

THE METABOLIC PROFILING OF HEPATOCELLULAR CARCINOMA

MOHAMED IDROOS FAHMY SHARIFF

IMPERIAL COLLEGE LONDON

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THESIS ABSTRACT

Hepatocellular carcinoma (HCC), the commonest primary liver tumour, is curable if detected early. It is often diagnosed late, carrying a devastating prognosis. In part, this is due to lack of accurate diagnostic markers. Using proton nuclear magnetic resonance (^1H NMR) spectroscopy and ultra performance liquid chromatography mass spectrometry (UPLC-MS), metabolic profiles of plasma, serum and urine were generated from an animal model of HCC and three cohorts of patients with HCC from Nigeria, Egypt and the UK.

Plasma ^1H NMR spectra from an animal model of HCC displayed decreases in lipoproteins, acetyl-glycoprotein, acetoacetate and glucose ($p \leq 0.001$). Blood ^1H NMR studies of Nigerian and Egyptian patients with HCC compared to healthy controls and those with cirrhosis revealed alterations in levels of LDL ($p = 0.002$, 0.12), VLDL ($p = < 0.001$, 0.77), lactate ($p = 0.03$, 0.12), *N*-acetylglycoproteins ($p = < 0.001$, 0.001) and acetoacetate ($p = 0.52$, 0.06). Using UPLC-MS blood profiling, lysophosphatidylcholine (24, 0, 0) (LPC), a major cell membrane component, was identified as altered between groups.

Urine ^1H NMR spectral profiles were distinguished in a Nigerian, mostly HBV-infected HCC cohort, with a sensitivity/specificity of 100%/93% and 89.5%/88.9% from healthy subjects and patients with cirrhosis. Urinary creatinine ($p < 0.001$, 0.06), carnitine ($p = 0.04$, < 0.001) and creatine ($p = 0.05$, 0.29) were contributory. In an Egyptian, mostly HCV-infected HCC cohort, sensitivity/specificity was 100%/94% and 81%/71%, displaying reduced creatinine ($p = 0.003$, < 0.001), glycine ($p = < 0.001$, 0.88), trimethylamine-*N*-oxide ($p = < 0.001$, 0.18), hippurate ($p = < 0.001$, 0.66), citrate ($p = < 0.001$, 0.12) and increased carnitine ($p = 0.29$, 0.30) and creatine ($p = 0.12$, 0.33) levels. In the UK cohort, sensitivity/specificity was 53.6%/96%. UPLC-MS of urine from the Nigerian HCC cohort identified elevated acetylcarnitine levels ($p = 0.07$, < 0.001).

Both in blood and urine, HCC induces a metabolic profile change that can distinguish patients from healthy or cirrhotic controls. Furthermore, there exists some similarity between distinct populations. Underlying pathways may include heightened cellular proliferation, altered cellular, aberrant lipid metabolism and cancer cachexia.

To Sammy, Tasir and Mikhail,

With love.

DECLARATION

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

Mohamed I.F.Shariff

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TABLE OF CONTENTS

THESIS ABSTRACT	2
DECLARATION.....	4
ACKNOWLEDGEMENTS	5
TABLE OF CONTENTS	6
LIST OF TABLES	15
LIST OF FIGURES	18
LIST OF ABBREVIATIONS	22
1 INTRODUCTION.....	24
1.1 Hepatocellular carcinoma.....	24
1.1.1 Epidemiology.....	24
1.1.2 Aetiology.....	25
1.1.3 Prognosis, surveillance and the importance of early tumour diagnosis.....	28
1.1.4 Diagnosis.....	29
1.1.5 Serum markers of hepatocellular carcinoma.....	30
1.1.6 Urinary markers of hepatocellular carcinoma.....	34
1.1.7 The origin and pathogenesis of hepatocellular carcinoma.....	37
1.1.8 Treatment options	39
1.2 Altered tumour metabolism	40
1.2.1 The “Warburg Effect” and glycolysis.....	40
1.2.2 Molecular effectors and tumour metabolism	42
1.2.3 Metabolite effects on carcinogenesis	44
1.3 Metabolic profiling.....	46
1.3.1 Proton nuclear magnetic resonance spectroscopy.....	47

1.3.2	Mass spectrometry	48
1.4	Rationale for study	50
1.5	Hypotheses	51
1.6	Overall Aims	51
2	METHODOLOGY	52
2.1	Principles of nuclear magnetic resonance spectroscopy	52
2.1.1	Nuclear magnetic resonance and the property of spin	52
2.1.2	Spin orientations and energy levels	52
2.1.3	Precession and the Larmor frequency	54
2.1.4	Nuclear magnetic resonance systems.....	56
2.1.5	Signal generation	57
2.1.6	Fourier transformation	58
2.1.7	Chemical shift	58
2.1.8	Coupling.....	59
2.1.9	Pulse sequences.....	59
2.1.10	Proton nuclear magnetic resonance spectroscopy processing	59
2.2	Principles of mass spectrometry	61
2.2.1	The ion source	61
2.2.2	Mass analysers	61
2.2.3	Ion detectors.....	62
2.2.4	Tandem mass spectrometry.....	62
2.2.5	Chromatography	62
2.2.6	Data pre-processing	63
2.3	Multivariate statistical analysis.....	66
2.3.1	Principal components analysis and outlier exclusion	67
2.3.2	Partial least squared discriminant analysis	67

3	PLASMA PROTON NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY PROFILING OF A RAT MODEL OF HEPATOCELLULAR CARCINOMA	69
3.1	ABSTRACT	69
3.2	INTRODUCTION	70
3.2	AIMS	71
3.4	METHODS	72
3.4.1	Fisher rat model of hepatocellular carcinoma.....	72
3.4.2	Sample preparation	73
3.4.3	Proton nuclear magnetic resonance spectroscopy.....	73
3.4.4	Spectral processing	74
3.4.5	Multivariate and univariate statistical analysis	75
3.5	RESULTS.....	76
3.5.1	Fisher rat weights.....	76
3.5.2	Proton magnetic resonance spectral profiles.....	77
3.5.3	Multivariate statistical analysis.....	80
3.5.4	Univariate statistical analysis.....	83
3.6	DISCUSSION	85
3.7	CONCLUSIONS.....	89
4	THE PLASMA AND SERUM METABOLIC PROFILING OF HEPATOCELLULAR CARCINOMA IN A NIGERIAN AND EGYPTIAN COHORT USING PROTON NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY	90
4.1	ABSTRACT	90
4.2	INTRODUCTION.....	91
4.3	METHODS	92
4.3.1	Patient and healthy volunteer selection.....	92
4.3.1	Sample collection.....	92
4.3.2	Blood laboratory tests	93

4.3.3	Sample preparation	93
4.3.4	Proton nuclear magnetic resonance spectroscopy.....	94
4.3.5	Data pre-processing	94
4.3.6	Multivariate analysis	97
4.3.7	Univariate analysis.....	97
4.4	RESULTS.....	98
4.4.1	Subject selection and demographics	98
4.4.2	Proton nuclear magnetic resonance spectroscopy.....	100
4.4.3	Multivariate statistical analysis.....	103
4.4.4	Univariate statistical analysis.....	106
4.5	DISCUSSION	110
4.6	CONCLUSIONS.....	115
5	BLOOD LIPID PROFILING OF HEPATOCELLULAR CARCINOMA USING ULTRA-PERFORMANCE LIQUID CHROMATOGRAPHY MASS SPECTROMETRY	116
5.1	ABSTRACT	116
5.2	INTRODUCTION.....	117
5.3	METHODS	119
5.3.1	Lipid extraction optimisation.....	119
5.3.2	Fisher rat model of hepatocellular carcinoma.....	119
5.3.3	Patient selection	119
5.3.4	Blood laboratory tests	120
5.3.5	Sample collection.....	120
5.3.6	Sample preparation	120
5.3.7	Chromatographic conditions.....	121
5.3.8	Mass spectrometry	121
5.3.9	Data pre-processing	122

5.3.10	Multivariate analysis	123
5.3.11	Univariate analysis.....	123
5.4	RESULTS.....	124
5.4.1	Patient and rat demographics	124
5.4.2	Lipid extraction optimisation.....	125
5.4.3	Positive mode lipid chromatograms.....	126
5.4.4	Multivariate statistical analysis.....	127
5.4.5	Lipid feature identification	128
5.4.6	Univariate statistical analysis.....	129
5.5	DISCUSSION	133
5.6	CONCLUSIONS.....	136
6	THE URINARY METABOLIC PROFILING OF HEPATOCELLULAR CARCINOMA USING PROTON NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY IN A NIGERIAN PATIENT COHORT.....	137
6.1	ABSTRACT	137
6.2	INTRODUCTION.....	138
6.3	METHODS	140
6.3.1	Patient selection	140
6.3.2	Sample collection.....	140
6.3.3	Proton magnetic resonance spectral processing.....	141
6.3.4	Multivariate statistical analysis.....	141
6.3.5	Univariate statistical analysis.....	142
6.4	RESULTS.....	143
6.4.1	Subject demographics	143
6.4.2	Serum biochemical analyses	145
6.4.3	Proton nuclear magnetic resonance spectroscopy.....	146
6.4.4	Multivariate data analysis	147

6.4.5	Univariate statistical analysis.....	149
6.5	DISCUSSION	151
6.6	CONCLUSIONS.....	154
7	THE URINARY METABOLIC PROFILING OF HEPATOCELLULAR CARCINOMA IN AN EGYPTIAN POPULATION USING PROTON MAGNETIC RESONANCE SPECTROSCOPY	155
7.1	ABSTRACT	155
7.2	INTRODUCTION.....	156
7.3	METHODS	158
7.3.1	Subject selection	158
7.3.2	Urine sample collection	158
7.3.3	Serum biochemical tests	158
7.3.4	Magnetic resonance spectral acquisition and processing.....	159
7.3.5	Multivariate statistical analysis.....	159
7.3.6	Univariate statistical analysis.....	160
7.4	RESULTS.....	161
7.4.1	Subject recruitment, exclusion and demographics.....	161
7.4.2	Serum biochemical analysis.....	163
7.4.3	Proton nuclear magnetic resonance spectroscopy.....	164
7.4.4	Multivariate statistical analysis.....	165
7.4.5	Male only comparisons	166
7.4.6	Univariate statistical analysis.....	166
7.5	DISCUSSION	170
7.6	CONCLUSIONS.....	175
8	URINARY METABOLIC PROFILING OF HEPATOCELLULAR CARCINOMA IN A UNITED KINGDOM COHORT USING PROTON NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY	176

8.1	ABSTRACT	176
8.2	INTRODUCTION	177
8.3	METHODS	179
8.3.1	Patient Selection.....	179
8.3.2	Sample collection.....	181
8.3.3	Sample preparation	181
8.3.4	Proton nuclear magnetic resonance spectroscopy.....	181
8.3.5	Spectral processing	182
8.3.6	Statistical Total Correlation Spectroscopy (STOCSY).....	182
8.3.7	Multivariate and univariate statistical analysis	183
8.4	RESULTS.....	185
8.4.1	Subject recruitment, exclusion and demographics.....	185
8.4.2	Serum biochemical analysis.....	187
8.4.3	Median spectra	188
8.4.4	Multivariate statistical analysis.....	188
8.4.5	Univariate statistical analysis.....	194
8.5	DISCUSSION	198
8.6	CONCLUSIONS.....	203
9	THE URINARY METABOLIC PROFILING OF HEPATOCELLULAR CARCINOMA IN A NIGERIAN PATIENT COHORT USING ULTRA HIGH PERFORMANCE LIQUID CHROMATOGRAPHY MASS SPECTROMETRY.....	204
9.1	ABSTRACT	204
9.2	INTRODUCTION	205
9.3	METHODS	209
9.3.1	Patient selection	209
9.3.2	Serum biochemical tests	209
9.3.3	Urine sample collection	209

9.3.4	Sample preparation	209
9.3.5	Chromatographic conditions	210
9.3.6	Mass spectrometry	210
9.3.7	Tandem mass spectrometry (MS-MS)	211
9.3.8	Data pre-processing	211
9.3.9	Multivariate analysis	212
9.3.10	Univariate analysis	212
9.4	RESULTS.....	213
9.4.1	Subject recruitment, exclusion and demographics.....	213
9.4.2	Positive ion mode data	217
9.4.3	Negative ion mode	221
9.4.4	Variable identification and authentication.....	224
9.4.5	Standard authentication	227
9.5	DISCUSSION	229
9.6	CONCLUSIONS.....	233
10	THE URINARY METABOLIC PROFILING OF HEPATOCELLULAR CARCINOMA USING ULTRA PERFORMANCE LIQUID CHROMATOGRAPHY MASS SPECTROMETRY IN A UK POPULATION.....	234
10.1	ABSTRACT	234
10.2	INTRODUCTION.....	235
10.3	METHODS	236
10.3.1	Patient selection	236
10.3.2	Blood laboratory tests	236
10.3.3	Urine sample collection and preparation	236
10.3.4	Chromatographic conditions	237
10.3.5	Mass spectrometry	237
10.3.6	Tandem mass spectrometry and feature identification	237

10.3.7	Data pre-processing	238
10.3.8	Multivariate analysis.....	238
10.3.9	Univariate analysis.....	238
10.4	RESULTS.....	239
10.4.1	Subject recruitment, exclusion and demographics.....	239
10.4.2	Serum biochemical analysis.....	244
10.4.3	Chromatographic data	246
10.4.4	Multivariate statistical analysis.....	247
10.4.5	Tandem mass spectrometry and univariate analysis.....	251
10.4.6	Univariate statistical analysis.....	253
10.5	DISCUSSION	254
10.6	CONCLUSIONS.....	257
11	CONCLUSIONS AND FUTURE WORK.....	258
11.1	FISHER RAT MODEL OF HCC	259
11.2	HUMAN BLOOD METABOLIC PROFILING STUDIES	260
11.3	HUMAN URINE METABOLIC PROFILING STUDIES.....	263
11.4	METABONOMICS IN OTHER CANCERS AND CACHEXIA.....	264
11.5	FUTURE DIRECTIONS	266
12	PUBLICATIONS AND PATENTS.....	268
12.1	ORIGINAL ARTICLES.....	268
12.2	PUBLISHED ABSTRACTS.....	268
12.3	BOOK CHAPTERS	268
12.3	REVIEW ARTICLES.....	269
12.4	OTHER NATIONAL AND INTERNATIONAL PRESENTATIONS	269
12.5	PATENTS FILED	269
13	REFERENCES.....	270

LIST OF TABLES

Table 1-1.	Diagnostic performance of serum markers of hepatocellular carcinoma.....	33
Table 1-2.	Comparison of nuclear magnetic resonance and mass spectrometry	47
Table 2-1.	Common nuclei studied using nuclear magnetic resonance spectroscopy.....	52
Table 3-1.	Fisher rat groups and interventions; groups 1 and 2 developed HCC.....	72
Table 3-2.	Fisher rat weights at time of sacrifice	76
Table 3-3.	Resonance identities in rat plasma spectra between 0.5 -4.5 ppm.....	79
Table 3-4.	Misclassification matrix for PLS-DA prediction of disease	80
Table 3-5.	Median metabolite levels in healthy and diseased animals.....	83
Table 4-1.	Metabolite peaks obscured by EDTA resonances.....	96
Table 4-2.	Spectral regions selected for multi- and univariate analyses	97
Table 4-3.	Parameters of Nigerian and Egyptian cohorts.....	99
Table 4-4.	Nigerian and Egyptian patient and healthy volunteer demographics	99
Table 4-5.	Biochemical analysis of all patients.....	100
Table 4-6.	Okuda stages of patients with hepatocellular carcinoma.....	100
Table 4-7.	Metabolite differences between groups.....	109
Table 4-8.	Metabolite alterations between groups: comparison to previous studies.....	111
Table 5-1.	Lipids.....	117
Table 5-2.	Patient demographics of UK and Nigerian cohort.....	124
Table 5-3.	Aetiologies of hepatocellular carcinoma and cirrhosis cohorts.....	124
Table 5-4.	Okuda stage of patients with hepatocellular carcinoma.....	125
Table 5-5.	Fisher rat weights at time of sacrifice.....	125
Table 5-6.	Discriminatory features identified by multivariate analysis	128
Table 6-1.	Nigerian subject demographics.....	144
Table 6-2.	Serum biochemical analyses.....	145

Table 6-3.	Validity values of partial-least squares discriminant analyses and diagnostic sensitivities and specificities.....	148
Table 7-1.	Egyptian subject demographics.....	162
Table 7-2.	Aetiology of liver disease in Egyptian patient cohorts.....	162
Table 7-3.	Serum biochemistry profiles of subject cohorts.....	163
Table 7-4.	Urine magnetic resonance metabolite resonances.....	164
Table 7-5.	Validity values of partial-least squares discriminant analyses.....	166
Table 7-6.	Discriminatory metabolites from Nigerian and Egyptian urine studies.....	171
Table 8-1.	UK patient demographics.....	185
Table 8-2.	Hepatocellular carcinoma and cirrhosis aetiological factors	186
Table 8-3.	Barcelona Clinic Liver Cancer stages of hepatocellular carcinoma	186
Table 8-4.	Alpha fetoprotein diagnostic sensitivity and specificity.....	187
Table 8-5.	Serum biochemistry profiles of subject cohorts.....	187
Table 8-6.	Misclassification matrix from partial least squared discriminant analysis....	193
Table 8-7.	Discriminatory metabolites comparison between subject cohorts.....	193
Table 9-1.	Comparison of subject demographics in ¹ H NMR and UPLC MS studies....	214
Table 9-2.	Demographic profiles for samples used in positive ion mode.....	215
Table 9-3.	Serum biochemistry profiles for samples used in positive ion mode.....	215
Table 9-4.	Demographic profiles for samples used in negative ion mode.....	216
Table 9-5.	Serum biochemistry profiles for samples used in negative ion mode.....	216
Table 9-6.	Positive ion mode discriminatory variables.....	220
Table 9-7.	Positive ion mode discriminatory variables comparisons generated by XCMS driven univariate analysis.....	220
Table 9-8.	Negative ion mode discriminatory variables	223
Table 9-9.	Negative ion mode discriminatory variables comparisons generated by XCMS driven univariate analysis.....	223

Table 9-10.	Variables selected for tandem mass spectrometry studies.....	224
Table 10-1.	Comparisons of subjects recruited for ¹ H NMR and UPLC MS studies.....	240
Table 10-2.	Demographic profiles for samples used in positive ion mode.....	241
Table 10-3.	Demographic profiles for samples used in negative ion mode.....	241
Table 10-4.	Positive ion mode aetiological factors.....	242
Table 10-5.	Negative ion mode aetiological factors.....	242
Table 10-6.	Positive ion mode Barcelona Clinic Liver Cancer staging.....	243
Table 10-7.	Serum biochemistry profiles for samples used in positive ion mode.....	244
Table 10-8.	Serum biochemistry profiles for samples used in negative ion mode.....	244
Table 10-9.	Serum alpha-fetoprotein diagnostic sensitivity and specificity.....	245
Table 10-10.	Positive ion mode misclassification matrix.....	249
Table 10-11.	Negative ion mode misclassification matrix.....	249
Table 10-12.	Discriminatory features identified from multivariate analysis.....	250

LIST OF FIGURES

Figure 1-1.	Estimated global age-standardised liver cancer incidence.....	25
Figure 1-2.	The “angiogenic switch” in HCC.....	38
Figure 1-3.	Warburg theory of heightened glycolysis in tumour cells.....	41
Figure 2-1.	Nuclei with spin align to a magnetic field.....	53
Figure 2-2.	Precession of protons aligned to magnetic field (B_0)	54
Figure 2-3.	Parallel and antiparallel orientation of protons.....	55
Figure 2-4.	Schematic diagram of a nuclear magnetic resonance system	56
Figure 2-5.	Radiofrequency pulse causing transverse net magnetisation	58
Figure 2-6.	Fourier transformation.....	58
Figure 2-7.	Multiple variables are generated from metabolic profiling studies	66
Figure 3-1.	Explanted liver from healthy control and rat with HCC.....	76
Figure 3-2.	Fisher rat plasma proton nuclear magnetic resonance spectra highlighting macromolecular suppression.....	77
Figure 3-3.	Effect of median fold change normalisation on spectral data.....	78
Figure 3-4.	Proton nuclear magnetic resonance spectra of rat groups.....	79
Figure 3-5A.	Principal components analysis of rat plasma	81
Figure 3-5B.	Partial least squared discriminant analysis of rat plasma.....	81
Figure 3-6A.	Principal components analysis of rat plasma	82
Figure 3-6B.	Partial least squared discriminant analysis of rat plasma.....	82
Figure 3-7.	Plasma relative intensities (integral values) of selected metabolites	84
Figure 3-8.	Lipid absorption and hepatic metabolism to lipoprotein.....	87
Figure 4-1.	Resonances from EDTA compared to an EDTA-free serum sample.....	95
Figure 4-2.	Representative plasma spectrum with EDTA exclusion.....	101

Figure 4-3.	Selected spectral regions of difference between median hepatocellular carcinoma, cirrhosis and healthy volunteer group spectra from combined Egyptian and Nigerian cohorts.....	102
Figure 4-4.	Multivariate analyses of combined Nigerian and Egyptian samples	104
Figure 4-5.	Multivariate analysis plots of Nigerian and Egyptian data.....	105
Figure 4-6.	Principal components analysis of male volunteer samples.....	106
Figure 4-7.	Univariate analysis of discriminatory metabolites.....	107
Figure 5-1.	Total ion chromatograms of the rat standard plasma after dichloromethane/methanol or methanol only extraction.....	126
Figure 5-2.	Nigerian, UK and rat serum and plasma total positive ion mode chromatograms following lipid extraction.....	127
Figure 5-3.	Principal components analysis of human and rat positive mode data.....	128
Figure 5-4.	Extracted ion chromatograms for mass 496.34.....	130
Figure 5-5.	Extracted ion chromatograms for mass 758.57.....	130
Figure 5-6.	Mass spectra of feature M496 T104.....	131
Figure 5-7.	Mass spectra of feature M759 T366.....	131
Figure 5-8.	Relative intensities of features M496 T104 (likely to be lysophosphatidylcholine (24, 0, 0)) and M759 T366 in UK, Nigerian and rat cohorts.....	132
Figure 5-9.	Schematic structure of lysophosphatidylcholine (C ₂₄ H ₅₀ NO ₇ P).....	135
Figure 6-1.	Urine magnetic resonance spectra displaying paracetamol metabolites.....	144
Figure 6-2.	Representative magnetic resonance urine spectra.....	146
Figure 6-3.	Magnetic resonance urine spectra (expanded region 3.0–4.2 ppm)	146
Figure 6-4.	Principal components analysis scores plots.....	148
Figure 6-5.	Univariate analysis of discriminatory metabolites creatinine, carnitine, creatine and acetone.....	150
Figure 7-1.	Urinary spectra of patient with glycosuria.....	161

