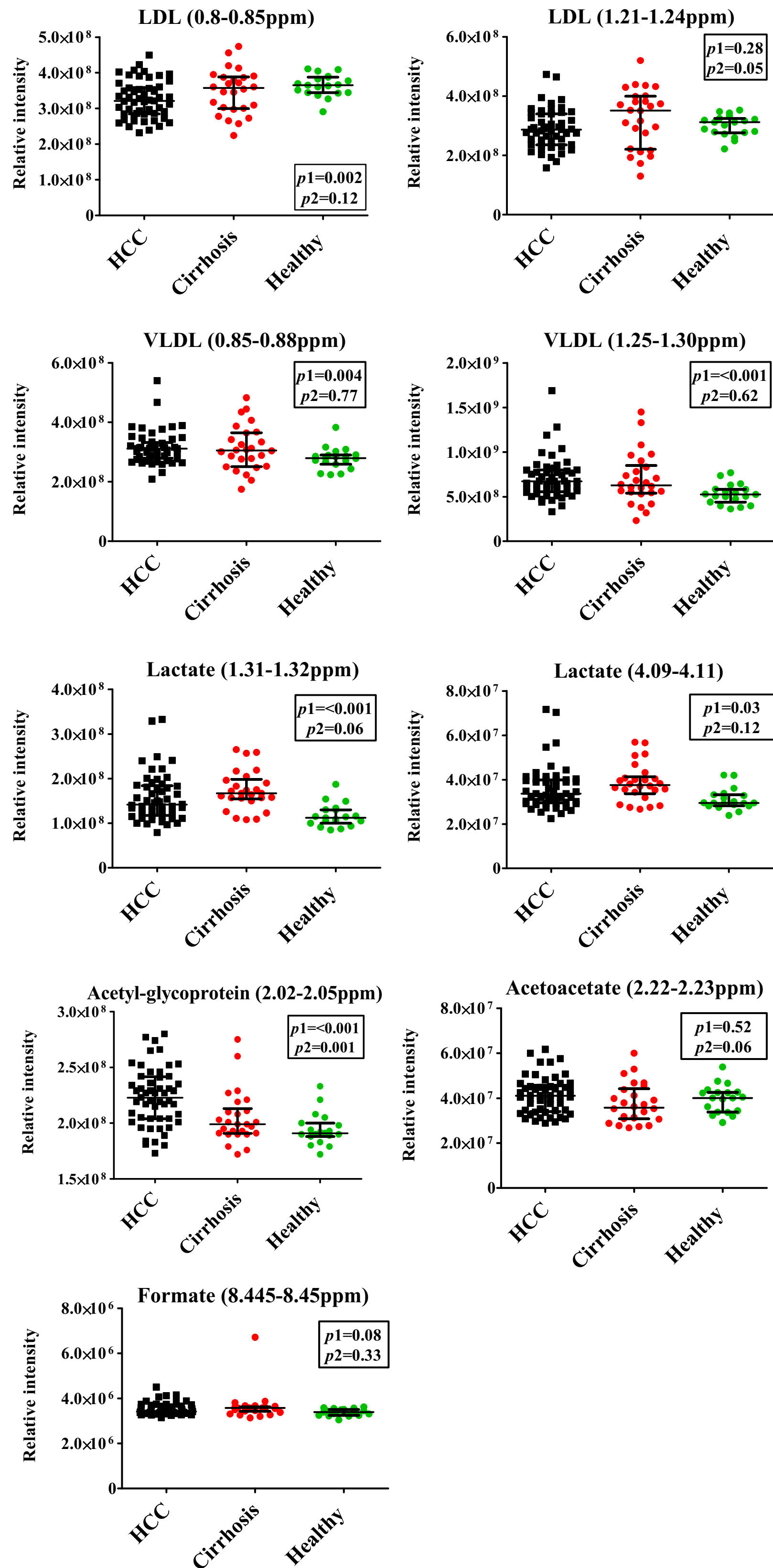

Figure 5 Univariate analysis of discriminatory metabolites. **Key:** *P*1 = *P*-value of HCC versus healthy control analyses; *P*2 = *P*-value of HCC versus cirrhosis analyses. Mann–Whitney tests of significance used for generation of *P*-values.

alcoholism. Wei and colleagues compared patients with HCC with those with HCV, and identified significant alteration in choline, valine and creatinine in the HCC groups.[13] Overexpression of metabolites, such as choline, has been found to be raised with a series of different tumours, and these are likely to represent non-specific serum markers.[35] Furthermore, these studies did not offer metabolic comparison with healthy controls. Gao and colleagues utilised ^{1}H NMR of serum from patients with HCC in comparison to patients with liver cirrhosis and healthy volunteers,[15] the results showing some similarities to those reported here. The Gao study did not clarify the patient age, gender or aetiology of liver disease. However, the metabolite signatures that we report here and those of the Gao study infer a significant influence of HCC upon lipid metabolism. Blood VLDL levels were elevated in patients with HCC, both in comparison to cirrhosis and healthy states in both studies.[15]