Figure 7-2.	Representative Egyptian subject urine spectra.....	164
Figure 7-3.	Principal components analysis scores plots Egyptian subject groups.....	165
Figure 7-4.	Univariate metabolite statistical analysis.....	168
Figure 7-5.	Schematic of glycine involvement in cellular methylation reactions	172
Figure 8-1.	Barcelona Clinic Liver Cancer algorithm.....	180
Figure 8-2.	UK patient demographics and dietary questionnaire	180
Figure 8-3.	Median proton nuclear magnetic resonance urine spectra.....	188
Figure 8-4.	Principal components analysis all UK urine samples	189
Figure 8-5.	Urine magnetic resonance spectra displaying marked glucose resonances....	190
Figure 8-6.	Urine magnetic resonance spectra displaying marked ethanol resonances....	190
Figure 8-7.	Principal components analysis following exclusions	191
Figure 8-8.	Partial least squared discriminant analysis of UK urine samples	192
Figure 8-9.	Median value scatter plots of discriminatory variables.....	192
Figure 8-10.	STOCSY analysis of region at 2.35 ppm, revealing correlation with Double doublet at 7.21 ppm.....	196
Figure 8-11.	Comparison of median spectra of creatinine (4.06 ppm).....	197
Figure 8-12.	Comparison of median spectra of <i>p</i> -cresol sulphate (7.21 ppm).....	197
Figure 9-1.	Ultra-performance liquid chromatography mass spectrometry total ion chromatograms from a patient with hepatocellular carcinoma	217
Figure 9-2.	Positive ion mode: principal components analysis.....	218
Figure 9-3.	Positive ion mode: principal components analysis following exclusions.....	219
Figure 9-4.	Negative ion mode: principal components analysis.....	222
Figure 9-5.	Negative ion mode: principal components analysis following exclusions....	222
Figure 9-6.	Total ion chromatogram and fragmentation spectrum of mass 204	225
Figure 9-7.	Total ion chromatogram and fragmentation spectrum of mass 229	226
Figure 9-8.	Relative concentration of variables M204-T67 and M229-T94.....	226

Figure 9-9.	Total ion chromatogram and mass spectrum of acetylcarnitine.....	228
Figure 9-10.	Fatty acid β -oxidation and carnitine and acetylcarnitine transport.....	230
Figure 9-11.	Altered glycolytic and fatty acid metabolism in tumour cells for the generation of cellular substrates.....	232
Figure 10-1.	Pie chart of aetiological factors (positive mode).....	243
Figure 10-2.	Representative positive and negative mode total ion chromatograms.....	246
Figure 10-3.	Principal components analysis of UK urine positive ion mode data	247
Figure 10-4.	Principal components analysis of UK urine positive and negative mode data.....	248
Figure 10-5.	Mass spectra of acetylcarnitine standard compared to subject samples.....	252
Figure 10-6.	Mass spectra of feature M187 T253, likely to be <i>p</i> -cresol sulphate	252
Figure 10-7.	Scatter charts displaying group comparisons for features M204 T61 (acetylcarnitine), M302 T334 (dimethylheptanoylcarnitine) and M187 T253 (<i>p</i> -cresol sulphate).....	253

LIST OF ABBREVIATIONS

1-D	One-dimensional
¹ H NMR	Proton nuclear magnetic resonance
AASLD	American Association for the Study of Liver Disease
ADP	Adenosine diphosphate
AFB1	Aflatoxin B1
AFP	α-fetoprotein
AFP-L3	<i>Lens culinaris</i> agglutinin-reactive AFP
AFU	Alpha-l-fucosidase
ATP	Adenosine triphosphate
BCLC	Barcelona Clinic Liver Cancer
CACT	Carnitine-acylcarnitine translocase
cGMP	Cyclic guanosine 3':5' monophosphate
CPMG	Carr-Purcell-Meiboom-Gill
CPT	Carnitine palmitoyltransferase I
DCP	Des gamma carboxyprothrombin
DEN	Diethylnitrosamine
EIC	Extracted ion chromatogram
ESI	Electrospray ionisation
FGF	Fibroblast growth factor
FID	Free induction decay
FT	Fourier transformation
GC	Gas chromatography
GNMT	Glycine N-methyltransferase
GPC	Glycerophosphocholine
GPC3	Glypican-3
HBsAg	Hepatitis B surface antigen
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HDL	High density lipoprotein
HIF 1	Hypoxia inducible factor
HILIC	Hydrophilic interaction chromatography
HIV	Human immunodeficiency virus
HSS	High strength silica
IQR	Interquartile ranges
JUTH	Jos University Teaching Hospital
LC	Liquid chromatography
LDL	Low density lipoprotein
LF	Larmor frequency
LPC	Lysophosphatidylcholine
MAT	Methionine adenosyltransferase
MS	Mass spectrometry
NAD	nicotinamide adenine dinucleotide
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NMOR	N-nitrosomorpholine
NOESY	Nuclear Overhauser enhancement spectroscopy

OLT	Orthotopic liver transplantation
OSC	Orthogonal signal correction
PC	Principal component
PC	Phosphocholine
PCA	Principal components analysis
PDGF	Platelet derived growth factor
PE	Phosphoethanolamine
PIVKA II	Prothrombin induced by vitamin K absence II
PLS-DA	Partial least squared discriminant analysis
PPAR α	Peroxisome proliferator-activated receptor α
Ppm	Parts per million
PSA	Prostate specific antigen
QC	Quality control
RD	Relaxation delay
RF	Radiofrequency
RP	Reversed phase
RPLC	Reversed phase liquid chromatography
SAH	S-adenosylhomocysteine
SAMe	S-adenosylmethionine
STOCSY	Statistical total correlation spectroscopy
TACE	Trans-arterial chemoembolisation
TCA	Tri-carboxylic acid
TGF α	Transforming growth factor α
TGF β	Transforming growth factor β
TGL	Triglycerides
TIGAR	TP53-induced glycolysis and apoptosis regulator20
TMA	Trimethylamine
TMAO	Trimethylamine-N-oxide
TOF	Time-of-flight
TSP	3-trimethylsilyl-(2,2,3,3- $^2\text{H}_4$)-1-propionate
UKCRN	UK Clinical Research Network
UPLC	Ultra performance liquid chromatography
US	Ultrasound
UTI	Urinary trypsin inhibitor
VEGF	Vascular endothelial growth factor
VLDL	Very low density lipoprotein

1 INTRODUCTION

1.1 HEPATOCELLULAR CARCINOMA

Hepatocellular carcinoma (HCC) is the commonest primary tumour of the liver. It is likely to originate from hepatic stem cells, although the exact lineage remains not well established (Mishra *et al.*, 2009; Sell, 2008). Internal and external stimuli, such as viral DNA integration, inflammation and fibrosis, are likely to induce alterations in tumour originator cells leading to apoptosis, cell proliferation, dysplasia and eventually, neoplasia. As with other malignancies, it is likely that genetic abnormalities give rise to uncontrolled cellular division through disruption of proliferative cellular pathways (Vogelstein and Kinzler, 2004).

1.1.1 Epidemiology

Hepatocellular carcinoma is the 3rd most common cause of cancer-related death, worldwide, causing 695, 000 deaths in 2008 (World Health Organisation, 2008). There is a distinct geographical variation in HCC incidence with 85% of cases occurring in the developing world where hepatitis B virus (HBV) is endemic. The highest incidence rates occur in sub-Saharan Africa and Southeast Asia. In 2008, there were 292, 966 cases in China alone (World Health Organisation, 2008) (**Figure 1-1**).

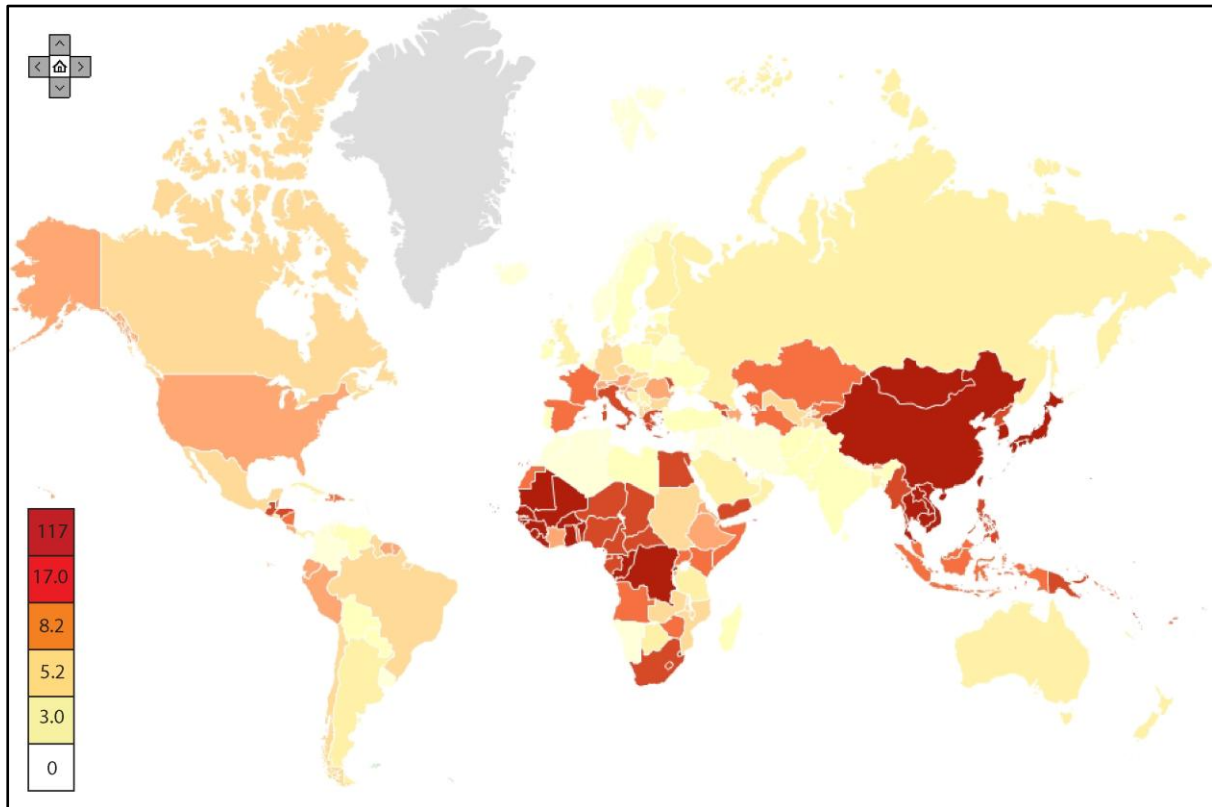


Figure 1-1. Estimated global age-standardised liver cancer incidence.

Key: Rates per 100,000, Adapted from (World Health Organisation, 2008).

In the developed world, the incidence of HCC, apart from in Japan (25,749 cases in 2008), is comparatively low, but there has been a steady overall increase across most western nations over the last two decades (World Health Organisation, 2008).

1.1.2 Aetiology

The prime risk factor for HCC is cirrhosis (Parkin *et al.*, 2001; Parkin, 2001; Parkin, 2006; Shariff *et al.*, 2009). Approximately 80 to 90% of HCCs occur on the background of chronic liver disease, although not all causes of cirrhosis carry the same risk. There is a 2:1 preponderance of male incidence of HCC and risk rises with age, with cases below 40 years being rare (El-Serag, 2011; World Health Organisation, 2008).

1.1.2.1 Viral hepatitis

Patients with viral hepatitis display heightened risk compared to other forms of chronic liver disease. Chronic HBV, in the presence of cirrhosis, increases the risk of HCC 1000-fold (Beasley *et al.*, 1981). Currently, HBV accounts for 50% of cases of HCC worldwide and is now classed as the world's most potent carcinogen after tobacco by the World Health Organisation (Parkin *et al.*, 2001; Parkin, 2001; Parkin, 2006). Hepatocellular carcinoma may also occur on the background of HBV without the presence of cirrhosis, implicating other mechanisms of oncogenesis other than chronic hepatic damage.

Chronic hepatitis C virus (HCV) causes cirrhosis in 30% of patients and 1 to 2% of these will develop HCC per year. Evidence of HCV infection are found in 80 to 90% of patients with HCC in Japan, 44 to 66% in Italy and 30 to 50% in the United States (El-Serag, 2011; El-Serag and Rudolph, 2007). Hepatocellular carcinoma, related to HCV infection, has become the fastest-rising cause of cancer-related death in the United States (El-Serag, 2011).

1.1.2.2 Aflatoxin B1

Aflatoxin B1 (AFB1) is a mycotoxin produced by a fungus of the *Aspergillus* spp., which contaminates a number of tropical and subtropical region staple foodstuffs (grain, corn, peanuts and legumes) (Groopman *et al.*, 1996; Qian *et al.*, 1994). Areas of high HCC incidence and high aflatoxin intake correspond to areas where HBV is endemic. Hepatitis B virus and AFB1 are therefore likely to interact synergistically increasing carcinogenic risk. At the molecular level, once ingested, AFB1 is metabolised to AFB1 –exo- 8,9-epoxide which can bind to DNA and may initiate carcinogenesis through an inactivating mutation of the third base codon 249 of the p53 tumour suppressor gene. This mutation is found in 30-60% of

HCC tumours in aflatoxin endemic areas endemic areas (Bressac *et al.*, 1991; Wild and Turner, 2002).

1.1.2.3 Non-alcoholic fatty liver disease

Non-alcoholic fatty liver disease (NAFLD), in the form of non-alcoholic steatohepatitis (NASH), has been implicated as a risk factor for HCC. This condition often occurs concurrently with features of the metabolic syndrome, including obesity, hypertension and diabetes mellitus. Studies from the United States have shown that historically no underlying risk factor for HCC was identifiable in 30-40% of cases, in the presence of so-called “cryptogenic” cirrhosis and it is likely that these cases were NASH associated (El-Serag and Rudolph, 2007). A retrospective Italian study of 641 cirrhosis-associated HCCs, observed that 44 cases were associated with cryptogenic cirrhosis and in this group the prevalence of obesity and diabetes mellitus was significantly raised (Bugianesi *et al.*, 2002). A Japanese cohort-study followed biopsy-proven NASH patients with advanced fibrosis and observed that the 5-year cumulative incidence of HCC was 7.6%. The burgeoning epidemic of worldwide obesity is likely to make NASH associated HCC a prevalent problem.

1.1.2.4 Alcohol

Heavy alcohol use (40 to 60g per day) is a risk factor for HCC (El-Serag, 2011). The risk is likely to arise through developing cirrhosis. Abstinence does not appear to affect HCC risk once cirrhosis is established, although alcohol has been shown to act synergistically, linearly increasing HCC risk, in the presence of other risk factors, particularly viral hepatitis (Donato *et al.*, 2002).

1.1.3 Prognosis, surveillance and the importance of early tumour diagnosis

The prognosis for patients with HCC remains poor, even in the developed world. In the United States, between 2001 and 2007, 5-year survival rates of those with a solitary HCC were 27%. This drops to 8.7% once the tumour has spread (U.S.National Institutes of Health, 2008).

Early diagnosis of HCC, when the tumour is amenable for surgical resection, ablation or liver transplantation, has been shown to vastly improve patient prognosis. Landmark trials by Mazzaferro and colleagues have established criteria for the selection of patients with HCC who benefit from orthotopic liver transplantation (OLT). If carefully selected, patients with early HCC carry similarly high post-transplant survival rates as for other indications for OLT (Mazzaferro *et al.*, 1996; Mazzaferro *et al.*, 2009). The survival benefit results from smaller tumour size and burden as well as the absence of micro-vascular invasion.

Population based studies have shown conflicting mortality benefit from HCC surveillance programmes. A Chinese study showed that the use of serum α -fetoprotein (AFP) with liver ultrasound surveillance resulted in a 3-year cumulative survival rate of 36% for surveyed patients and 0% for non-surveyed patients ($p<0.0001$) (Yuen *et al.*, 2000). A randomised control trial of nearly 19,000 HBV-infected patients in China showed that 6-monthly serum AFP measurements and liver ultrasound resulted in a 37 % reduction in HCC-related mortality (Zhang *et al.*, 2004). A further randomised control trial of HBV infected patients in China was undertaken in 5000 patients but only using 6-monthly AFP. Tumours were diagnosed earlier but there was no mortality benefit (Chen *et al.*, 2003). Nonetheless, international societies recommend 6-monthly screening by ultrasound (US) +/- serum AFP in

all patients with cirrhosis (Bruix and Sherman, 2005; Bruix and Sherman, 2011; Ryder, 2003).

1.1.4 Diagnosis

Clinical symptoms and signs of HCC are often absent or non-specific. Most cases occur in patients with established cirrhosis and may cause hepatic decompensation, but equally, HCC may lie silent in those with compensated liver disease. The key and as yet, unmet need is to identify small tumours, amenable to curable treatments, in nodular cirrhotic liver parenchyma. In developing countries, medical advice is often sought late resulting in delayed presentation with end-stage signs of a palpable hepatic mass, haemoperitoneum or decompensation with portal hypertensive complications.

Diagnosis is based on the fact that HCCs are highly arterialised, in contrast to the remainder of the liver. The most recent American Association for the Study of Liver Disease (AASLD) guidelines require the presence of features typical of HCC (arterial hypervascularity and venous phase washout) in just one imaging modality for lesions >1 cm (Bruix and Sherman, 2011). Previous guidelines suggested that diagnosis was made by the confirmation of two contrast-enhanced imaging modalities (ultrasound, computed tomography or magnetic resonance imaging) with characteristic features or one imaging modality suggestive of HCC with an AFP level of >400 ng mL⁻¹ (Bruix and Sherman, 2005).

Surveillance for HCC is recommended using US at 6 to 12 month intervals. The use of AFP for surveillance is not recommended unless US is unavailable according to the AASLD guidelines, although this remains controversial (El-Serag, 2011).

1.1.5 Serum markers of hepatocellular carcinoma

1.1.5.1 Serum α -fetoprotein

Serum AFP is the most widely used marker of HCC. It is a fetal glycoprotein which is synthesized *in utero* by the embryonic liver, cells of the vitelline sac and the fetal intestinal tract. Serum AFP is usually undetectable in healthy adults (Gomaa *et al.*, 2009). The production of AFP by HCC cells has been seen as confirmation that the tumour arises from hepatic stem cells as a form of maturation arrest, akin to an embryonic state (Sell, 2008). The AFP gene is located on chromosome 4 (4q11 – 4q13) and is part of the albimunoid super-family of genes which includes the albumin gene (Debruyne and Delanghe, 2008). The function of AFP remains unclear, with some studies suggesting a role in transport of certain molecules such as bilirubin, fatty acids, retinoids, and others a role in immunosuppression (Debruyne and Delanghe, 2008).

Not all HCCs secrete AFP and its diagnostic accuracy is variable. A meta-analysis of AFP for HCC surveillance found that it displayed a sensitivity of 39% to 65% and a specificity of 76% to 94% for tumour diagnosis (Daniele *et al.*, 2004). The cut-off level of AFP was important in determining the diagnostic power. A cut-off of 20 ng mL⁻¹ resulted in a sensitivity of 64% and specificity of 91% (Sherman *et al.*, 1995), while a cut-off of 400 ng mL⁻¹ resulted in a sensitivity of 17% and specificity of 99% (Trevisani *et al.*, 2001). Values of over 400 ng mL⁻¹ are generally considered diagnostic of HCC, although only about 20% of patients with HCC display values this high. Furthermore, patients with chronic viral hepatitis may display a raised AFP during viral flares without the presence of HCC. In a study of 290 Chinese patients with chronic HBV, 44 were found to have elevated serum AFP levels (>20 ng mL⁻¹) and only six (13%) had HCC. The remaining 38 had elevated serum AFP either due to viral flares or unknown causes (Lok and Lai, 1989). Trevisani and colleagues also

observed that an AFP elevation in non-infected patients could be more indicative of HCC when compared to infected patients (Trevisani *et al.*, 2001).

It would appear logical that if AFP is specific to HCC then targeted immunotherapy may be effective in selectively neutralising HCC cells. This, however, is not the case. Because AFP is a “self” antigen, usually secreted *in utero*, targeted therapy may result instead in autoimmunity. Furthermore, Butterfield and colleagues conducted two clinical trials of infused AFP immunostimulant peptides and AFP-stimulated dendritic cells (Butterfield, 2007). Both were unsuccessful in showing any tumour-regressive effects, albeit in patients with advanced disease. In the latter study, there was serological evidence of increased interferon gamma producing AFP-specific T cells, perhaps suggesting that an AFP HCC vaccine is conceivable but immunomodulatory therapy remains unlikely.

1.1.5.2 Lens culinaris agglutinin-reactive AFP

Lens culinaris agglutinin-reactive AFP (AFP-L3) is a glycoform variant of AFP and is expressed as a percentage of the total AFP level. It can be detected in approximately one third of patients with small HCCs (<3cm) when cut-off levels of 10% to 15% are used. At higher cut-off levels of >15%, AFP-L3 displays a sensitivity of 75% to 96.9% and specificity of 90% to 92% (Khien *et al.*, 2001; Kumada *et al.*, 1999). The usefulness of this marker is limited as studies have only been conducted in East Asian populations in whom AFP levels are already raised.

1.1.5.3 Des gamma carboxyprothrombin

Des gamma carboxyprothrombin (DCP) is an abnormal prothrombin protein and is also known as prothrombin induced by vitamin K absence II (PIVKA II). It is produced as a result

of an acquired defect in the post-translational carboxylation of the prothrombin precursor in malignant cells, the gene responsible being *gamma-carboxylase* (Grizzi *et al.*, 2007). In several large studies, serum DCP was found to display poor diagnostic sensitivity (48% to 62%) but good specificity (81% to 98%) for HCC (Grizzi *et al.*, 2007; Marrero *et al.*, 2003). A study comparing the performance characteristics of AFP, DCP and Lens culinaris agglutinin-reactive AFP in the diagnosis of HCC observed that DCP was significantly better than the other markers in differentiating HCC from cirrhosis, with a sensitivity of 86% and a specificity of 93% (Volk *et al.*, 2007). There are conflicting reports, however, with a study by Nakamura and colleagues reporting that the efficacy of DCP was lower than that of AFP in the diagnosis of small tumours, although higher than that of AFP for large tumours (Nakamura *et al.*, 2006).

1.1.5.4 Alpha-l-fucosidase

Alpha-l-fucosidase (AFU) is a glycosidase found in cellular lysosomes and increased activity is found in patients with HCC. Studies of its diagnostic accuracy have displayed high sensitivity (82%) and specificity (70.7 – 85.4%) (Ishizuka *et al.*, 1999; Tangkijvanich *et al.*, 1999; Wang and Cao, 2004). A comparative study of AFP and AFU in an Egyptian cohort found AFU to have a higher sensitivity (81.8% vs. 68.2%) but lower specificity (55% vs. 75%) with a combined AFP + AFU sensitivity of 88.6% (el-Houseini *et al.*, 2005). Unfortunately, AFU has been found to be elevated in other tumours and is therefore not specific to HCC (Gomaa *et al.*, 2009).

1.1.5.5 Glypican-3

Glypican-3 (GPC3) is a heparin sulphate proteoglycan and has been shown to be capable of promoting the proliferation of tumour cells by modulating Wnt pathways and affecting cellular adhesion. As a tumour marker, GPC3 expression has been shown to be elevated in HCC tissue and in serum of 40 to 53% of patients with HCC (el-Houseini *et al.*, 2005). A summary of the performance of the various serum markers is displayed in **Table 1-1**.

Table 1-1. Diagnostic performance of serum markers of hepatocellular carcinoma.

Serum marker	Sensitivity (%)	Specificity (%)
AFP	39 – 65	79 - 94
AFP-L3	75 - 97	90 - 92
DCP	48 - 62	81 - 98
AFU	82	71 - 85

1.1.6 Urinary markers of hepatocellular carcinoma

The concept of urinary markers for HCC is not new and dates back to initial studies in the 1970s. For any urine biomarker three central attributes are necessary for it to fulfil. First, the biomarker, if produced pre-renally, needs to be small enough and of the correct ionic charge to be filtered by the renal glomerulus and not re-absorbed by the tubules. Therefore, it has to be roughly less than 20 kDa in atomic weight. Second, the marker should be specific to the cancer in question and not secondary to the effects of cancer on general physiology. Finally, the marker should be secreted in adequate amounts for accurate, repeatable detection in early disease. Large, complex proteins are unlikely to enter the urinary stream, so are not candidates for urinary biomarkers.

1.1.6.1 Nucleosides

Studies in the 1970s observed elevated levels of the methylated purines 7-methylguanine, 1-methylguanine, *N*-dimethylguanine, 1-methylhypoxanthine and adenine in the urine of patients with HCC. In 1976, it was found that urine levels of cyclic guanosine 3':5' monophosphate (cGMP) were elevated in rats with transplanted liver and renal tumours (Criss and Murad, 1976). In 1982, Dusheiko and colleagues, found parallels in human studies, observing elevated urinary cGMP levels in patients with HCC (Dusheiko *et al.*, 1981). In the same study, cGMP was also elevated in the urine of patients with liver disease and other non-HCC tumours, reducing the specificity of the marker considerably.

In 1986, Tamura and colleagues observed that urinary levels of pseudouridine, a *C*-glycoside isomer of the nucleoside uridine, to be elevated in patients with HCC (Tamura *et al.*, 1986). When combined with serum AFP, sensitivity for HCC detection was 83%. Disappointingly,

this marker was also non-specific and found to be similarly elevated in patients with other malignancies such as non-Hodgkin's lymphoma.

In a Taiwanese patient study it was observed that the urinary nucleosides adenosine, cytidine and inosine were elevated in patients with HCC (Jeng *et al.*, 2009). When combined with serum AFP, sensitivity for tumour diagnosis was 80%. The study was flawed in that controls consisted of healthy patients with no liver disease and ideally the finding should have been confirmed in comparison to a group of patients with cirrhosis.

1.1.6.2 Transforming growth factor α and β

Transforming growth factor α and β (TGF α and β) have both been detected in the urine of patients with HCC. The first report was from 1990, observing elevated TGF α levels in urine (Kato *et al.*, 1990). In 1991, a TGF-related protein was found in HCC patient urine and this was confirmed as TGF β 1 in 1997 by the same group (Chuang *et al.*, 1991) (Tsai *et al.*, 1997). In these studies, TGF β 1 correlated with prognosis and survival. A functional link was attractive as TGFs are known to stimulate non-transformed cells reversibly to grow as colonies *in vitro*.

1.1.6.3 Neopterin

In 1998, a study performed in Japan found neopterin, a protein now known to be released from macrophages following inflammatory stimulation, to be elevated in the urine of patients with advanced HCC (Daito *et al.*, 1992; Kawasaki *et al.*, 1988). Similar to other potential markers, neopterin has since been shown to be elevated in a number of malignancies and pro-inflammatory conditions such as human immunodeficiency virus (HIV) related disease, reducing its validity as a specific marker for HCC (Sucher *et al.*, 2010).

1.1.6.4 Polyamines

The polyamines, organic compounds containing two or more amine groups, include putrescine, spermine, and spermidine. Their exact cellular role is unclear but they are required for cellular proliferation. Putrescine acts on S-adenosylmethionine (SAME), a methylating molecule, to produce spermine which in turn acts on further SAME molecules to produce spermidine (Wishart *et al.*, 2007). Antionella and colleagues reported increased urinary levels of free and acetylated polyamines using HCC patients compared to healthy controls and patients with cirrhosis (Antoniello *et al.*, 1998), although the sensitivity of these markers was found not to be high enough for early tumour detection.

1.1.6.5 Urinary trypsin inhibitor

Urinary trypsin inhibitor (UTI) is a 25 kDa protein thought to be produced by hepatocytes. In 2004, an ELISA-based study observed that urinary UTI was elevated in patients with HCC, albeit not significantly when compared to patients with cirrhosis (Lin *et al.*, 2004). Follow-up studies have found correlations with severity of liver disease and patient prognosis in general but not specifically with HCC (Kikuchi *et al.*, 2009).

1.1.7 The origin and pathogenesis of hepatocellular carcinoma

Genetic profiling studies of HCC tissue have shown several genes to be disrupted through somatic mutations, chromosomal disruption and epigenetic aberration through methylation abnormalities including *p53*, *Rb1*, *β-catenin*, *CMYC* and *survivin*. The WNT-β catenin pathway is the most commonly disrupted pathway, usually as a result of mutations in *CTNNB1*, *AXIN1* genes, *CDH1* epigenetic silencing and changes in expression of WNT receptors from the Frizzles Family (Minguez *et al.*, 2009). Activation of the pathway induces translocation of β-catenin into the nucleus where it regulates specific oncogenes such as *CMYC* and *CCND1*. An initial somatic mutation in an oncogene or tumour suppressor gene is likely to generate a clonal expansion of cells which then have the potential, through further “proliferation advantageous” mutations and chromosomal disruptions, to develop into pre-neoplastic lesions. These lesions, often <1 cm, have been identified in patients with cirrhosis and have been sub-classified into low or high grade dysplastic nodules (Fernandez *et al.*, 2009). The former carry a low risk and the latter a very high risk, of malignant transformation.

1.1.7.1 The “angiogenic switch”

Dysplastic nodules are hypoechoic on ultrasound imaging and derive their blood supply from the portal vein. Established HCC displays typical arterial phase uptake on contrast imaging. At a critical point an “angiogenic switch” is activated which stimulates arterial neo-vascularisation of the nodule and development of an established HCC (**Figure 1-2**). Japanese groups have identified this as a critical moment before which total cure with resection is likely and after which prognosis deteriorates rapidly (Takayama *et al.*, 1998).

Certain factors may contribute to “neo-angiogenesis” of HCCs. Vascular endothelial growth factor (VEGF) and platelet derived growth factor (PDGF) have been implicated as angiogenesis modulators. HCC cell lines may produce VEGF by themselves and increased concentration of VEGF in the serum of patients with HCC has been correlated with outcome after surgical resection (Armengol *et al.*, 2004; Poon *et al.*, 2004). Hypoxia inducible factor (HIF 1), a factor commonly expressed in HCC and heavily influential upon cellular metabolism, has been shown to induce expression of VEGF. A number of oncogenes have also been implicated in angiogenesis such as *ras* and *myc* (Vogelstein and Kinzler, 2004).

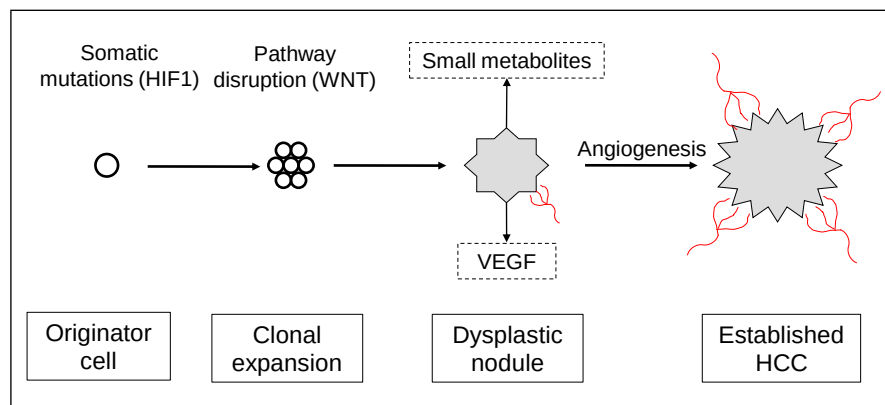


Figure 1-2. The “angiogenic switch” in HCC.

It has been shown that chemotherapeutics active against HCC such as the multikinase inhibitor sorafenib, exert their effects through inhibition of pro-angiogenic factors such as VEGF and PDGF, establishing neo-angiogenesis as a major therapeutic target in HCC (Fernandez *et al.*, 2009). With the onset of neo-angiogenesis, there is likely to be a rapid change in the metabolism of tumour cells and also the surrounding stroma (Fraisl *et al.*, 2008). The importance of the interaction between tumour and stromal cells is becoming increasingly recognised. Vizan and colleagues, studied the metabolic adaption of endothelial cells, to stimulation by VEGF and fibroblast growth factor (FGF). Glycogen synthesis, the pentose cycle and glycolytic pathways were shown to be essential for endothelial cell

proliferation and inhibition of these pathways decreased endothelial cell viability and migration (Vizan *et al.*, 2009). The interaction of cellular metabolism and neo-angiogenesis is therefore crucial to tumour development.

1.18 Treatment options

Hepatocellular carcinoma is curable. The Barcelona Clinic Liver Cancer (BCLC) algorithm is the most widely recognised staging and treatment guidelines (Bruix and Sherman, 2011). Early tumours (single lesions or three lesions all less than 3cm) are amenable to resection, especially if in non-cirrhotic livers, or OLT, in those that are cirrhotic. Indeed, for those tumours less than 2cm, radio-frequency ablation has been shown to be curative (Livraghi *et al.*, 2008). In those patients with intermediate stage multinodular tumours, trans-arterial chemoembolisation (TACE) is recommended. In resource poor settings, simpler, inexpensive techniques, such as ethanol injection, are highly effective in causing tumour necrosis. In those patients with advanced tumours, not amenable to any of the options above, sorafenib, an oral multikinase inhibitor has been shown to provide a survival benefit of 10.7 months versus 7.9 months (Llovet *et al.*, 2008).

1.2 ALTERED TUMOUR METABOLISM

There is increasing evidence that altered metabolism in tumour cells is both a cause and effect of carcinogenesis. Tumour cells require increased amounts of energy and substrates for *de novo* synthesis of nucleotides, lipids, and proteins for rapid proliferation. Otto Warburg, in the 1920s, pioneered the theory of altered tumour metabolism. Recent evidence both supports and disputes his original conclusions.

1.2.1 The “Warburg Effect” and glycolysis

In 1924, Warburg, through placing a section of rat carcinoma in nitrogen-saturated Ringer’s solution (to simulate anaerobic conditions), observed that the tumour could be transplanted to a live donor if sugar was included in the Ringer’s solution, but not if the solution was left plain (Warburg *et al.*, 1924). Following this work, Warburg discovered that even in the presence of oxygen, cancer cells preferentially metabolise glucose by glycolysis as oppose to oxidative phosphorylation, a vastly more inefficient route for energy production. He hypothesised that the increase in glycolysis under normal oxygen conditions arose from a deficiency in the mitochondrial oxidative phosphorylation (Warburg, 1958) (**Figure 1-3**). He thus established that tumour cells take up glucose at high rates to fuel heightened glycolysis. Indeed, it is upon this basis that tumours can be identified with glucose-labelled positron emission tomography (Ariff *et al.*, 2009). Glycolysis generates adenosine triphosphate (ATP) with lower efficiency but at a faster rate than oxidative phosphorylation, which may be of benefit for rapidly dividing cells. The role of mitochondria in tumour cells is contentious. Primary defects in oxidative phosphorylation (which occurs within the mitochondrial membrane) have been invoked to explain the Warburg phenomenon because tumour mitochondria are often small, lack cristae and are deficient in the β -F1 subunit of the ATPase (Kroemer and Pouyssegur, 2008; Lopez-Rios *et al.*, 2007). However, many groups have

demonstrated that tumour cell mitochondria are actually functional and even Warburg admitted that despite their high glycolysis rate, oxygen consumption by cancer cells is not diminished (Weinberg and Chandel, 2009). Furthermore, HCC is a highly vascular tumour that, certainly in the early stages, is likely to be adequately supplied with oxygenated blood. Importantly, glycolysis also provides intermediates for the pentose phosphate pathway and subsequent biosynthesis of nucleic acids. Which of these functions heightened glycolysis serves is, as yet, unresolved.

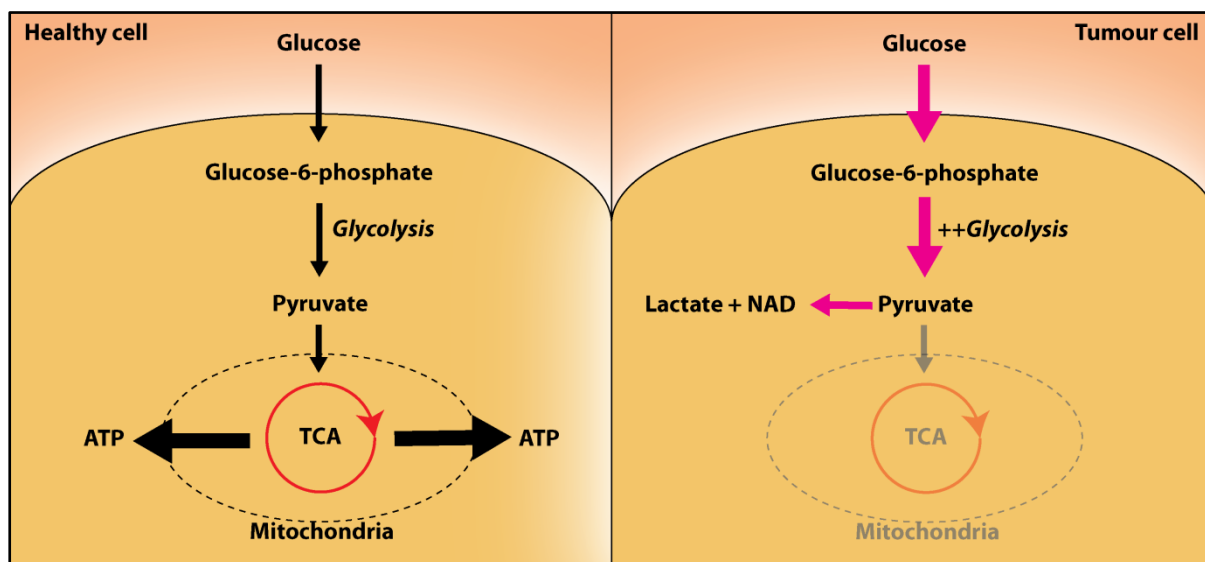


Figure 1-3. Warburg theory of heightened glycolysis in tumour cells.

Key: TCA, tri-carboxylic acid cycle; ATP, adenosine triphosphate; NAD, nicotinamide adenine dinucleotide.

There is now some consensus that the major role of heightened glycolysis in tumour cells is to provide substrates to the pentose phosphate pathway for nucleotide synthesis, rather than energy provision in the form of ATP (Vander Heiden *et al.*, 2009; Weinberg and Chandel, 2009). In essence, the tumour is maximising production of cellular constituents for proliferation at the expense of energy production.

Glutamine, through entering the TCA cycle via a glycolysis-independent pathway, may also provide an alternative route to traditional oxidative phosphorylation for energy production. In vitro studies have identified the importance of glutamine as an important substrate for tumour and in particular, hepatoma cells. Reitzer and colleagues, in 1979, observed that glutamine and not glucose, was the major energy source for cultured HeLa cells (Reitzer *et al.*, 1979). Glutamine is converted to α -ketoglutarate through glutaminolysis, to fuel the TCA cycle. It has also been shown that the higher the level of aspartate aminotransferase activity, a catalyst of glutaminolysis, the higher the rate of cellular proliferation (Sheid *et al.*, 1965). Furthermore, Ehrlich rat hepatoma cells oxidise glutamine at higher rates than any other amino acid (Lazo, 1981; Weinberg and Chandel, 2009).

Both in the presence of tumour and as a stimulant factor, altered lipid metabolism has been implicated. The rate of HCC occurrence in the presence of non-cirrhotic NAFLD is increasingly described, suggesting carcinogenic mechanism in the presence of cellular accumulated lipids other than fibrosis and inflammation. A possible mechanism for this is through TNF α activation, the major molecule involved in hepatic inflammation, which has been shown to promote hepatic insulin resistance which in turn promotes lipid accumulation. Furthermore, TNF and IL-6 are both involved in the activation of JAK/STAT and ERK pathways, which are both crucial in the development of HCC in an obese rat model (Bechmann *et al.*, 2012).

1.2.2 Molecular effectors and tumour metabolism

Several oncogenes and tumour suppressor genes have been implicated in altered tumour metabolism. Sequential mutations are common in HCC and two effectors in particular, HIF 1 and p53, may be responsible for some of the metabolic changes arising in HCC.

1.2.2.1 Hypoxia inducible factor

Hypoxia inducible factor 1 is a heterodimeric protein complex transcription factor that is activated by hypoxic, inflammatory, metabolic and oxidative stress (Harris, 2002; Kroemer and Pouyssegur, 2008; Weinberg and Chandel, 2009). The HIF 1 heterodimeric complex (HIF 1 α + HIF 1 β) is stabilised at low oxygen levels, but degraded by the proteasome in normoxic conditions. The HIF 1 heterodimer stimulates glycolysis by increasing the expression of pro-glycolytic uptake enzymes and transport molecules, such as GLUT 1 and hexokinase (Weinberg and Chandel, 2009). HIF 1 β deficient hepatoma cells grown as solid tumours in mice were found to have reduced rates of growth and glycolytic intermediates compared to wild type hepatoma cells (Griffiths *et al.*, 2002). It would therefore appear that HIF 1 may play a central role in the Warburg model. However, HIF 1 is only stable in hypoxic conditions and Warburg's model describes heightened glycolysis in normoxic conditions. Only a minority of cancers display aberrant HIF 1 function in normoxia, such as renal cell carcinoma (Semenza, 2007). The role of HIF 1 in HCC is still under investigation but a number of recent studies, mostly in animal models, have observed high HIF 1 activity and its downstream counterparts, such as GLUT1, in hepatoma cells (Amann *et al.*, 2009; Wang *et al.*, 2009; Yao *et al.*, 2009).

1.2.2.2 p53

Tumour suppressor genes, such as p53, have also been implicated in alterations in metabolism. Inactivation of p53 can cause the Warburg phenomenon. p53 positively regulates the expression of the protein synthesis of cytochrome c oxidase 2, which is required for the assembly of the oxidative phosphorylation enzyme, cytochrome c oxidase (Matoba *et al.*, 2006) and also negatively regulates phosphoglycerate mutase, a key glycolytic enzyme (Kondoh *et al.*, 2005). In addition, p53 transcriptionally activates TP53-induced glycolysis

and apoptosis regulator (TIGAR) an inhibitor of phosphofructokinase activity which in turn lowers the level of fructose 1,6-biphosphate which acts as an allosteric activator of glycolytic enzymes (Bensaad *et al.*, 2006).

These examples illustrate the evidence that genetic alteration through tumour-driven mutation can affect metabolism. It is likely that many genes and proteins are involved in altered tumour metabolism, with a few taking a lead role.

1.2.3 Metabolite effects on carcinogenesis

Metabolites can affect carcinogenesis and may not be mere by-products of cellular reactions. Lactate, thought to be a “waste” product of glycolysis, may be such a signal. Lactate may stimulate HIF 1 independently of hypoxia (Hsu and Sabatini, 2008) and may condition the tumour environment and suppress anticancer immune effectors (Koukourakis *et al.*, 2006; Kroemer and Pouyssegur, 2008; Swietach *et al.*, 2007).

HIF 1 can also be stimulated by the build up of TCA cycle intermediates, fumarate and succinate. This is evidenced through tumourogenic germline mutations of TCA cycle enzymes fumarate hydratase and succinate dehydrogenase, resulting in an accumulation of fumarate and succinate which competitively inhibit the α -ketoglutarate-dependent HIF-1 α prolyl hydroxylase, the enzyme that targets HIF 1 for destruction (Gottlieb and Tomlinson, 2005).