Low density lipoprotein levels, conversely, were reduced in our study. Acetoacetate, a by-product of fatty acid oxidation, was elevated in patients with HCC. It is increasingly recognised that the liver, as a central hub of lipid metabolism, may alter its production of VLDL as a result of disease.[36] This is of particular importance in the presence of HCV particles which utilise altered VLDL particles as a transport and translocation facilitator thereby affecting blood levels.[37] It is less well documented how HCC may affect this pathway. Intuitively, it would be expected that as a tumour grows in an already diseased cirrhotic liver, functionality decreases and lipid production does so as well. The results in this study are therefore counter-intuitive, with raised VLDL and reduced LDL levels. Gao and colleagues offer little explanation of why this would occur in their study, stating that HCC and cirrhosis merely enhance lipid metabolism. The genetic changes that occur in HCC are diverse and can affect many pathways.[38] It is possible that one of these affected pathways may affect lipid metabolism and promote the production of VLDL. A candidate may be Peroxisome proliferator-activated receptor α (PPARα), a nuclear transcription factor, which, if activated, is known to decrease hepatic VLDL secretion and enhance clearance.[39] It is also plausible that peripheral VLDL breakdown, via the lipolytic pathway, is reduced. If this were the case then less LDL would be formed, as seen here. This may be affected through a down-regulation of lipolytic enzymes, such as hepatic or lipoprotein lipase, the interplay of which is highly complex in lipid metabolism.[36]

A more robust argument for the observed rise in metabolites that we observed may be explained by the Warburg phenomenon, which highlights the preferential metabolism of glucose by anaerobic glycolysis in tumour cells.[40] Glycolysis produces energy at a higher rate than oxidative phosphorylation in cancer cells, albeit at the compromise of metabolic efficiency.[41] The heightened rate of anaerobic metabolism may be a favourable trait for a rapidly proliferating tumour.[42] The shift in metabolism causes a rise in by-products of anaerobic respiration, such as lactate, which was significantly raised in the HCC group.

The increase in VLDL that we noted may be a consequential effect of alternative energy metabolism in HCC. Hepatic VLDL is produced by fatty acid esterification with glycerophosphate, a by-product of anaerobic glycolysis.[43] Hepatic VLDL secretion may be the inappropriate response from the tumour's anaerobic respiration, leading to global lipid mobilisation for the lipolytic pathway. The result of the pathway is supported by the observed increase in acetoacetate in the HCC group. Acetoacetate is a ketone body, which together with acetone and beta-hydroxybutyrate, is formed as a by-product of beta-oxidation.[44] The rise in ketone bodies was also observed in the Liu study and may indicate a globally heightened lipolytic pathway in the HCC group as a consequence of abnormal anaerobic respiration.[34]

In our study, formate levels were elevated in patients with HCC. This metabolite is produced from the folate cycle in hepatic embryonic cells. In conjunction with the abnormal rise of AFP, an embryonic glycoprotein detectable in HCC, the increase in formate is an unsurprising result of liver tumorigenesis.[45]

N-Acetylglycoproteins were increased in patients with HCC in our study. These represent "acute phase protein" fragments of glycoproteins, such as α_1-acid glycoprotein, haptoglobin, transferrin and fibrinogen. Hepatocytes are known to secrete these molecules under a number of different stressful stimuli including cancer.[46,47] A NMR study by Bell and colleagues, comparing patients with different malignancies to matched healthy controls, observed this resonance to display large variations in amplitude in the blood of cancer patients, compared to healthy volunteers.[17] In HCC on the background of a cirrhotic liver, it may be that hepatic function is preserved to an extent, so as to secrete this molecule as a stress response.

Our study characterises a certain metabolic trait in patients with HCC and cirrhosis, which can be distinguished from the healthy population. This finding is extremely relevant to the current investigative changes for HCC in clinical practice. If the metabolites that characterise HCC are incorporated into a testable profile, they may be given a simple scoring system to identify both the presence and severity of HCC by a blood test. While identifying AFP is no longer the recommended guideline for HCC diagnosis, a minimally invasive serum marker for cancer is extremely useful, particularly in a developing world scenario where management of advanced tumours is limited. Early identification through a simple serum investigation may be an important step in addressing the global HCC burden.

In conclusion, this study has produced results which may provide insight into the altered lipid pathways

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induced by Warburg's phenomenon of anaerobic respiration in HCC. This is the first blood profiling NMR study to look at two ethnically diverse patient populations and find results that independent of genetics, diet and underlying disease aetiology, all factors that have limited the meaningfulness and translatability of previous studies. Our previous urinary metabolic profiling studies in the same populations have thrown up a different series of metabolites present in the urine, which delineate HCC distinct from cirrhosis and non-cirrhotic liver disease.[21–23] This highlights that parallel investigations on different body fluids are valuable in the search for new biomarkers of liver cancer, and that ultimately, a combined biomarker panel from blood, urine and possibly stool may be a useful avenue for future research.

CONFLICTS OF INTEREST

The authors have none to declare.

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AUTHOR'S CONTRIBUTIONS

The study was conceived and overseen by SDT-R, EO, IW, IJC, RW and EH. MIFS, NGL, AIG and MMEC conducted the study, while NGL, AIG, EB and MMEC were responsible for sample collection in-country, transport and processing. MIFS undertook the analyses, which were verified by NGL, EH and SDT-R. The paper was written primarily by MIFS, JUK and SDT-R, but all authors contributed to the writing of the manuscript and approved the final version

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