Through high-throughput liquid-and-gas-chromatography-based mass spectrometry of urine and plasma from patients with prostate carcinoma, Sreekumar and colleagues identified Sarcosine (Sreekumar *et al.*, 2009), a metabolite derivative of glycine, as a marker of the

cancer. Furthermore, exogenous addition of sarcosine to tumour cells, or knockdown of sarcosine degrading enzymes, caused a shift of benign prostatic cells into a malignant phenotype. Although promising, sarcosine has not lived up to this promise in follow up studies by other groups. Jentzmik and colleagues found that urinary sarcosine could not reliably differentiate prostate carcinoma from benign prostatic disease after digital rectal examination (Jentzmik *et al.*, 2010) and Struys and colleagues observed that serum sarcosine levels were not discriminatory between men with metastatic prostate cancer, those with an elevated prostate specific antigen (PSA) and healthy controls (Struys *et al.*, 2010).

1.3 METABOLIC PROFILING

Metabolic profiling is a general term encompassing “metabonomics”, which is the study of global metabolic responses to physiological, drug and disease stimuli (Nicholson and Lindon, 2008) and “metabolomics”, which aims to characterise and quantify all the small molecules in biofluid sample (Fiehn, 2001). With careful attention to study design, true biomarkers of disease can be identified. It is well recognised, however, that gender, age, diurnal rhythm, diet and ethnicity can affect and bias metabolite profiles (Holmes *et al.*, 2008; Psihogios *et al.*, 2008; Slupsky *et al.*, 2007). The largest study of urine metabolic profiles involved over 4,500 volunteers from Japan, China, UK and the USA (Holmes *et al.*, 2008). Geographical and dietary differences appeared to be highly discriminatory, above that of ethnicity and population. Indeed, recruits from the UK and USA were poorly distinguished while those from South East Asia and the Western population were easily distinguished. Furthermore, Japanese individuals domiciled in the US were well discriminated from those in native Japan, promoting the divergence of geography and diet and negating that of race. Vegetarians were also discriminated from non-vegetarians. Metabolites discriminatory in these studies included those closely linked to diet such as creatine and TMAO. Bearing this in mind, metabolic profiling study designs should try and compare dietary homogenous populations.

The most commonly used methods of metabolite characterisation are proton nuclear magnetic resonance (^1H NMR) spectroscopy and mass spectrometry (MS). These techniques are complimentary and each has advantages and disadvantages (**Table 1-2**). Sensitivity of MS is high, in some forms of gas chromatography (GC)-MS reaching femtomolar levels, but samples are degraded during the run and metabolite identification can be challenging (Want *et al.*, 2005; Want *et al.*, 2007). Nuclear magnetic resonance spectroscopy displays lower sensitivity (nano to milli-molar) but samples remain intact and NMR spectral profiles have

been extensively categorised making metabolite identification more straightforward (Holmes *et al.*, 1997; Nicholson *et al.*, 1995; Wishart *et al.*, 2007).

Table 1-2. Comparison of nuclear magnetic resonance and mass spectrometry.

Variable	NMR	MS
Sensitivity	Lower than MS (nanomolar)	Higher than NMR (picomolar)
Sample degradation	No	Yes
Reproducibility	High	Moderate
Metabolite identification	Well categorised	Labour intensive

1.3.1 Proton nuclear magnetic resonance spectroscopy

Nuclear magnetic resonance refers to the behaviour of nuclei subjected to a magnetic field. Hydrogen is the most abundant atom in living organisms and using high power magnetic fields on *in vitro* samples high resolution metabolic spectra can be obtained with clearly defined metabolite peaks of small molecules (<2 kDa). Comprehensive metabolic profiles have been generated from biofluids including urine (Holmes *et al.*, 2008; Williams *et al.*, 2009), serum (Bertini *et al.*, 2009; Gao *et al.*, 2008; Gao *et al.*, 2009; Nicholson *et al.*, 1995), bile (Khan *et al.*, 2005) and intact tissue (Yang *et al.*, 2007).

1.3.1.1 Proton nuclear magnetic resonance spectroscopy and hepatocellular carcinoma

There have been two human studies of HCC using *in vitro* ^1H NMR spectroscopy of tissue samples. Yang and colleagues, using *in vitro* magic-angle spinning ^1H NMR spectroscopy, observed that compared to normal adjacent liver tissue, HCC tissue had elevated levels of

lactate, glutamate, glycine, leucine, alanine, choline metabolites and phosphorylethanolamine, with reduced levels of triglycerides, glucose and glycogen (Yang *et al.*, 2007). Soper and colleagues were able to distinguish normal liver tissue from HCC and cirrhotic tissue with an accuracy of 98% and 92%, respectively although no description of discriminatory metabolites was provided (Soper *et al.*, 2002).

Gao and colleagues utilised 600 MHz ^1H NMR spectroscopy of serum to distinguish cirrhosis and HCC from healthy control specimens, observing that cirrhosis and HCC sera had higher levels of acetate, *N*-acetylglycoproteins, pyruvate, glutamine, alpha-ketoglutarate, glycerol, tyrosine, 1-methylhistidine and phenylalanine, together with lower levels of low-density lipoprotein, unsaturated lipids, isoleucine, valine, acetoacetate, creatine, choline and unsaturated lipids (Gao *et al.*, 2009).

1.3.2 Mass spectrometry

Mass spectrometry has been utilised for metabolic profiling since the 1970s (Pauling *et al.*, 1971). Metabolites, or their constituent fragments, are detected and distinguished by their molecular weight and ionic charge. Due to their complex nature, biological fluids require separation prior to mass spectrometric analysis to achieve detection of as many metabolites as possible. The most common separation methods are GC or liquid chromatography (LC). Gas chromatography requires extensive sample pre-treatment and derivatisation steps. In contrast, LC requires minimal sample preparation and is immediately amenable to biofluid analysis. Ultra performance LC (UPLC) utilises separation columns with much smaller particle size packing material (1.4 -1.7 μm) than traditional columns permitting the injection of liquids at pressures exceeding 10 000 psi thus allowing for improved metabolite resolution. Once

ionised, the particles are detected usually by a time-of-flight (TOF) analyser, which allows the detection of analytes over the range of m/z 50 – 1000 Da.

1.3.2.1 Mass spectrometry and hepatocellular carcinoma

Mass spectrometry has been widely used for metabolic profiling in cancer, including prostate (Sreekumar *et al.*, 2009) and colorectal carcinoma (Qiu *et al.*, 2009). Yin and colleagues identified dihydrosphingosine and phytosphingosine as potential serum metabolite biomarkers of HCC using reversed phase (RP) LC and hydrophilic interaction chromatography (HILIC) MS (Yin *et al.*, 2009). Recently, Chen and colleagues analysed serum and urine samples from 82 patients with HCC and compared these profiles to patients with benign liver tumours and healthy volunteers (Chen *et al.*, 2011). Forty three serum and 31 urine metabolites were differentially present in samples of patients with HCC. These included bile acids, free fatty acids, inosine and histidine.

Wu and colleagues reported a urinary GC-MS study of 20 HCC patients which identified a marker panel of 18 metabolites discriminating HCC and healthy Chinese controls (Wu *et al.*, 2009). This panel included octanedioic acid, glycine and hypoxanthine. In the same year, Chen and colleagues utilised HILIC and RPLC in a comparison of 21 urine samples of patients with HCC to 24 healthy volunteer samples (Chen *et al.*, 2009). In this set, hypoxanthine, creatinine, betaine, carnitine, acetylcarnitine, leucylproline and phenylacetylglutamine were altered between groups.

1.4 RATIONALE FOR STUDY

1. Mortality from HCC remains extremely high, especially in the developing world where surveillance US is not affordable and patients subsequently present late with advanced, incurable disease. There is an urgent unmet need of effective and minimally invasive biomarkers of HCC. A urine test for HCC would be invaluable in this respect.
2. There is limited evidence that HCC induces an altered metabolic phenotype which can be detected in the blood and urine of patients. Thus far, all metabolic profiling studies of HCC have been undertaken in patients from the same country and with the same aetiology of liver disease. Furthermore, the underlying mechanism of how and why the tumour causes these metabolic alterations is not clear.
3. Biomarkers of HCC perform poorly and are confined to blood tests which may be prohibitively expensive and unavailable in parts of the developing world. Alterations in the urine metabolic profiles due to HCC may be amenable for development into a non-invasive test for HCC.

1.5 HYPOTHESES

1. The presence of HCC results in altered blood and urinary metabolic profiles which can be characterised using ^1H NMR and LC/MS.
2. Metabolic blood and urine profiles of HCC can be modelled to allow accurate disease diagnosis.

1.6 OVERALL AIMS

1. To characterise blood ^1H NMR and LC/MS metabolic profiles of the HCC in a Fisher rat model of HCC.
2. To characterise blood ^1H NMR and LC/MS metabolic profiles of HCC in aetiologically-and culturally-diverse human populations (Nigeria, Egypt and UK).
3. To evaluate the diagnostic accuracy of blood and urine metabolic profiles in discriminating samples from patients with HCC from cirrhosis and healthy controls.

2 METHODOLOGY

2.1 PRINCIPLES OF NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

2.1.1 Nuclear magnetic resonance and the property of spin

Nuclear magnetic resonance refers to the behaviour of atoms subjected to a magnetic field, first described in 1946 by Bloch and Purcell (Gadian, 1995; Keeler, 2005). Atoms with an odd mass number such as proton (^1H), phosphorus-31 (^{31}P) and carbon-13 (^{13}C) possess the quantum property of “spin” and behave as dipoles aligning along the axis of an applied magnetic field (**Table 2-1**). As hydrogen is the most abundant element in organic materials, it is the most commonly studied nuclei in human biofluid NMR.

Table 2-1. Common nuclei studied using nuclear magnetic resonance spectroscopy.

Nucleus	Spin quantum number	Natural abundance (%)
^1H	$\frac{1}{2}$	99.98
^{13}C	$\frac{1}{2}$	1.1
^{19}F	$\frac{1}{2}$	100
^{31}P	$\frac{1}{2}$	100

2.1.2 Spin orientations and energy levels

A spinning charge, such a proton, generates a magnetic field and the resulting “spin magnet” has a magnetic moment (μ) proportional to its spin. Nuclei with spin possess a certain number of orientations dependent on the formula: $2I+1$; where I = overall spin. Therefore, hydrogen nuclei, with a $\frac{1}{2}$ integer overall spin, will have two possible orientations. (**Figure 2-1**). In the presence of an external magnetic field (B_0) the protons behave like tiny bar magnets and align along the field. Because the nuclei have spin and obey the laws of quantum mechanics, the magnetic moment of the nuclei will align parallel or anti-parallel to the applied field.

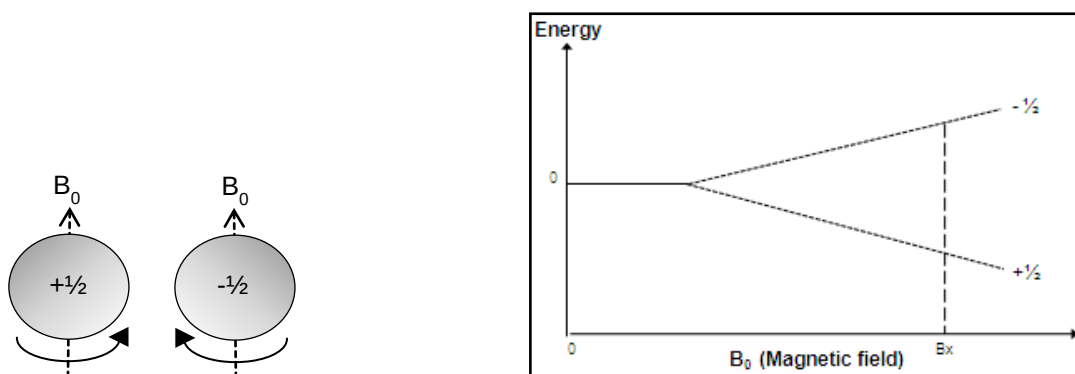


Figure 2-1. Nuclei with spin align to a magnetic field.

If the proton spin is aligned parallel with the field, a lower energy state is required, $+1/2$, while those protons that spin anti-parallel to the field, $-1/2$, require a higher energy state. In the absence of an external magnetic field there is no overall magnetisation of the nuclei as an equal number of opposing spinning protons cancel each other out.

The difference in energy states between the two orientations of the proton is very small and is proportional to the strength of the applied magnetic field (B_0). If there is no magnetic field, the difference in energy states is zero, while the difference increases with the strength of the external field (**Figure 2-1**). Strong magnetic fields are required for high resolution magnetic resonance spectroscopy. Magnetic field strength is measured in Tesla (T) and the earth's magnetic field is approximately 10^{-4} T at ground level. In comparison, magnetic resonance spectrometers use powerful magnets with field strengths from 1 to 20 T.

2.1.3 Precession and the Larmor frequency

Because protons possess angular momentum, they do not align themselves completely and instead “precess” around an axis aligned with the field (**Figure 2-2**). The frequency with which the proton precesses depends on the strength of the magnetic field and the gyro-magnetic ratio of the nucleus and is described by the Larmor frequency (LF), equation:

$\omega_0 = \gamma B_0$; where ω_0 is angular frequency (LF) and γ is the gyro-magnetic ratio.

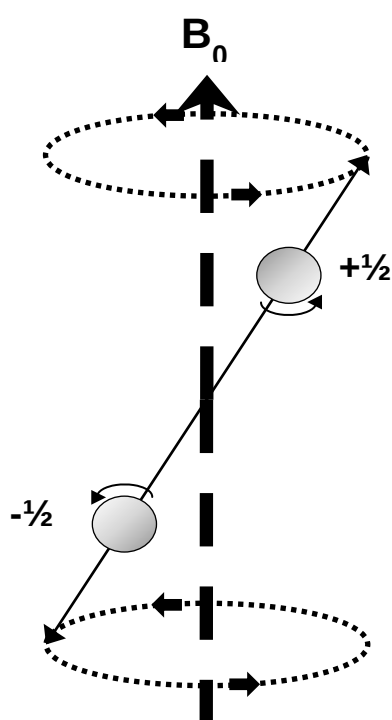


Figure 2-2. Precession of protons aligned to magnetic field (B_0).

Therefore, protons will precess faster with an increasing strength in the magnetic field. If the sample is irradiated with a radiofrequency pulse at the same frequency as LF, the protons absorb the energy and are promoted to the less favourable anti-parallel energy state ($-1/2$). This energy absorption is called “resonance” because the frequency of irradiation energy is the same as, or resonates, with that of the precession frequency.

Within a given experimental sample there will be a high number of protons, typically 10^{18} or greater. The process of NMR involves all of these protons. At room temperature, with no externally applied magnetic field, nuclei will be distributed evenly throughout all spin states. Since the energy difference between opposing spin states is comparatively small, energy from thermal collision will be enough to promote some nuclei into higher energy states. Therefore, the number of protons in each state will be almost equal, giving no net magnetisation of the sample. With the application of a magnetic field, even if very strong, the number of protons aligned with the field is only slightly greater than those opposing the field (**Figure 2-3**). This results in a net magnetisation of the sample in the direction of the field.

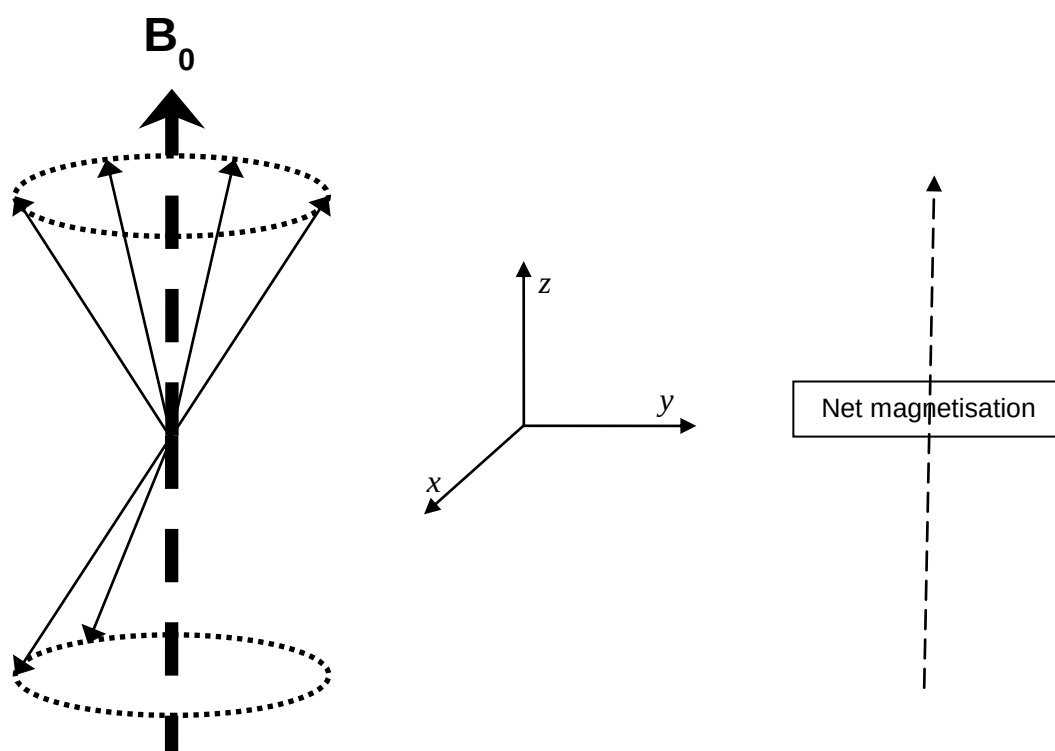


Figure 2-3. Parallel and antiparallel orientation of protons.

In the presence of B_0 , a small excess of protons align with the field than against it resulting in a net magnetisation in the direction B_0 .

2.1.4 Nuclear magnetic resonance systems

Magnetic fields of the strength used for *in vitro* high resolution NMR spectroscopy can only be generated by superconducting magnets. The field is generated by passing a current through a wire coil. The wire is super-cooled to <6 K and is composed of filaments of one metal or alloy embedded in another (solenoid: typically tin, copper and niobium) which allows its conductive resistance to be reduced to almost 0, thereby allowing high currents to flow and perpetually persist. The wire coil is immersed in liquid helium which in turn is surrounded by a bath of liquid nitrogen (77 K) to prevent the helium from evaporating. Passing vertically through the centre of the machine is a room temperature zone into which the sample is placed (Figure 2-4).

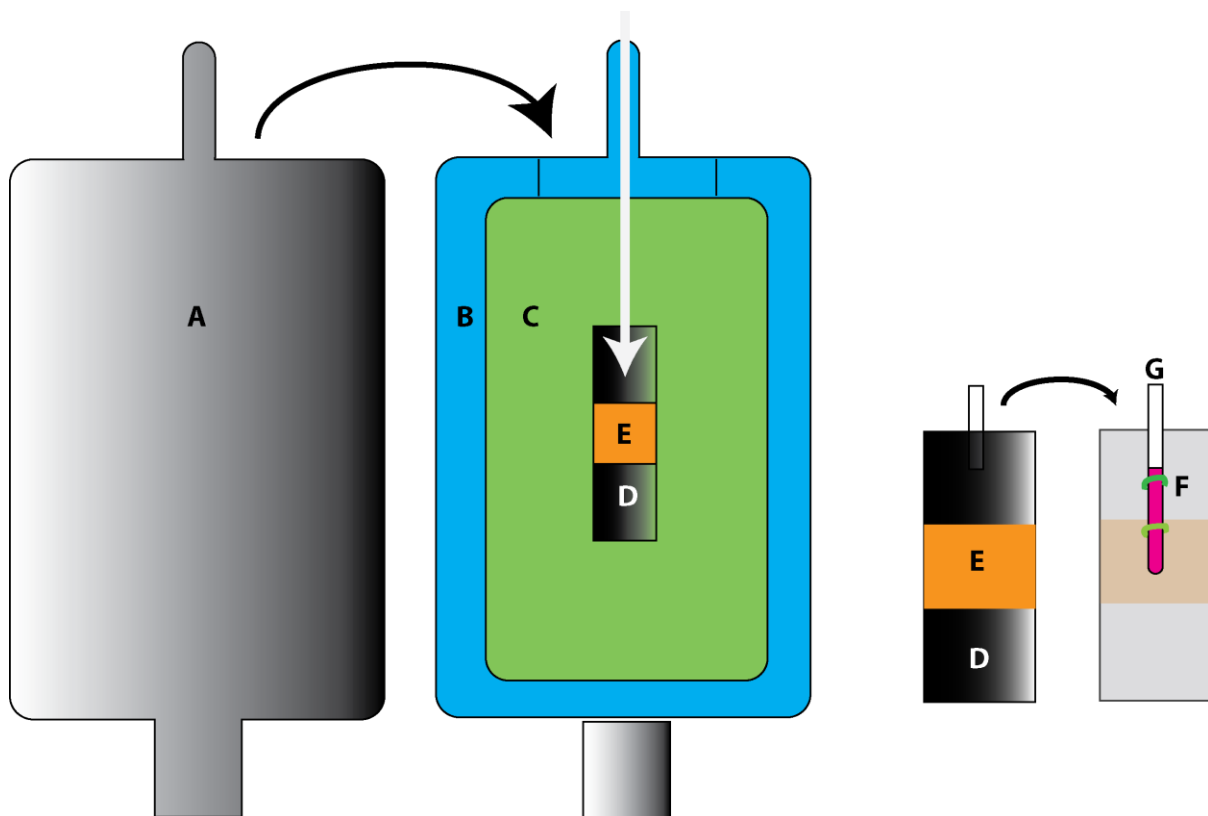


Figure 2-4. Schematic diagram of a nuclear magnetic resonance system.

Key: A, outer shell; B, liquid nitrogen; C, liquid helium; D solenoid wire coil; E, shim coils; F, receiver and radiofrequency pulse coils; G, biofluid sample.

2.1.4.1 Shimming

To obtain precision the magnetic field needs to be as homogenous across the sample as possible. The magnet alone, even though it generates a very strong field, is not able to achieve this. Shim coils are small wire coils placed strategically around the sample, through which current is transmitted, generating small, local magnetic fields which can be individually altered to provide a near homogenous field.

2.1.4.2 Tuning and locking

“Locking” the field is performed to compensate for small, undesired fluctuations in field homogeneity. This is achieved by continuous monitoring of a designated signal (usually deuterated hydrogen) and adjusting the field by small amounts to keep this signal at the same frequency.

2.1.5 Signal generation

Given that the net magnetisation of the sample is in the z-plane (B_0), it is impossible to detect a signal as the NMR receiver coils lie in a different plane to that of the large, externally applied magnetic field. If the net magnetic moment component were tilted, for example by 90° , then a signal could be detected by a receiver coil. This “tilting” is achieved by irradiating the protons with a radiofrequency (RF) pulse at the LF (**Figure 2-5**). The absorption of energy has two effects. First, more protons are promoted to the higher energy state (opposing B_0) and this therefore decreases net magnetisation in the direction of B_0 . Second, the RF pulse causes the protons to precess in phase, which results in the alignment of the charge and therefore a net magnetisation in the y-plane which can be detected by receiver coils inside the magnet.

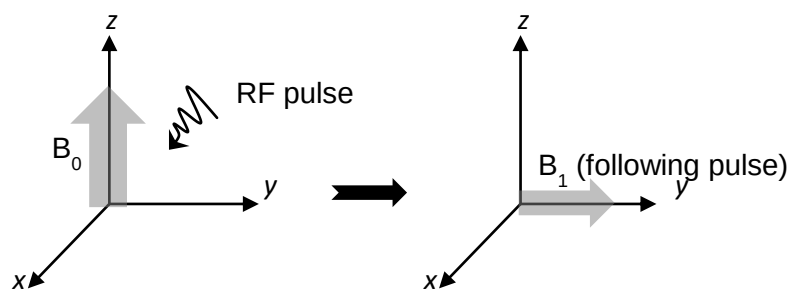


Figure 2-5. Radiofrequency pulse causing transverse net magnetisation.

2.1.6 Fourier transformation

The signal generated is referred to as a free induction decay (FID) and contains all the time-dependent proton resonances from the samples. This is not possible to interpret so is transformed from a time-dependent to a frequency-dependent signal through the mathematical process of Fourier transformation (**Figure 2-6**).

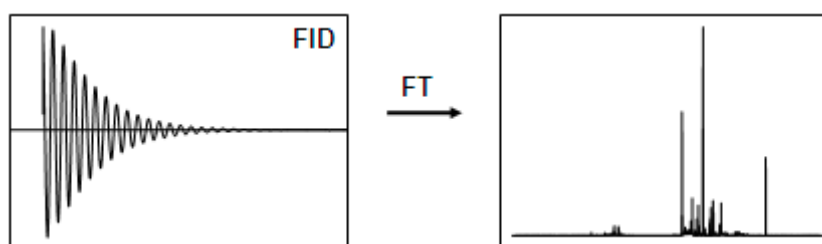


Figure 2-6. Fourier transformation.

Key: FID, free induction decay; FT, Fourier transformation.

2.1.7 Chemical shift

The molecular surroundings of each proton are different due to interactions from other protons, bonds and electron shielding. Thus, the resonance frequency of protons will also be different. After Fourier transformation, the proton resonances are measured according to their

frequencies. This is converted to a chemical shift parts per million (ppm) scale by subtracting a reference frequency (a molecule with a known, stable resonance frequency) and dividing by that frequency.

2.1.8 Coupling

Some molecules generate a single resonance (singlet). Protons may interact with other closely bonded protons to produce two or more resonances (doublets or multiplets). This feature, known as *J-J* coupling, can be used for metabolite identification, either through calculation of chemical structure depending on the coupling profiles or with known multiplet patterns occurring at particular resonances.

2.1.9 Pulse sequences

Characteristics of the molecular surrounding of the proton can also be manipulated by using certain RF pulse sequences and strengths. For example, a nuclear Overhauser enhancement spectroscopy (NOESY) sequence which uses three sequential 90° pulses will allow the detection of higher and lower molecular weight samples, while that of the Carr-Purcell-Meiboom-Gill sequence (one 90° pulse and a repetitive loop of 180° pulses) suppresses larger weight molecular resonances and accentuates lower weight species.

2.1.10 Proton nuclear magnetic resonance spectroscopy processing

2.1.10.1 Phase correction

Protons will resonate in the form of a sine wave and each of these waves will possess a phase. Not all the protons in the samples will possess the same phase. Therefore, when the FID is Fourier transformed, some resonances will display positive peaks, some will display negative peaks and some in between. By multiplying the spectrum by a phase correction, which can be

applied to the whole spectrum, all peaks can be corrected to positive and symmetrical line shapes.

2.1.10.2 Baseline correction

Baseline errors can be corrected using automated or manual software so that all spectra have a baseline starting at a y -value of 0.

2.1.10.3 Exponential weighting or line-broadening functions

To reduce the contribution of background noise in the spectrum, the FID can be multiplied by a weighting or “line-broadening” function. This has the effect of truncating the FID tail, so that resonances from noise are not Fourier transformed resulting in an increased signal to noise ratio.

2.1.10.4 Zero-filling

The NMR signal is collected as a series of data points by the receiver coil. By adding an equal number of zeros to the original number of data points (done simply by the computer) the number of data points increases, which in turn improves line shapes in the resultant NMR spectrum. This manipulation has no effect signal resolution.

2.2 PRINCIPLES OF MASS SPECTROMETRY

The premise of MS is simple: measure the weight of an ion to determine what it is. This is no different for high or low molecular weight MS. Thus the first principal is the generation of ions into a gaseous state from the compound of interest. Ions are generated so they can be manipulated by electromagnetic fields and passed through the MS instrument.

2.2.1 The ion source

The ion source serves to create ions. There are many different methods of ionisation including chemical, electrical, desorption and field. Some cause extensive compound fragmentations (hard ionisation) and some less so (soft ionisation). In liquid state samples, the liquid is introduced into the ion source as nebulised gas droplets which are subsequently ionised. For all the studies presented in this thesis, electrospray ionisation (ESI) was used. Electrospray ionisation, developed by Fenn (Fenn, 2002), is well adapted to UPLC-MS and functions by passing a large voltage through the tip of an introducing nozzle through which the dissolved analytes pass. This effects the transformation of the liquid into charged droplets which evaporate solvent incrementally increasing the charge density across the particle. At a critical point like charges in the droplets repel resulting in the formation of ions.

2.2.2 Mass analysers

Once the ions are formed they need to be separated according to their masses. More accurately, the system bases this differential on the ion's mass: charge ratio (m/z). All mass analysers use static or dynamic electric and magnetic fields alone or combined. The quadrupole analyser is made up of four rods arranged circumferentially around the ion path. Using the stability of trajectories in oscillating fields to separate the ions, the quadrupole can be manipulated to selectively allow ions of a particular m/z ratio through or selectively allow

lighter ions through before heavier ones. The TOF analyser is a flight tube in which there is no electro-magnetic field. Here, ions are separated according to their velocities after they have been accelerated by a preceding electric field. The mass analysers used in the experiments described in this thesis consisted of a quadrupole followed by TOF tube (Q-TOF).

2.2.3 Ion detectors

The ions are detected at the end of the of the ion path once they discharge upon the detector. Many types of detector exist but all use the same principal of determination dependent on ion charge, weight or velocity. Therefore final readings are a measurement of total ion count.

2.2.4 Tandem mass spectrometry

Many metabolites may have the very similar masses, even to one decimal place. Tandem mass spectrometry, also known as fragmentation or MS-MS, can be undertaken to identify molecules. The instrument will contain a collision cell, usually placed after the quadrupole, which is filled with an inert gas such as argon. Ions can be selectively passed into the cell whereupon a variable voltage from 5 to 30 eV is applied which has the effect of fragmenting the ion into its constituent ions. Most ions will produce a signature fragmentation pattern which may be identifiable using databases or standards.

2.2.5 Chromatography

Chromatographic separation of a sample is usually performed prior to MS by passing the sample over a chromatographic column. The chemical attributes of the column cause certain molecules to stick and others to flow through. Inorganic and organic solvents are used at varying proportions to elute molecules at different times, generating a chromatogram of a

sample. The column can be very tightly packed with retention compounds allowing for higher injection pressures (high or ultra-high pressures) subsequently producing higher resolution chromatograms (Want *et al.*, 2007). Chromatographic separation also ensures a steady stream of molecules entering the ion source, rather than large batches. If too many molecules enter together then ionisation will be inefficient due to a limited amount of charge available to all species. This phenomenon is known as “ion suppression” and can have deleterious effects on sensitivity.

2.2.6 Data pre-processing

The datasets produced are complex. Each sample will provide both chromatographic retention time and mass spectrometry data. Prior to any form of comparative analysis it is essential that the data are aligned so that an analyte with a particular m/z and retention time has the same m/z and retention time in all the samples. Correction of m/z drift is straightforward with the continuous direct injection of a known reference standard, such as leucine-enkephalin, m/z 556.2771, utilised as a “mass lock”. Retention time drift, due to sample composition, column variability with time and environmental factors, is more difficult to correct for. Many software programmes are now available for data processing to resolve this problem. For these experiments the data processing software XCMS was used (Scripps Center for Mass Spectrometry, La Jolla, San Diego, California, USA) (Smith *et al.*, 2006) (<http://metlin.scripps.edu/download>). XCMS performs three functions resulting in comparable data information:

- i. Peak detection. The algorithm used is based on cutting the data into slices a fraction of a mass unit ($0.1\ m/z$) and operating on individual slices in the chromatographic

time domain. The signal is determined by taking the maximum intensity at each time point in the slice (Smith *et al.*, 2006).

- ii. Peak matching. After identifying peaks they must be matched across all samples. Overlapping fixed-interval bins of 0.25 m/z width are generated to match peaks. Because the bins are overlapping, the algorithm counts the same peak twice and subsequently removes peak groups originating from overlapping bins resulting in a single peak per bin. Once peaks have been matched by mass, they are matched by retention time. This is performed by calculating the overall distribution of peaks in chromatographic time and identifying the boundaries of regions where many peaks have similar retention times. The distribution of peaks or “meta-peaks” are defined in a fixed interval in which all peaks are placed into a group (Smith *et al.*, 2006).
- iii. Retention time correction. From the initial grouping, the algorithm then identifies those peak groups in which very few samples have no peaks assigned and very few samples have more than one peak assigned. The algorithm calculates the median retention time and deviation from median for every sample in that group. Consequently, a detailed, non-linear retention time deviation contour can be built for each sample and peak groups can be corrected for retention time differences (Smith *et al.*, 2006).

The resulting data matrix displays sample identification (observations) against unique mass (in Da) / retention time (“T” in sec) “features” (variables), each number corresponding to the intensity for that feature in a particular sample. The software also performs a simple univariate *t*-test between two selected groups identifying those features the levels of which

are significantly different between those two groups. The features are listed in order of significance and accompanying extracted ion chromatograms (EIC) are generated to aid visualisation. Ultra-performance liquid chromatography with XCMS data processing has been validated for use in metabolic profiling experiments (Nordstrom *et al.*, 2006).

2.3 MULTIVARIATE STATISTICAL ANALYSIS

The data generated by multi-sample NMR spectroscopy and LC-MS studies can generate 30,000 variables (**Figure 2-7**). Much of these data, however, are collinear. As analyses are often untargeted, in that it is not known what differences may be present between groups, an alternative method of statistical comparison is required. Multivariate analysis allows the important differences between groups of data to be rapidly visualised reducing multidimensional data to two or three variables. Furthermore, the differences between patient groups may be characterised by a group of metabolite ratios rather than a single metabolite.

Multivariate statistical analysis in the form of principal components analysis (PCA) and partial least squared discriminant analysis (PLS-DA) were used for analyses in the studies presented in this thesis (Trygg *et al.*, 2007).

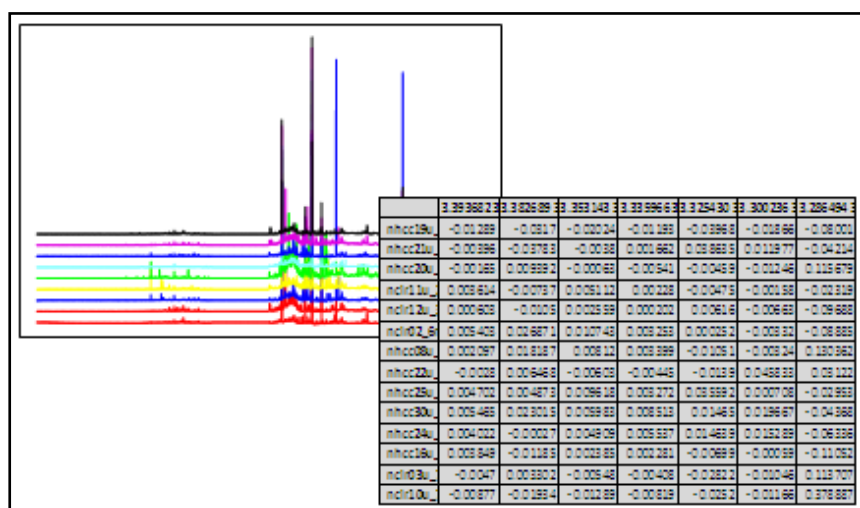


Figure 2-7. Multiple variables are generated from metabolic profiling studies.

2.3.1 Principal components analysis and outlier exclusion

Principal components analysis is an unsupervised analytical tool that provides an overview of complex data through an examination of the covariance structure, highlighting sample outliers and clustering. This is done by converting each sample into a coordinate in n -dimensional space based on its metabolic profile and calculating the factors (which are combinations of these vector co-ordinates) which contribute to the greatest variation across a group of samples. Orthogonal factors, or principal components (PC), are then plotted against each other. Essentially, new variables (principal components) are created based on the variance between metabolite profiles between samples and the samples plotted as coordinates with the new variables acting as x , y and occasionally, z axes. In this manner, samples which are similar cluster and those that are different spread apart. A measure of distribution, akin to Gaussian distribution, can be overlaid to identify those samples that are outside of the 95% confidence level of distribution (the Hotelling's or Mahalanobis' distance). These samples are classed as outliers and it is likely that they contain an abnormally high or low level of a particular metabolite making them unrepresentative of the group. Furthermore, because PCA is a measure of variation, these samples are likely to influence multivariate analysis, heavily weighting principal components. It is therefore reasonable to exclude these samples on the basis of unfair bias.

2.3.2 Partial least squared discriminant analysis

Partial least squared discriminant analysis is a supervised analytical method which relates metabolite data to class membership, elucidating separation between the groups. As with PCA, each sample is placed in n -dimensional space based on its metabolic profile, but instead of variation between samples, variation between groups is modelled allowing the elucidation of variable differences between groups. Furthermore, models can be created to test the

accuracy of this multivariate model in predicting the origin of a new sample. These statistical techniques can be applied to data matrices generated from ^1H NMR and UPLC-MS. Both produce matrices with hundreds to thousands of variables whereby multivariate analyses allow visualisation of two or three variables (principal or partial least squared components), far more interpretable to the investigator.

3 PLASMA PROTON NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY PROFILING OF A RAT MODEL OF HEPATOCELLULAR CARCINOMA

3.1 ABSTRACT

Introduction: There are several rat models of HCC, however, only two studies have previously characterised the ^1H NMR spectroscopic changes in HCC and these were of tumour tissue. There have been no reports of ^1H NMR spectroscopy analysis of blood from rat models of HCC. Quinolones are known to reduce the inflammatory component of liver fibrosis reducing end-stage complications. The primary aim of this study was to characterise the blood metabolic profile of HCC in a rat model of HCC and the secondary aim was to evaluate the effect of the quinolone, norfloxacin on metabolic profiles. **Methods:** HCC was induced in 10 Fisher rats by administration of intra-peritoneal diethylnitrosamine (DEN) and oral *N*-nitrosomorpholine (NMOR) and plasma was collected upon sacrifice. Five of these rats were concomitantly administered oral norfloxacin. Six Fisher non-treated rats acted as healthy controls. Proton NMR spectra were acquired for all samples using a 600 MHz NMR system and results were analysed by visual comparison of median spectra and multivariate analysis. **Results:** Control animals were 120 g heavier than diseased counterparts. Proton NMR spectra from diseased rats displayed significant decreases in lipoproteins, unsaturated fatty acids, acetyl-glycoprotein, acetoacetate, and glucose ($p \leq 0.001$). Plasma citrate and formate levels were increased ($p = 0.02$). Norfloxacin appeared to abrogate this effect slightly. **Conclusion:** The spectral profiles of plasma in rats with HCC display marked changes with relation to lipid metabolism and cellular turnover. Norfloxacin appears to moderate these metabolic alterations to a small degree.

3.2 INTRODUCTION

Human ^1H NMR spectroscopic studies of blood in patients with HCC have identified a number of altered metabolites, implicating changes in hepatic function, lipid metabolism and bile acid metabolism (Gao *et al.*, 2008; Yin *et al.*, 2009). Many rat models of HCC exist, however there are no reports of ^1H NMR spectroscopic studies of rat serum or plasma. To characterise the serum or plasma metabolic changes in an animal model of HCC would provide valuable translatable information to human disease.

There have been two reports of rat HCC tissue ^1H NMR metabolite profiling studies. Tesiram and colleagues performed a meticulous study using ^1H NMR spectroscopy to investigate the changes of lipid metabolites in a Fisher rat model of hepatic adenoma induced with a choline deficient diet (Tesiram *et al.*, 2005). It was demonstrated that a substantial increase in glycerol backbone-containing phospholipids increased throughout adenoma development. Hepatocellular carcinoma did not develop in these animals but changes described in adenomas are likely to share similarities with HCC. A second ^1H NMR spectroscopy study, performed in male Sprague-Dawley rats with DEN induced HCC, reported raised low, very low and high density lipoprotein (LDL, VLDL and HDL) levels in addition to alterations in branch chain amino acids, acetate, glutamine, choline, trimethylamine-*N*-oxide, glycine, glucose and glycogen (Wang *et al.*, 2011). Whether these metabolic profiles are reflected in blood has not previously been investigated using ^1H NMR spectroscopy.

Quinolones are administered to patients with decompensated cirrhosis who develop spontaneous bacterial peritonitis in an antimicrobial capacity. It has also been reported that quinolones improve survival and decrease incidence of end-stage complications of liver disease, such as hepatorenal syndrome (Fernandez *et al.*, 2007). Their effect might be

mediated through a decrease in gut bacterial translocation reducing the pro-inflammatory effectors of liver fibrosis (Gomez-Hurtado *et al.*, 2011).

3.2 AIMS

The primary aim of this study was to ascertain whether altered metabolic profiles could be characterised in a rat model of HCC using ^1H NMR spectroscopy. The secondary aim was to investigate the effect of the quinolone, norfloxacin, on HCC-induced ^1H NMR plasma profile changes.

3.4 METHODS

3.4.1 Fisher rat model of hepatocellular carcinoma

All Fisher rat plasma samples were kindly provided by Dr Fatma Mohamed and Professor Rajiv Jalan, Royal Free Hospital, University College London. Hepatocellular carcinoma was induced by the administration of two potent carcinogens, DEN (Sigma-Aldrich, Gillingham, UK) and NMOR (Sigma-Aldrich, Gillingham, UK). This model was chosen owing to the rapid rate of development of HCC (14 weeks) allowing for rapid assessment of blood profiles of disease. Male Fisher rats had been divided into three groups: Group 1 ($n = 5$) consisted of rats which received 100 mg kg^{-1} intra-peritoneal DEN and NMOR at 80 ppm in drinking water *ad libitum* until sacrifice at 14 weeks; Group 2 ($n = 5$) also received the same doses of DEN and NMOR in addition to $20 \text{ mg kg}^{-1}/\text{day}$ gavaged norfloxacin (Dr Mohamed and Professor Jalan, who donated the plasma samples, are investigating the effects of antibiotics on liver inflammation); Group 3 ($n = 6$) received no treatment and acted as healthy controls (**Table 3-1**). All rats in groups 1 and 2 developed multifocal HCC, group 1 displaying a greater extent of liver inflammation than group 2. All rats in group 3 had normal liver histology. All animals were sacrificed at 14 weeks whereupon blood was venesected from the abdominal aorta into heparin containing tubes and livers extracted for histological analysis. All samples were centrifuged and snap frozen in liquid nitrogen and kept at -80°C until analysis.

Table 3-1. Fisher rat groups and interventions; groups 1 and 2 developed HCC.

Intervention	Group 1	Group 2	Group 3
<i>n</i>	5	5	6
Diethylnitrosamine (DEN)	100 mg kg^{-1}	100 mg kg^{-1}	0
N-nitrosomorpholine (NMOR)	80 ppm in water	80 ppm in water	0
Norfloxacin	0	$20 \text{ mg kg}^{-1}/\text{day}$	0

3.4.2 Sample preparation

Samples were prepared according to standard validated protocols (Beckonert *et al.*, 2007). Samples were thawed at room temperature and 200 μ L were transferred into a 1.5 mL Eppendorf (Cambridge, UK) tube to which 400 μ L NaCl / D₂O (90% / 10%) were added. External reference standards, such as 3-trimethylsilyl-(2,2,3,3-²H₄)-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 line width. The mixture underwent centrifugation for 5 min at 13, 000 rpm and 550 μ L of supernatant were transferred to Norell, 5 mm 507-HP-7 NMR tubes (Norell, Landisville, New Jersey, USA) ready for ¹H NMR analysis.

3.4.3 Proton nuclear magnetic resonance spectroscopy

All samples were run at the department of Biomolecular Medicine, Imperial College London. Samples were run in a random non-grouped order under automation on a Bruker Ultrashield PlusTM 600 NMR system operating at 600.22 Hz ¹H frequency (Bruker Biospin, Rheinstetten, Germany) fitted with a super-cooled probe head, containing the receiver and radiofrequency coils, cooled to almost 0 K by use of liquid helium (CryoprobeTM, Bruker Biospin, Rheinstetten, Germany). The system was tuned, matched and frequency locked onto proton 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. Pulse programme and acquisition parameters were set according to optimised protocols for blood from the department of Biomolecular Medicine, Imperial College London. Spectra were acquired using NOESY and Carr-Purcell-Meiboom-Gill sequences at 300 K. One-dimensional (1-D) NOESY pulse sequence was used 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, 64 free induction decays (FIDs) were collected into 32, 000 data points with a spectral width of 20 ppm. Proton NMR spectra were also acquired using a CPMG-presat sequence (Beckonert *et al.*, 2007) to attenuate broad signals from macromolecules such as protein and lipid. For CPMG-presat experiments, the following pulse sequence was used: -RD-90°-(t -180°- t) $_n$ -ACQ, where t is the spin echo delay, n is the number of loops and 180° corresponds to a 180° RF pulse. Here, RD = 2 s , t = 0.0002 s , n = 160 , acquisition time = 2.73 s and for each sample, 128 FIDs were collected into 32, 000 data points with a spectral width of 20 ppm. A line broadening function of 0.3 Hz was applied prior to Fourier transformation. Proton NMR spectra were manually phased, baseline corrected and referenced to α -glucose at 5.23 ppm in TOPSPIN v2.0 (Bruker Biospin, Rheinstetten, Germany). Spectral peaks were assigned with reference to the literature (Holmes *et al.*, 2008; Nicholson *et al.*, 1995; Wishart *et al.*, 2007).

3.4.4 Spectral processing

Proton NMR spectra were exported to MATLAB R2010 (MathWorks, Natick, Massachusetts, U.S.A) and to avoid influence on analyses from water suppression aberration, the water region from 4.5 - 6 ppm was excluded from all spectra. Data were normalised using median fold-change normalisation to remove the effect of inter-experimental variability. Median spectra for both groups were generated to allow direct visual comparison of average spectra and allow the selection of regions that were visually divergent, in addition to those identified by multivariate analysis, for peak integration.

3.4.5 Multivariate and univariate statistical analysis

Full resolution data were used for analyses. Data matrices were generated in MATLAB containing ppm variables as columns and sample identities as rows. Matrices were exported to SIMPCA-P v12 (Umetrics, Umea, Sweden). After water exclusion, this amounted to a matrix, for both NOESY and CPMG experiments, of 31,181 variables and 16 observations. Data were mean-centred and 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, 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 are generated through seven-fold cross validation. In this method, every 7th sample is 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 is repeated for all the samples (grouping the 2nd, 9th, 16th and 3rd, 10th, 17th and so on) until all the samples have been excluded once. The results are averaged to produce a model which is 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. Comparison of integrals was performed using GraphPad Prism (La Jolla, California, USA) using Mann-Whitney non-parametric tests of significance, as normal Gaussian distribution could not be assumed, *p*-values of <0.05 were considered significant.

3.5 RESULTS

3.5.1 Fisher rat weights

Table 3-2 records the median weights of the three groups of rats. Healthy controls were 120 g heavier than their liver disease counterparts. Macroscopic appearances of healthy and diseased liver of the groups are shown in **Figure 3-1**.

Table 3-2. Fisher rat weights at time of sacrifice.

	Group 1 (HCC)	Group 2 (HCC + Norfloxacin)	Control group
Median weight (g)	336.0	335.5	455.5

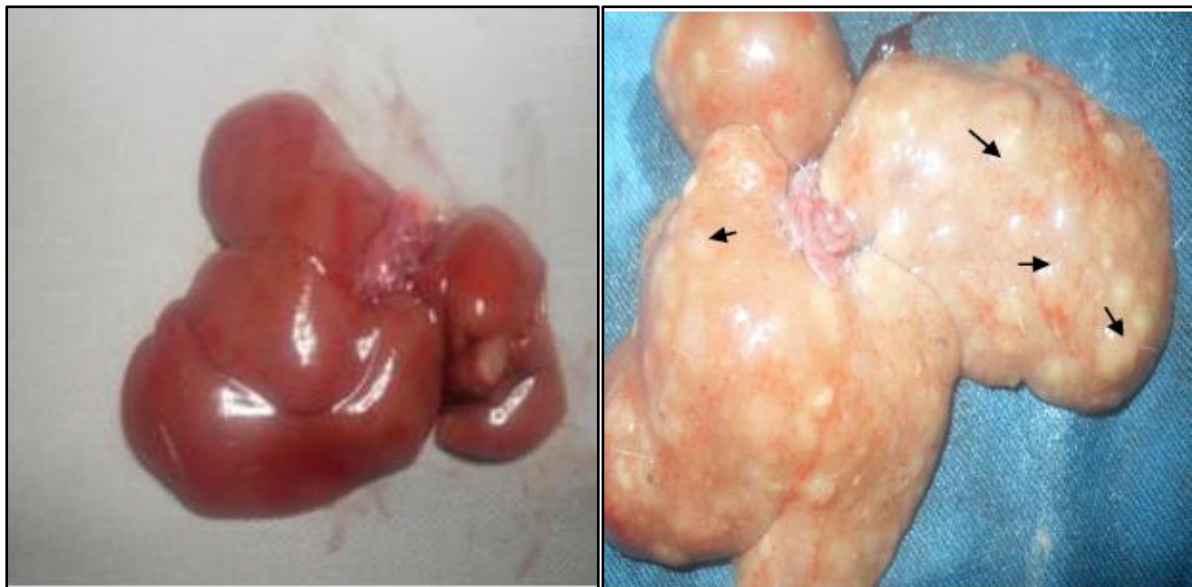


Figure 3-1. Explanted liver from healthy control and rat with HCC.

*Key: Arrows indicate areas of HCC nodularity.
(Courtesy of Dr Fatma Mohamed).*

3.5.2 Proton magnetic resonance spectral profiles

Figure 3-2 displays the same plasma sample after NOESY and CPMG acquisitions. This highlights the effect of macromolecular suppression allowing smaller molecules to be detected. **Figure 3-3** shows the effect of normalisation on a segment of spectra, reducing the effect of variability, not due to inherent sample qualities, but instead due to variability of experimental conditions.

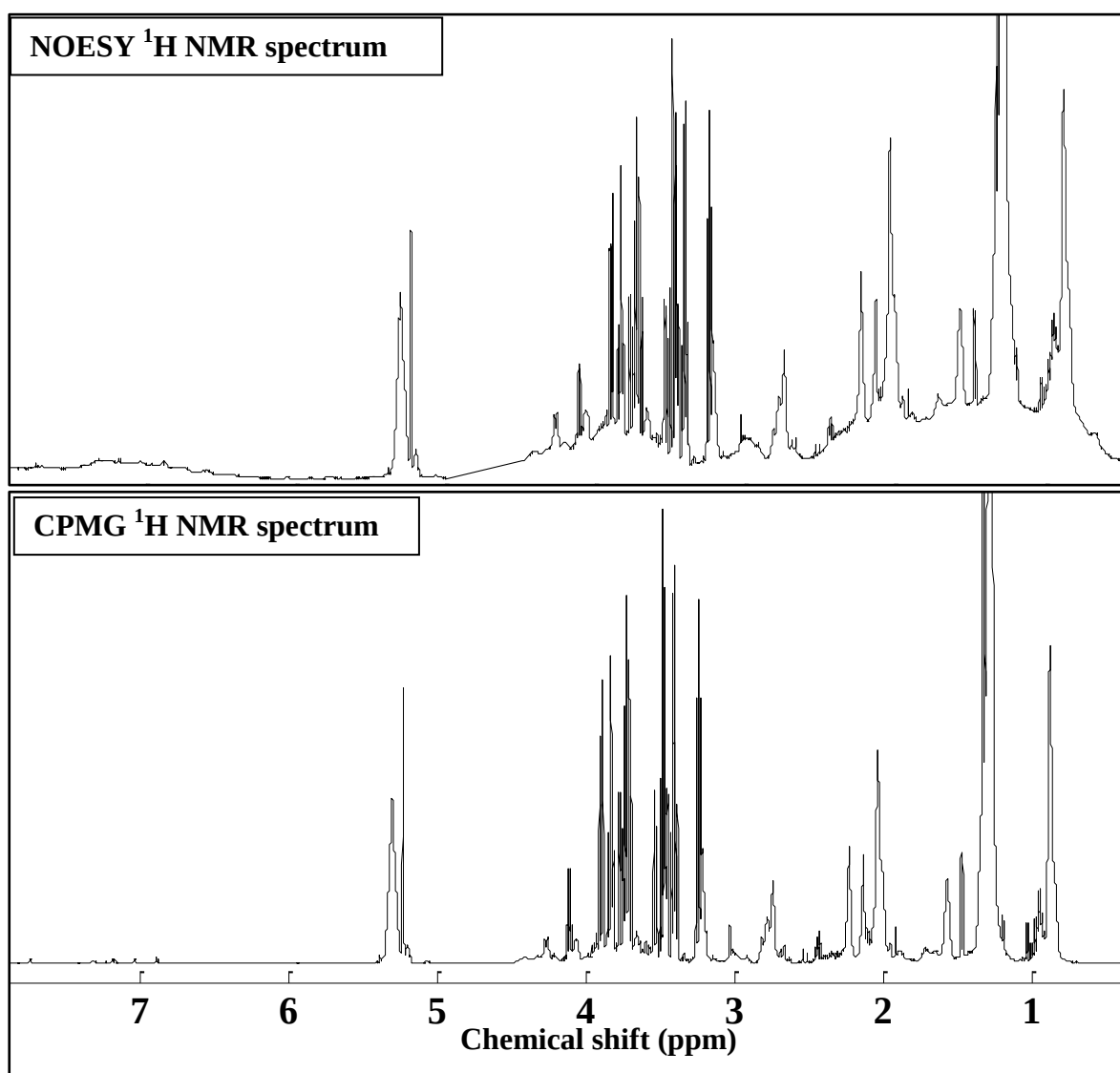


Figure 3-2. Fisher rat plasma proton nuclear magnetic resonance spectra highlighting macromolecular suppression.

Top pane: NOESY and bottom pane: CPMG ^1H NMR spectra.

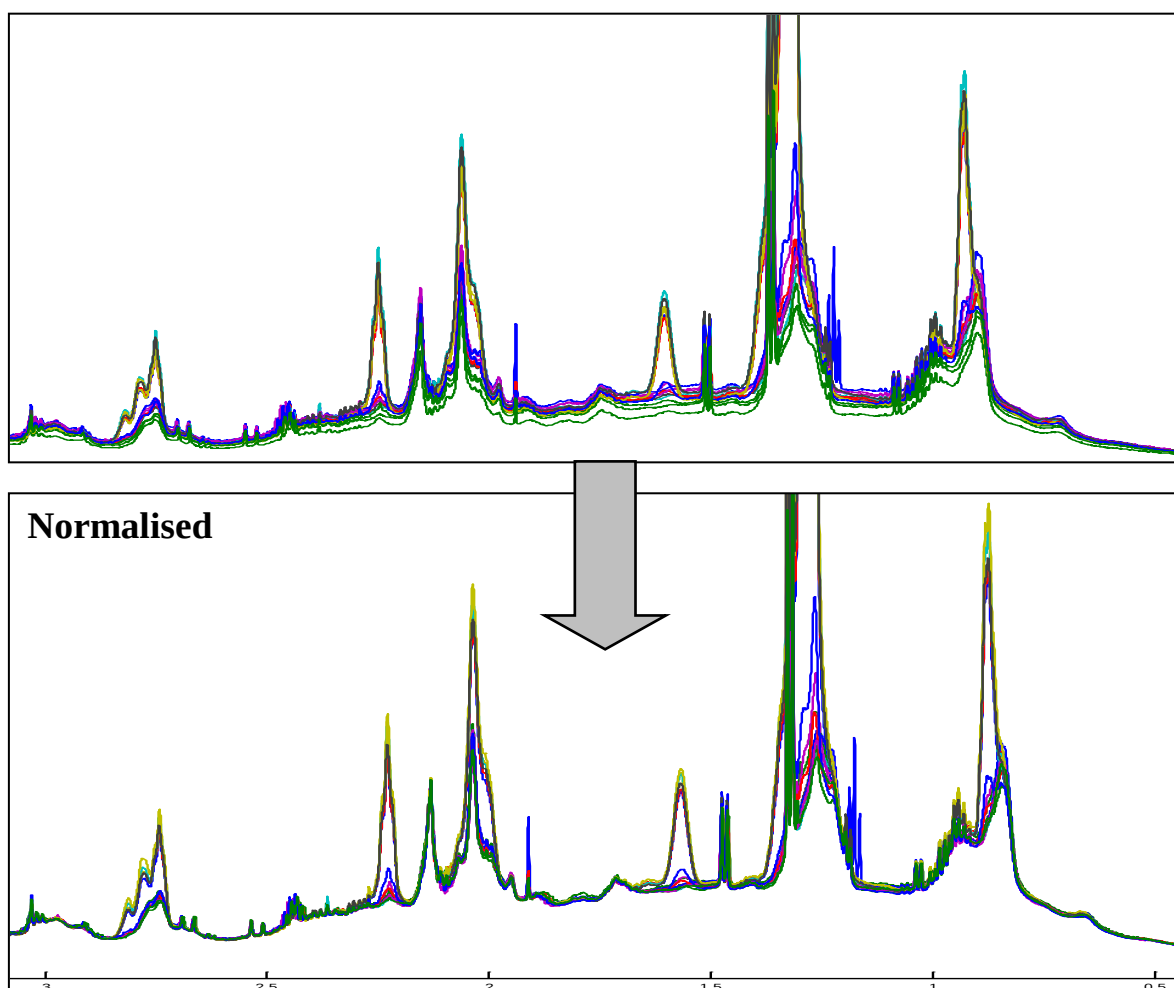


Figure 3-3. Effect of median fold change normalisation on spectral data.

Key: Different coloured spectra represent individual samples.

Figure 3-4 displays the median spectra of animals which developed HCC (groups 1 and 2) compared to healthy controls (group 3). A number of spectral regions displayed high divergence between groups and these are recorded in **Table 3-3**. It is noteworthy that the same areas in NOESY and CPMG acquired spectra were altered. This is likely due to the fact that the dominant signals from certain molecules occur in both studies and are relatively unaffected by the macromolecular suppression from a CPMG pulse sequence.

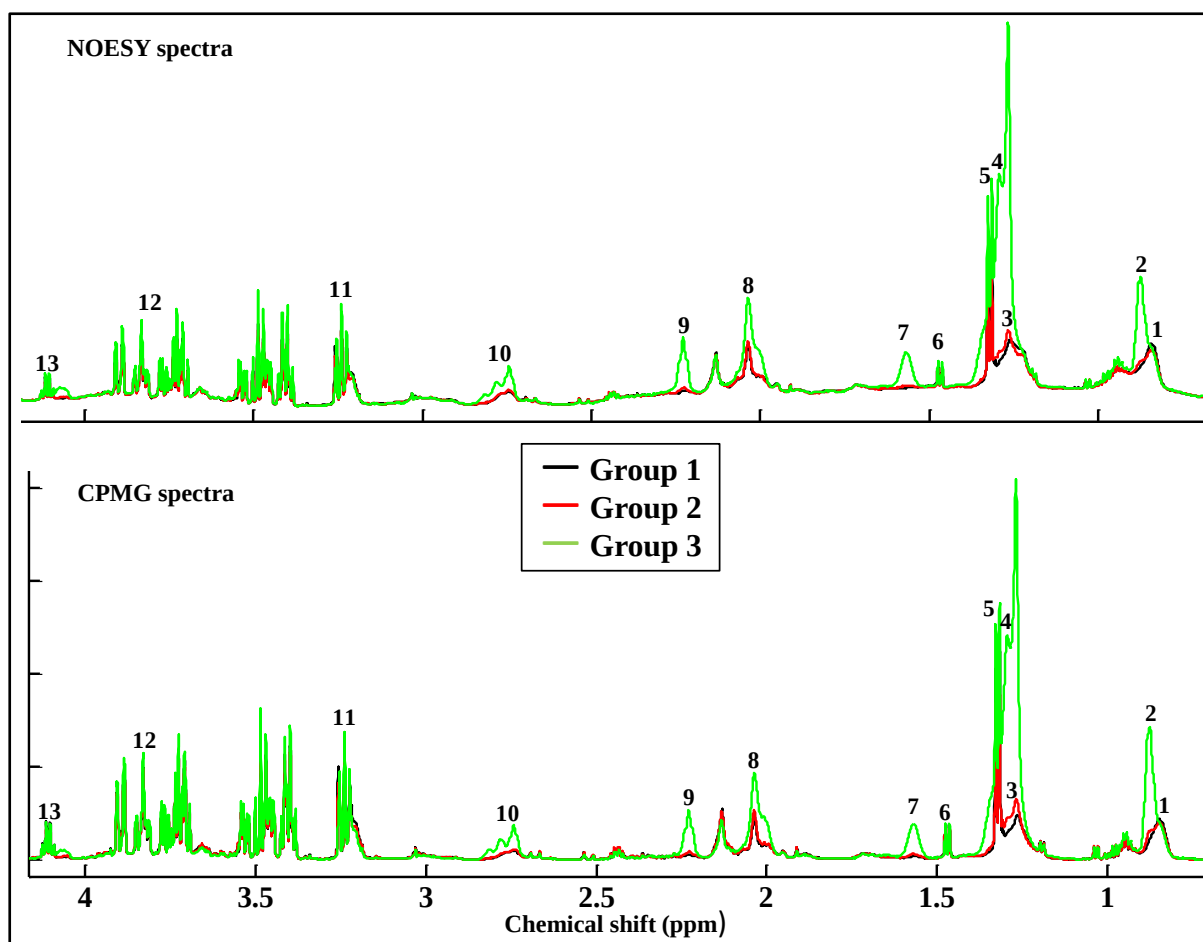


Figure 3-4. Proton nuclear magnetic resonance spectra of rat groups.

Key: Median spectra of HCC (Group 1); HCC + norfloxacin (Group 2); healthy controls (Group 3).

Table 3-3. Resonance identities in rat plasma spectra between 0.5 -4.5 ppm.

Label	Chemical shift (ppm)	Multiplicity	Molecule
1	0.84	Multiplet (broad)	Low density lipoprotein (CH ₃)
2	0.87	Multiplet (broad)	Very low density lipoprotein (CH ₃)
3	1.22	Multiplet (broad)	Low density lipoprotein (CH ₂)
4	1.25	Multiplet (broad)	Very low density lipoprotein (CH ₂)
5	1.33	Doublet	Lactate (CH ₃)
6	1.46	Doublet	Alanine
7	1.57	Multiplet (broad)	Lipid (CH ₂)
8	2.04	Singlet	Acetyl-glycoprotein
9	2.23	Multiplet (broad)	Lipid (CH ₂)
10	2.69	Multiplet	Lipid (CH ₂)
11	3.24	-	Glucose moieties and amino acids
12	3.8	-	Glucose moieties
13	4.11	Quadruplet	Lactate (CH)

3.5.3 Multivariate statistical analysis

Multivariate analysis scatter plots are displayed in **Figures 3-5 and 3-6** depict NOESY and CPMG studies. Clear clustering can be seen causing separation of rats with HCC (groups 1 and 2) and healthy controls. This is interpreted as the greatest variation between samples (from all 3 groups) accounting for most of the variation between animals with HCC and those without. In alternative terms, those animals with HCC have more similar plasma metabolite profiles than those without. In both NOESY and CPMG studies, groups 1 (no norfloxacin) and 2 (norfloxacin) are poorly separated by multivariate analysis, implying that blood from these animals possess similar plasma profiles. Using the supervised method of PLS-DA, whereby models are generated to identify those multivariate components which describe most variation between groups, data were generated for CPMG and NOESY studies for combined groups 1 and 2 (those animals with HCC) versus group 3 (healthy controls) (**Figures 3-5 and 3-6**). The R^2 (goodness of fit) and Q^2 (goodness of prediction) were greater than 0.95 in both studies, suggesting very different plasma metabolic profiles between HCC and non-HCC animals. This is reflected in the misclassification matrix and sensitivity and specificity of HCC diagnosis in **Table 3-4**, illustrating 100 % sensitivity and specificity of HCC diagnosis, for both NOESY and CPMG studies. In keeping with the visual similarity between median spectral comparisons of NOESY and CPMG data, loadings plots of PLS-DA components identified the same spectral regions contributing most to multivariate models. As a result, only CPMG data were used for subsequent univariate analyses.

Table 3-4. Misclassification matrix for PLS-DA prediction of disease.

	Predicted as HCC	Predicted as No HCC	Total	Sensitivity / Specificity
HCC	10	0	10	100% / 100%
No HCC	0	6	6	

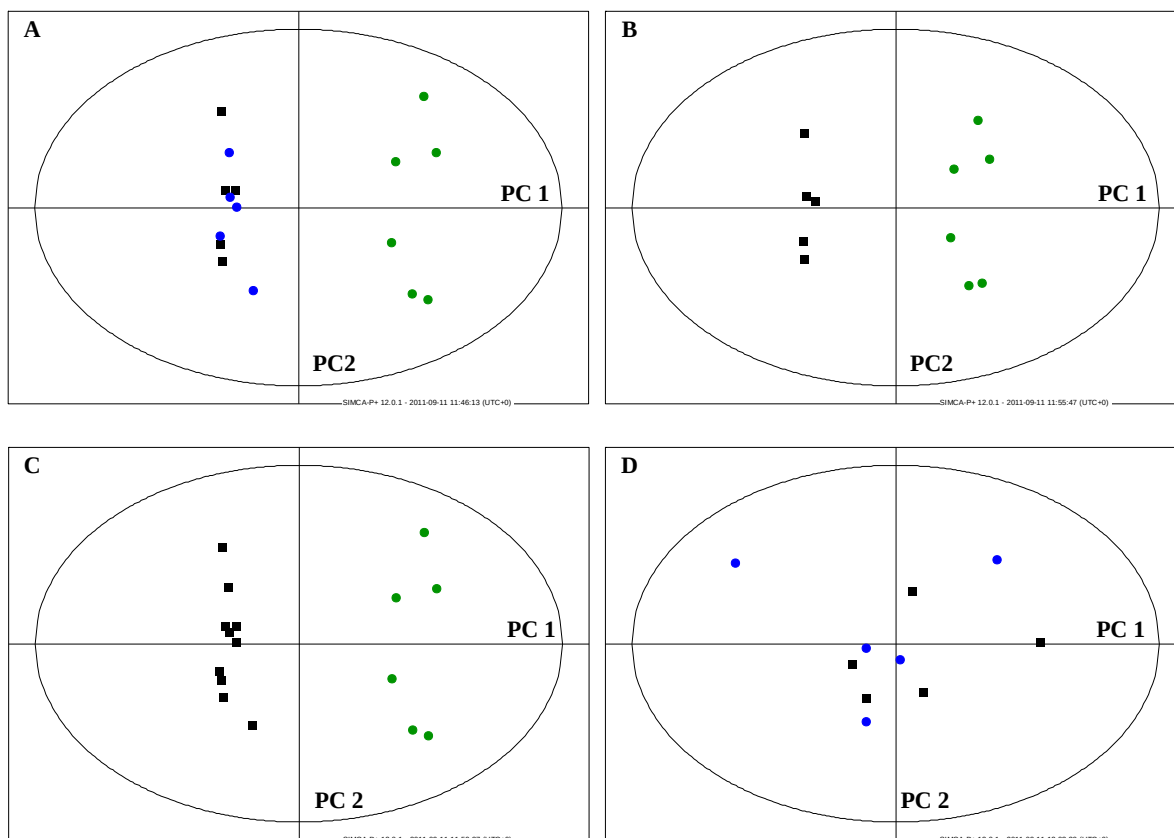


Figure 3-5A. Principal components analysis of rat plasma.

Key: Data from NOESY analysis. A. Groups 1 (■), 2(●) and 3(●); B. Group 1 and group 3; C. Groups 1 and 2 combined and group3; D. Groups 1 and 2. PC = Principal Component

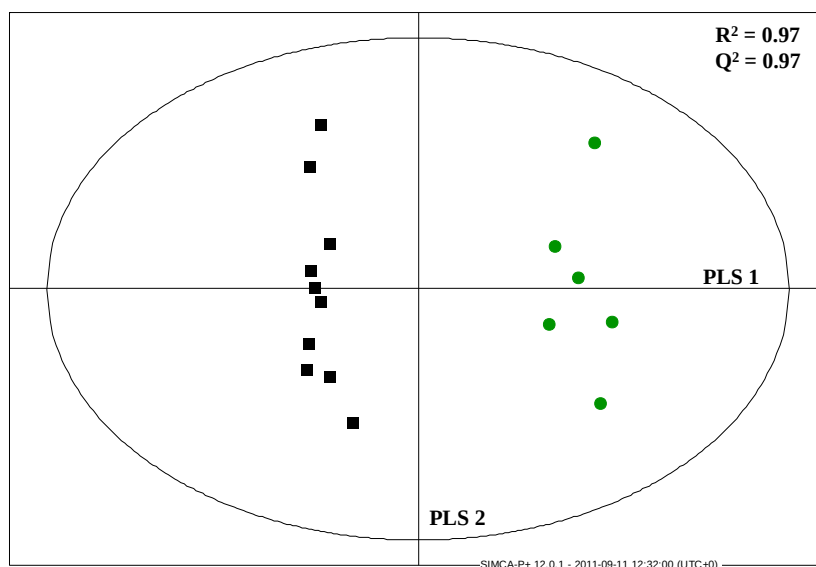


Figure 3-5B. Partial least squared discriminant analysis of rat plasma.

Key: Data from NOESY analysis. HCC + norfloxacin (■) and healthy controls (●).

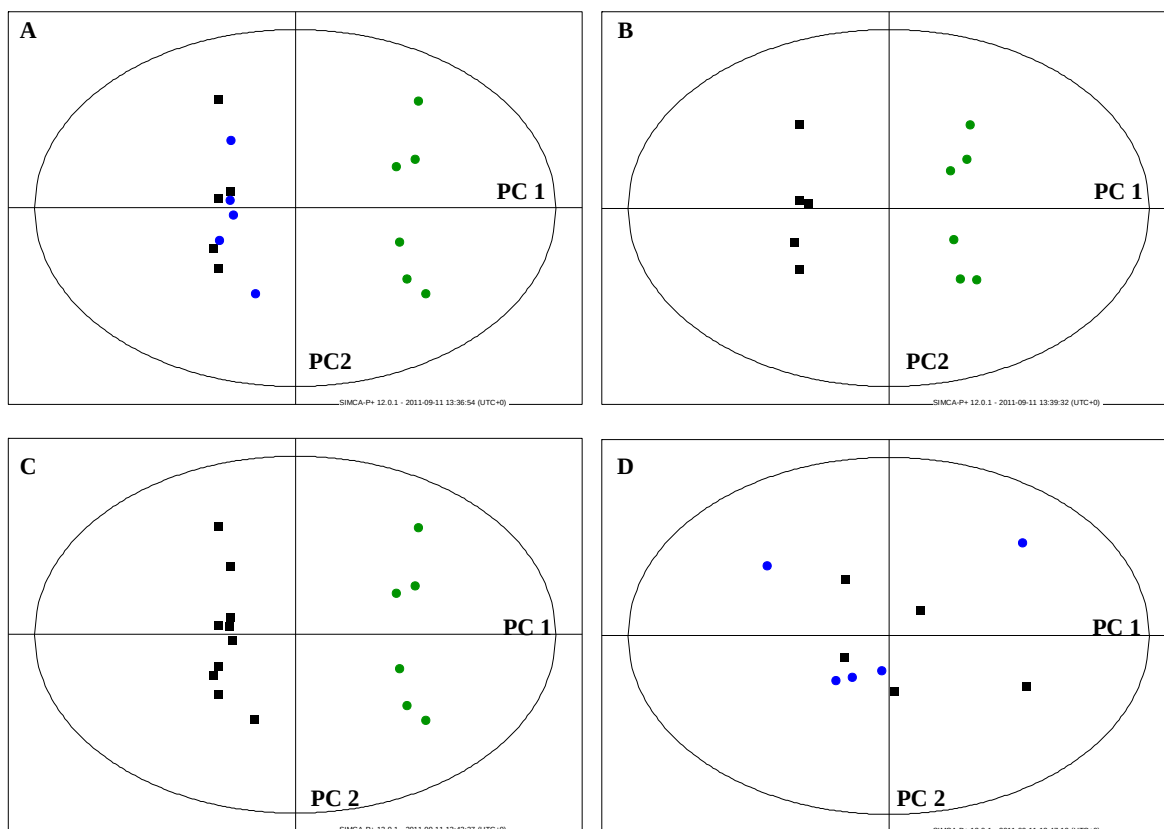


Figure 3-6A. Principal components analysis of rat plasma.

Key: Data from CPMG analysis. A. HCC (■), HCC + norfloxacin (●) and healthy controls (●); PC = Principal Component.

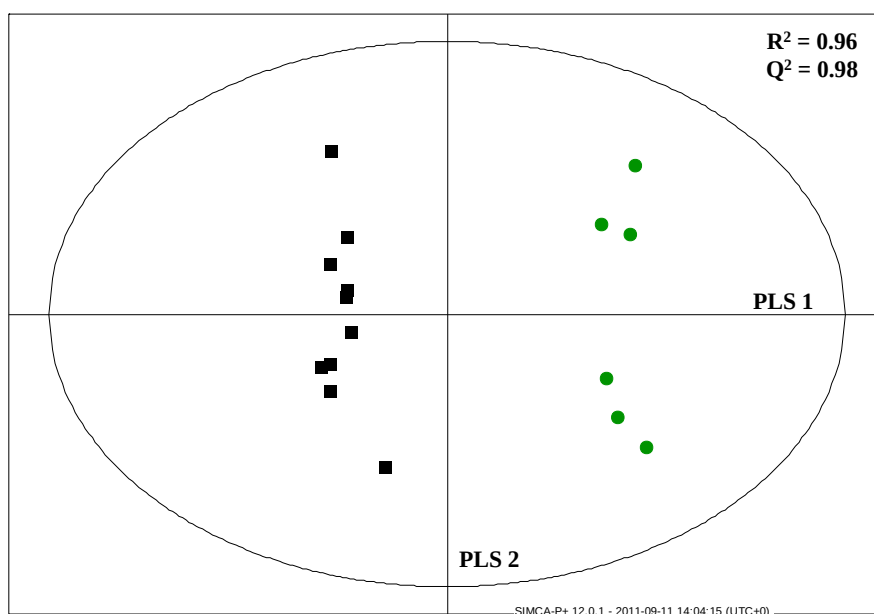


Figure 3-6B. Partial least squared discriminant analysis of rat plasma.

Key: Data from CPMG analysis. HCC + norfloxacin (■) and healthy controls (●).

3.5.4 Univariate statistical analysis

Variables from CPMG PLS-DA models and from visual comparison of median ^1H NMR spectra that contributed most to variation between those animals with (groups 1 and 2) and those without HCC (group 3), are tabulated and graphically illustrated below (**Table 3-5**) (**Figure 3-7**). Plasma LDL, VLDL, acetyl-glycoprotein, α -glucose and unsaturated fatty acids were all significantly reduced in rats with HCC ($p =$ or <0.001). Plasma citrate and formate were significantly increased in animals with HCC ($p = 0.02$).

Table 3-5. Median metabolite levels in healthy and diseased animals.

Chemical shift (ppm)	Molecule	HCC	HCC + Nor.	Controls	<i>p</i> -value
0.80 – 0.85	LDL (CH_3)	1.70×10^8	1.48×10^8	1.67×10^8	^a 0.55, ^b 0.60
0.86 – 0.89	VLDL (CH_3)	9.58×10^7	1.37×10^8	6.41×10^8	^a 0.03, ^b 0.004
1.24 -1.28	LDL (CH_2)	2.05×10^8	2.66×10^8	1.35×10^9	^a 0.10, ^b 0.004
1.28 – 1.31	VLDL (CH_2)	1.48×10^8	2.60×10^8	1.09×10^9	^a 0.03, ^b 0.004
2.03 – 2.06	Acetyl-glycoprotein	1.41×10^7	2.27×10^7	3.51×10^8	^a 0.06, ^b 0.004
2.50 – 2.52	Citrate	6.14×10^8	5.69×10^6	5.01×10^6	^a 0.15, ^b 0.13
2.22 – 2.24	Acetoacetate	1.41×10^7	2.27×10^7	1.41×10^8	^a 0.008, ^b 0.004
5.22 – 5.24	α -glucose	1.27×10^8	1.59×10^8	1.50×10^8	^a 1.0, ^b 0.004
5.25 – 5.38	Unsaturated lipid	1.59×10^8	6.32×10^8	6.32×10^8	^a 0.1, ^b 0.008
8.44 – 8.45	Formate	570,230	512,847	429,393	^a 0.8, ^b 0.03

Key: p-values calculated using Mann-Whitney non-parametric tests of significance, ^a and ^b corresponds to HCC vs. HCC + Norfloxacin and HCC vs. Controls, respectively. Nor., norfloxacin; LDL, low density lipoprotein; VLDL, very low density lipoprotein.

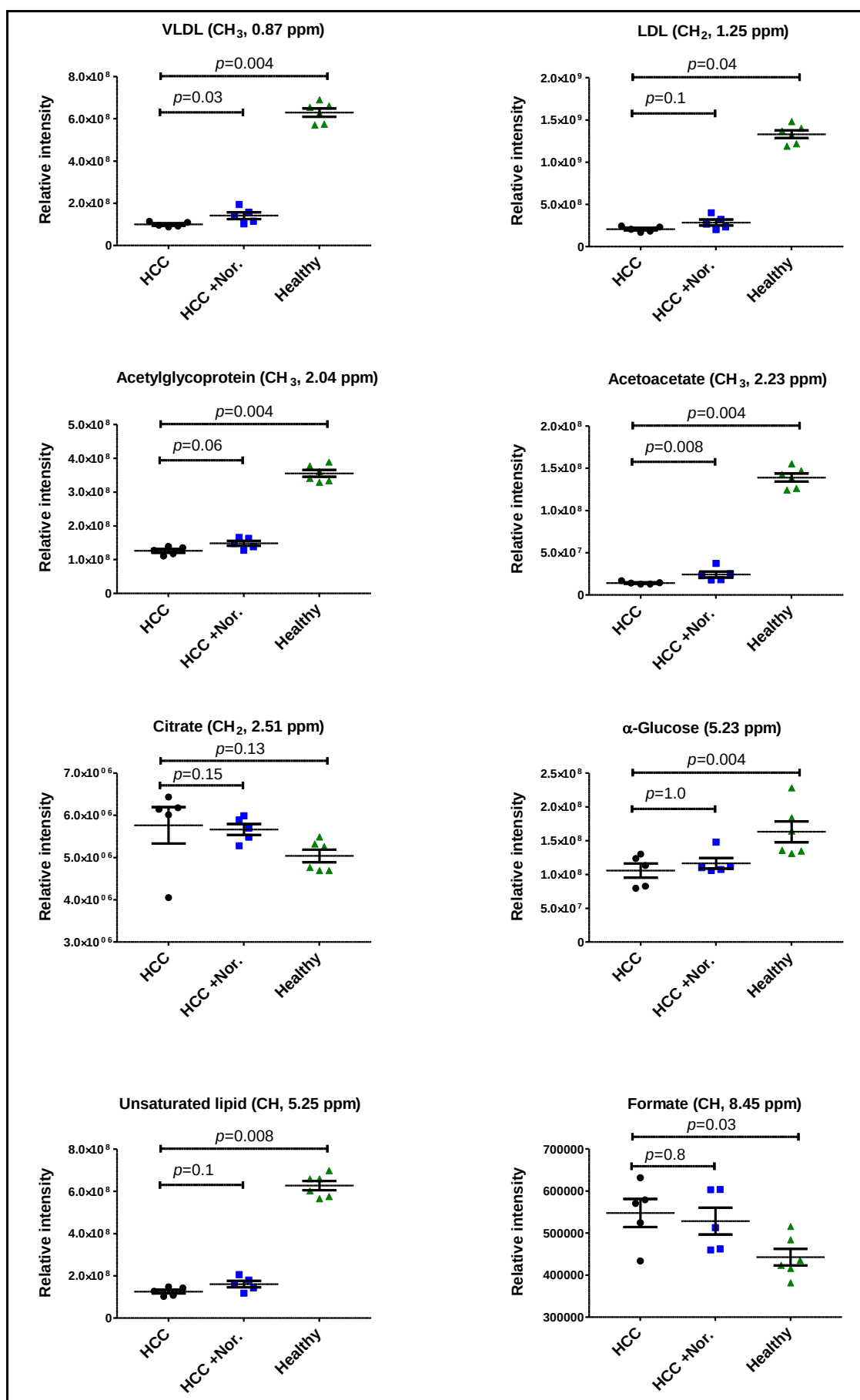


Figure 3-7. Plasma relative intensities (integral values) of selected metabolites.

3.6 DISCUSSION

The aims of this study were to investigate the plasma ^1H NMR metabolite changes that occur in the presence of HCC, and also to evaluate the effect of norfloxacin upon these profiles. Certainly, the metabolite profiles of those animals with HCC differed greatly from healthy control animals, mainly with respect to lipid lipoprotein associated species, displaying clear separation on PCA plots and excellent classification by PLS-DA. The animals with HCC, however, were over 100 g lighter than healthy controls and this is clearly a significant factor which influences interpretation of the findings. It is not clear, however, how this weight difference explains the specific alteration in metabolites.

Low density lipoprotein, VLDL and unsaturated fatty acids were significantly reduced in animals with tumours. This suggests several possible explanations. Firstly, the absorption of fat (via chylomicrons – see **Figure 3-8**) may be reduced, reducing the provision of lipid for production of VLDLs by the liver, although this would be unlikely as vastly reduced absorption would have been required to cause the results seen. Secondly, the findings may indicate cancer cachexia through an increased basal metabolic rate, which is defined as loss of skeletal muscle mass, with or without loss of body fat (Tisdale, 2010). Cancer cachexia is a complex condition likely to be cytokine mediated and indicative of end-stage disease (Tisdale, 2010). There are conflicting findings that support and argue against this: firstly, there is little evidence of increased muscle breakdown in rats with HCC, with no rise in plasma creatine or creatinine. Secondly, if fat were being actively metabolised it would be mostly through β -oxidation in mitochondria, a by-product of which is acetoacetate (acetic acid). Acetoacetate plasma levels were significantly reduced in animals with HCC which indicates reduces fatty acid metabolism, not in keeping with the use of fatty acids as an energy substrate. Ketoacids, however, are not definitive markers of fat metabolism and it may

be the case that fat is being mobilised for energy production in animals with higher basal metabolic rates, a hall mark of cancer cachexia. These results may also indicate deteriorating liver function. In addition to its multiple roles of coagulation cascade component, bile acid and vitamin production, the liver produces VLDL particles which are then circulated in the blood providing tissues with fatty acid substrates (**Figure 3-8**). Given the complexity of lipid metabolism, it would be incorrect to presume that reduced production of VLDL particles results in reduced production of LDL particles. Low density lipoprotein is formed by the catabolism of VLDL but is dependent on several factors such as hepatic function and peripheral lipase activity. It may be that the functional status of the liver, induced by tumour burden and inflammation caused by the carcinogens DEN and NMOR is reduced, causing the majority of lipid alteration in this model. It is also possible that the lipids are being used to fuel cellular division, providing the substrate for cell membranes in rapidly dividing tumours. If this were the case, then the by-products of fatty oxidation would not be raised as seen here. Finally, DEN is a potent carcinogen and its effect on lipid metabolism, aside from effects on liver inflammation, are not known. Animal studies have shown that DEN induces lipid peroxidation, which may be a factor in interpreting the results presented here (Jayakumar *et al.*, 2011). The effects of NMOR on lipid metabolism are not known.

The findings presented here do parallel those of a recent study undertaken by Wang and colleagues, where male Sprague-Dawley rat liver tumour homogenate, induced by DEN, were analysed using ^1H NMR spectroscopy (Wang *et al.*, 2011). A raised level of LDL, VLDL and choline was observed in tumours suggesting their increased incorporation into the tumour mass. Tesiram and colleagues observed substantial increases in glycerol backbone-containing phospholipids in choline-deficient Fisher rats with hepatic adenomas (Tesiram *et al.*, 2005). In this study, diseased rats were lighter than healthy controls but the growth

characteristics of both groups were similar suggesting an alteration in fat storage rather than muscle development.

Acetylglycoproteins are known to be produced by hepatocytes and released in response to stressful stimuli and can be induced by a number of different inflammatory conditions including cancer (Baumann *et al.*, 1983; Wang *et al.*, 2006). Acetylglycoproteins were significantly reduced in animals with HCC which could also imply a decreased functional status of hepatocytes due to disease. The macroscopic appearance of the rat livers, almost completely infiltrated with multi-nodular tumour, would support the theory that less functional tissue remains to produce hepatic proteins.

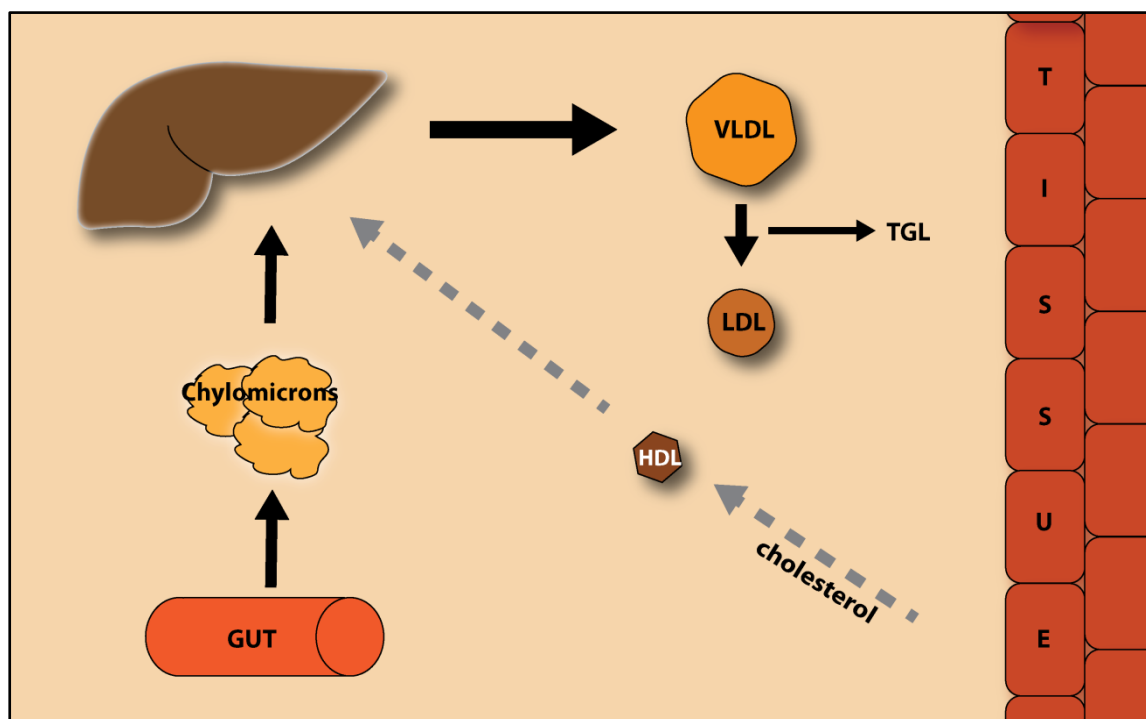


Figure 3-8. Lipid absorption and hepatic metabolism to lipoprotein.

Key: VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; TGL, triglycerides.

Blood glucose levels, as measured in the glucose moieties (3.0 – 3.5ppm) and α -glucose (5.23ppm) were significantly reduced in animals with HCC. Hypoglycaemia is a well recognised paraneoplastic phenomena of HCC purported to be related to the secretion of insulin-like growth factors (Yamaguchi *et al.*, 1998). It is also a manifestation of cancer cachexia of which these animals are likely to have been sufferers.

The effect of norfloxacin on plasma NMR metabolic changes was modest, with phenotypes (all DEN/NMOR + norfloxacin treated animals also developed HCC) and metabolic profiles more similar to DEN/NMOR animals than controls. There is no presentable evidence that this slight alteration is due to reduced bacterial translocation and liver inflammatory status in this study. Furthermore, DEN is dependent upon the action of cytochrome P450 enzymes for conversion to its active metabolites (Salcido-Neyoy *et al.*, 2009) and norfloxacin has been shown to suppress the activities of these enzymes (McLellan *et al.*, 1996). The results observed here may therefore be due to enzyme inhibition rather than alterations in bacterial translocation.

3.7 CONCLUSIONS

The spectral profiles of plasma in rats with HCC display marked changes with relation to lipid metabolism and cellular turnover. Norfloxacin appears to abrogate these effects slightly. The results of this study have provided an insight to the altered hepatic lipid metabolism in the presence of HCC. Interpretation must be cautious, given the small sample number. The ^1H NMR changes in lipid profiles are likely due to a combination of cancer cachexia and reduced liver function although certain corroboratory findings, such as an increase in ketoacids, is missing. Further studies are required to characterise lipid subspecies. Low density lipoprotein, VLDL and unsaturated fatty acids cover a broad range of lipid subspecies and it would be important to identify whether any particular species is associated with HCC. For this, a more sensitive method of lipid analysis is required, such as liquid chromatography mass spectrometry LC-MS. Similar studies also need to be undertaken in the human population to test the translatability of findings.

4 THE PLASMA AND SERUM METABOLIC PROFILING OF HEPATOCELLULAR CARCINOMA IN A NIGERIAN AND EGYPTIAN COHORT USING PROTON NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

4.1 ABSTRACT

Introduction: Previous studies have observed alterations in the ^1H NMR blood spectral profiles of patients with malignancy. No study has examined the blood metabolic profiles of HCC in two diverse populations. Presented here is a ^1H NMR spectroscopy study of serum and plasma from patients recruited from Nigeria (mostly HBV infected) and Egypt (mostly HCV infected). The aim of this study was to identify differences that occur in the serum and plasma spectra of patients with HCC using ^1H NMR spectroscopy. **Methods:** Patients with HCC (53) and cirrhosis (26) and healthy volunteers (19) were recruited from two countries: the University of Jos, Nigeria (2007 – 2008) and The National Liver Institute, Shebeen El Kom, Egypt (2004 – 2007). Participants provided serum or plasma samples. All samples were analysed as a group cohort using 600 MHz ^1H NMR spectroscopy using NOESY pulse sequences. Comparison of median group spectra and multivariate analysis were performed to identify regions of difference. **Results:** Non-significant age differences were present between the HCC and healthy volunteer group (50 years versus 40 years, $p=0.09$) but not between HCC and cirrhosis groups (49.5 years, $p=0.31$). Alterations between patients with HCC, healthy volunteers and patients with cirrhosis were detected in levels of LDL ($p=0.002$ and 0.12), VLDL ($p<0.001$ and 0.77), lactate ($p=0.03$ and 0.12), *N*-acetylglycoproteins ($p<0.001$ and 0.001) and acetoacetate ($p=0.52$ and 0.06). **Conclusions:** Differences were present in the serum and plasma of patients with HCC but whether this is due to confounding from age differences or complex alterations in lipid metabolism is debatable.

4.2 INTRODUCTION

The previous chapter described the plasma ^1H NMR spectral changes due to HCC in a Fisher rat model of the malignancy. Whether the same holds true for human disease remains poorly investigated. The vastly increased 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.

One previous study, performed in a Chinese population has utilised ^1H NMR of serum to discriminate patients with HCC ($n = 39$) from patients with cirrhosis ($n = 36$) (Gao *et al.*, 2009). 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 ($(\text{CH}_2)_n$), measured by 400 MHz ^1H NMR spectroscopy, as a sensitive test for cancer (Fossel *et al.*, 1986). 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 (Bell *et al.*, 1988; Holmes *et al.*, 1988; Okunieff *et al.*, 1990; Wilding *et al.*, 1988).

The aim of the study presented here was to investigate whether serum and plasma ^1H NMR profiles are different in patients with HCC compared to patients with cirrhosis and healthy volunteers.

4.3 METHODS

4.3.1 Patient and healthy volunteer selection

Subjects were recruited in two cohorts from Jos University Teaching Hospital (JUTH) 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: US, computed tomography (CT) or magnetic resonance imaging (MRI). Cirrhosis was diagnosed on clinical findings, by the presence of portal hypertension (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 (Okuda *et al.*, 1985). This scoring method was chosen out of necessity as other, more comprehensive scoring tools, such as the Barcelona Clinic Liver Cancer staging algorithm, require WHO performance status, presence of portal vein invasion and encephalopathy as criteria, which were not recorded for most of the patients in this study.

4.3.1 Sample collection

Nigerian blood samples were collected between 2007 and 2008 and Egyptian samples between 2004 and 2007. Random, non-fasted 5 mL blood samples were venesected into either plain serum or EDTA containing sterile tubes and placed immediately on ice or into a refrigerator at 4 °C. Samples were centrifuged within 1 – 2 hrs 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 ethylenediaminetetraacetic acid (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 ^1H NMR metabolic profiles from serum and plasma, allowing the two to be compared with relative assurance (Deprez *et al.*, 2002; Teahan *et al.*, 2006; Wedge *et al.*, 2011). These studies highlight the fact that clinical differences between groups were profoundly more influential than spectral differences between EDTA plasma and plain serum samples.

4.3.2 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, 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.

4.3.3 Sample preparation

Samples were prepared according to the same protocol outlined in **Chapter 3**, using NaCl / D₂O solution. Samples were analysed on the same day as preparation.

4.3.4 Proton nuclear magnetic resonance spectroscopy

All Nigerian samples were run in a random, non-grouped order on 29.10.2009. All Egyptian samples were run on 02.12.2010. Proton NMR spectroscopy has been found to display 95% concordance of results, even if different NMR systems are used (Keun *et al.*, 2002). All samples were run at the department of Biomolecular Medicine, Imperial College London. Nigerian and Egyptian samples were run on two Bruker Ultrashield PlusTM 600 NMR systems operating at 600.29 - 600.44 MHz ¹H frequency (Bruker Biospin, Rheinstetten, Germany). Spectral acquisition parameters, generation of 90° pulse and pulse sequences were as described in **Chapter 3**. Spectra were acquired using 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 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 (Holmes *et al.*, 2008; Nicholson *et al.*, 1995; Wishart *et al.*, 2007).

4.3.5 Data pre-processing

Spectra were exported to MATLAB R2010 (MathWorks, Natick, Massachusetts, U.S.A) and the water region from 4.5 - 6 ppm was excluded. As the concentration of EDTA varied between the serum and plasma samples, regions were excluded where it resonates 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 (**Table 4-1**). Furthermore, the effect of EDTA on other molecules, in terms of spectral resonance or peak shift, were found to be negligible (Barton *et al.*, 2010). Peaks from EDTA are displayed in **Figure 4-1**, in comparison to an EDTA-free sample. 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.

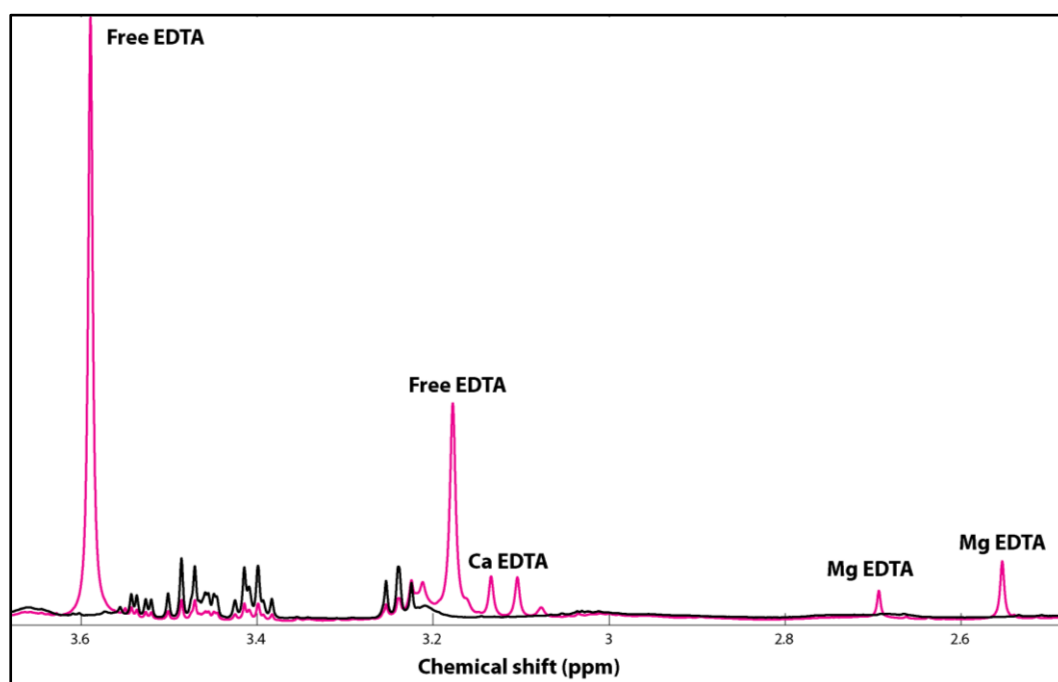


Figure 4-1. Resonances from EDTA compared to an EDTA-free serum sample.

Key: EDTA plasma sample (-), EDTA free serum sample (-).

Table 4-1. Metabolite peaks obscured by EDTA resonances.

Obscuring signal	Chemical shift (ppm)	Metabolites with peaks in this region	Additional clear peaks (ppm)
Free EDTA	3.6	B-Aminobutyrate	1.33/2.5
		Valine	0.99/1.04/2.28
		Sarcosine	2.7
		Acetylcarnitine	Nil
		γ -Hydroxybutyrate	2.2
		Threonine	1.32
		<i>myo</i> -Inositol	3.28
		Glycerol	3.87
Free EDTA	3.2	Glycerophosphorylcholine	3.95/4.30
		Carnitine	Nil
		Choline	3.50/4.05
		Anserine	7.1
		Tyrosine	3.94/6.87
		Arginine	1.73/1.92/1.68
		Glucose	5.23
		Histidine	3.98/7.02/7.73
		B-Alanine	2.50-2.60
		Creatine	3.93
Ca-EDTA	3.06-3.017	Creatinine	4.05
		Tyrosine	3.94/6.87
		Ornithine	Nil
		Cysteine	3.9
		B-Aminoisobutyrate	1.2/2.6-2.7
		Citrulline	1.57/1.86/3.70
		<i>N</i> - α -Acetylhistidine	7.08/7.83
		3-Methylhistidine	7.01/7.61/7.77
		cis-Aconitate	Nil
		Citrate	Nil
Mg-EDTA	2.7		

Adapted from (Barton et al., 2010).

Metabolites with no other spectral resonances are in bold.

4.3.6 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 4-2**. The integral areas of these regions were recorded in a data matrix and exported to SIMCA (Umetrics, Umea, Sweden) for PCA and PLS-DA as described in earlier chapters. 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.

Table 4-2. 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	<i>N</i> -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

4.3.7 Univariate analysis

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

4.4 RESULTS

4.4.1 Subject selection and demographics

A total of 98 volunteers were recruited for study, 56 from Nigeria and 42 from Egypt (**Table 4-3**). Subjects were recruited in three cohorts, 53 patients with ultrasound or computed tomography proven HCC (29 Nigerian + 24 Egyptian); 26 patients with clinically-confirmed cirrhosis with features of portal hypertension, but no HCC (12 Nigerian + 14 Egyptian); and 19 healthy subjects with no history of liver disease (15 Nigerian + 4 Egyptian). All patients, except one, in the Nigerian HCC and cirrhosis groups were HBsAg positive (ELISA, 3rd generation). The single non-HBV patient with cirrhosis was also anti-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 induced liver disease. All healthy volunteers were HBsAg and anti-HCV antibody negative with no history of liver disease. The Nigerian and Egyptian groups were combined as the number of healthy volunteers from Egypt was low (4). If country-specific analyses were undertaken the number of HCC and cirrhosis samples for the Egyptian group (24 and 14) would have vastly outnumbered the healthy controls leading to inevitable bias of results.

Subject demographics are displayed in **Tables 4-4 and 4-5**. 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 years for patients with HCC ($p=0.09$). There were fewer males in the healthy volunteer group (42% versus 70% in the HCC group, $p=0.052$). Median serum AFP levels were significantly higher (1198 IU mL^{-1}) in patients with HCC compared to those with cirrhosis and healthy volunteers (5.61 and 1.44, $p<0.001$). Of note, if an AFP cut off 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. Stages of HCCs are shown in **Table 4-6**.

Table 4-3. Parameters of Nigerian and Egyptian cohorts.

Parameter	Nigerian cohort	Egyptian cohort
<i>n</i>	56	42
	HCC(29), Cirrhosis(12), Healthy(15)	HCC(24), Cirrhosis(14), Healthy(4)
HBV	HCC (29), Cirrhosis (12)	0
HCV	0	HCC (23), Cirrhosis (14)
Collection date	2007-2008	2004-2007
Plain serum	8	42
EDTA plasma	48	0
NMR system	600 MHz	600 MHz
Acquisition date	29.10.2009	02.12.2010

Table 4-4. Nigerian and Egyptian patient and healthy volunteer demographics.

Characteristic	HCC	Cirrhosis	Healthy Controls	<i>p</i> -value
<i>n</i>	53	26	19	-
Median Age (years) (range)	50	48.5	40	^a 0.09 and ^b 0.31
Male (%)	37 (70%)	18 (69%)	8 (42%)	^{ai} 0.052 and ^{bi} 1.0
Nigerian	29	12	15	-
Egyptian	24	14	4	-

Key: Mann-Whitney non-parametric comparisons of HCC versus healthy (^a) and versus cirrhosis (^b) and Fisher's exact test for categorical variables (^{ai} and ^{bi}).

Table 4-5. Biochemical analysis of all patients.

Test (range)	HCC	Cirrhosis	Healthy Controls	<i>p</i> -values (Mann-Whitney)
Serum Samples (n)	53	26	19	-
AFP (IU mL⁻¹)	1198	5.61 ⁺	1.44 ⁺	^a and ^b <0.001*
Creatinine (mmolL⁻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).

Table 4-6. Okuda stages of patients with hepatocellular carcinoma.

Stage	Number
1	8
2	25
3	16
Unknown due to insufficient data*	4
Total	53

Key: *Due to samples not tested for bilirubin or albumin or no documentation of ascites.

4.4.2 Proton nuclear magnetic resonance spectroscopy

A representative NOESY plasma spectrum is displayed in **Figure 4-2** with indication of which regions were excluded 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 LDL at 0.8 ppm and 1.21 ppm and VLDL at 0.85 ppm and 1.25 ppm was poor, although could discernibly be distinguished. This was evident in median spectra (**Figure 4-3**).

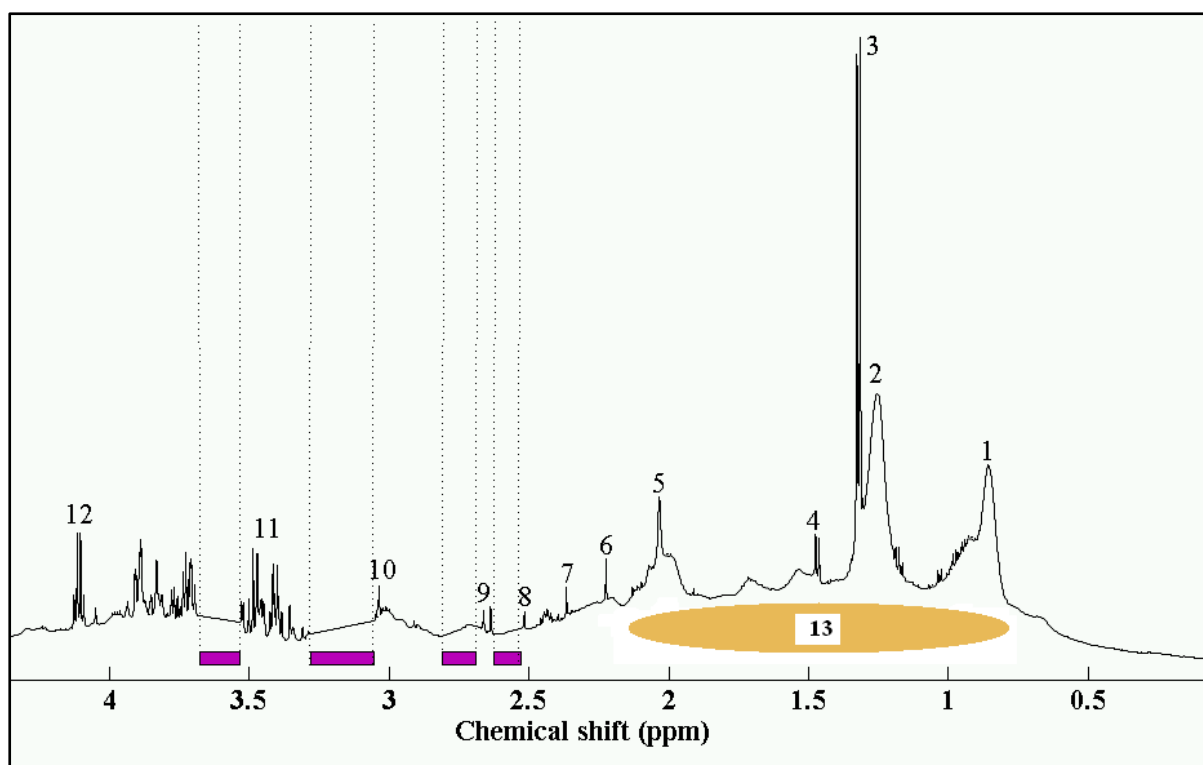


Figure 4-2. Representative plasma spectrum with EDTA exclusion.

Key: 1 and 2 - LDL/VLDL, 3 - lactate (CH₃), 4 - alanine, 5 - N-acetylglycoproteins, 6 - acetoacetate, 8 and 9 - citrate, 10 - creatinine, 11 - glucose resonances, 12 - lactate (CH) and 13 - albumin and albumin-bound fatty acids (Nicholson et al., 1995). Purple bars indicate areas of EDTA resonance exclusion.

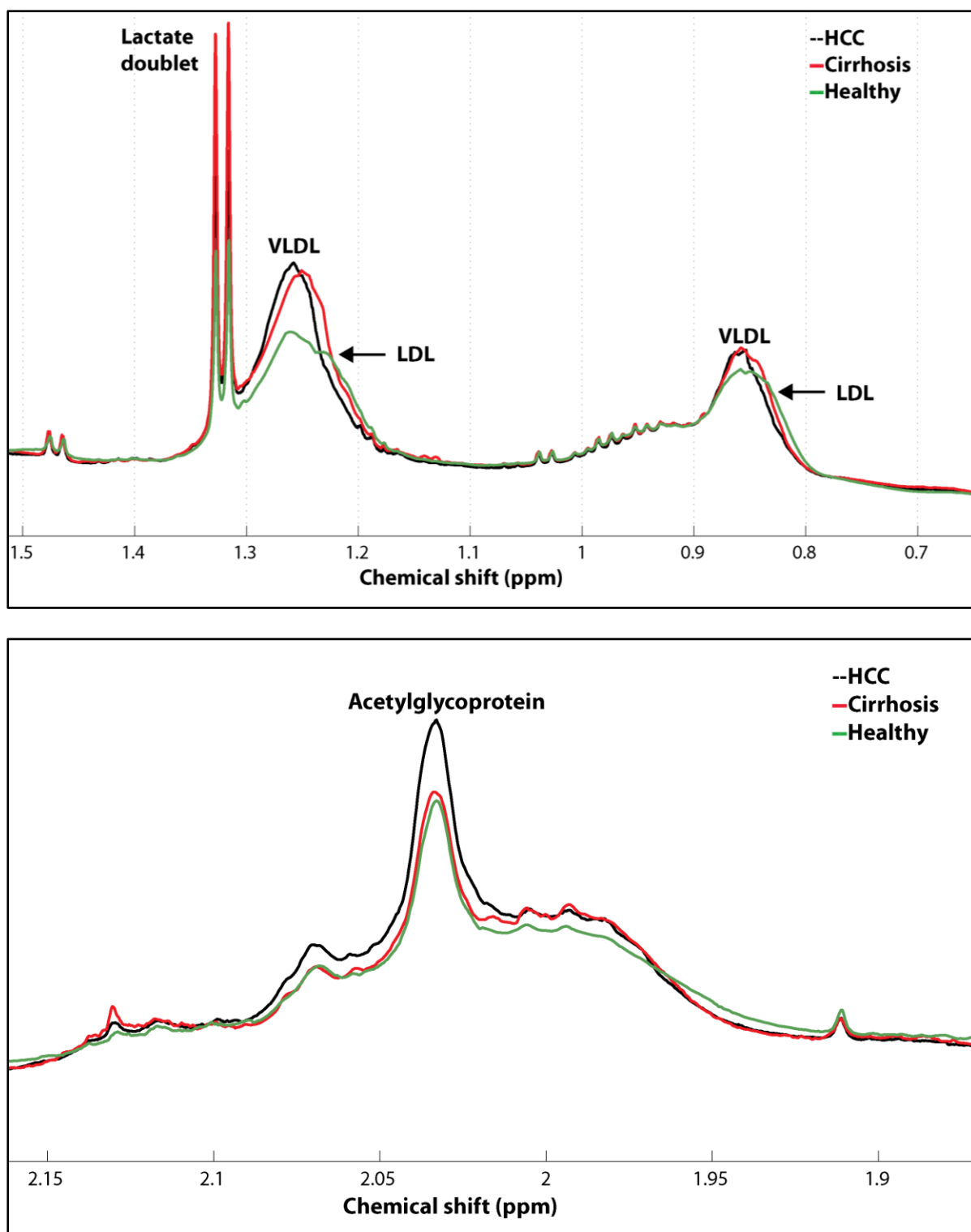


Figure 4-3. Selected spectral regions of difference between median hepatocellular carcinoma, cirrhosis and healthy volunteer group spectra from combined Egyptian and Nigerian cohorts.

4.4.3 Multivariate statistical analysis

Principal components analysis and PLS-DA was 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 4-2**). Principal components analysis of all groups (**Figure 4-4A**) displays clustering of healthy and patient groups suggesting that that variance between the groups accounts for most variance between these metabolite regions. Supervised PLSD-DA was undertaken and is displayed for HCC and healthy volunteer and HCC and cirrhosis groups in **Figures 4-4B and 4-4C**. Clustering can certainly be seen and goodness of fit of the models was good ($R^2=0.87$ and 0.7). Goodness of prediction or Q^2 levels was low: 0.22 and 0.25 . **Figure 4-5** 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 4-6**. 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.

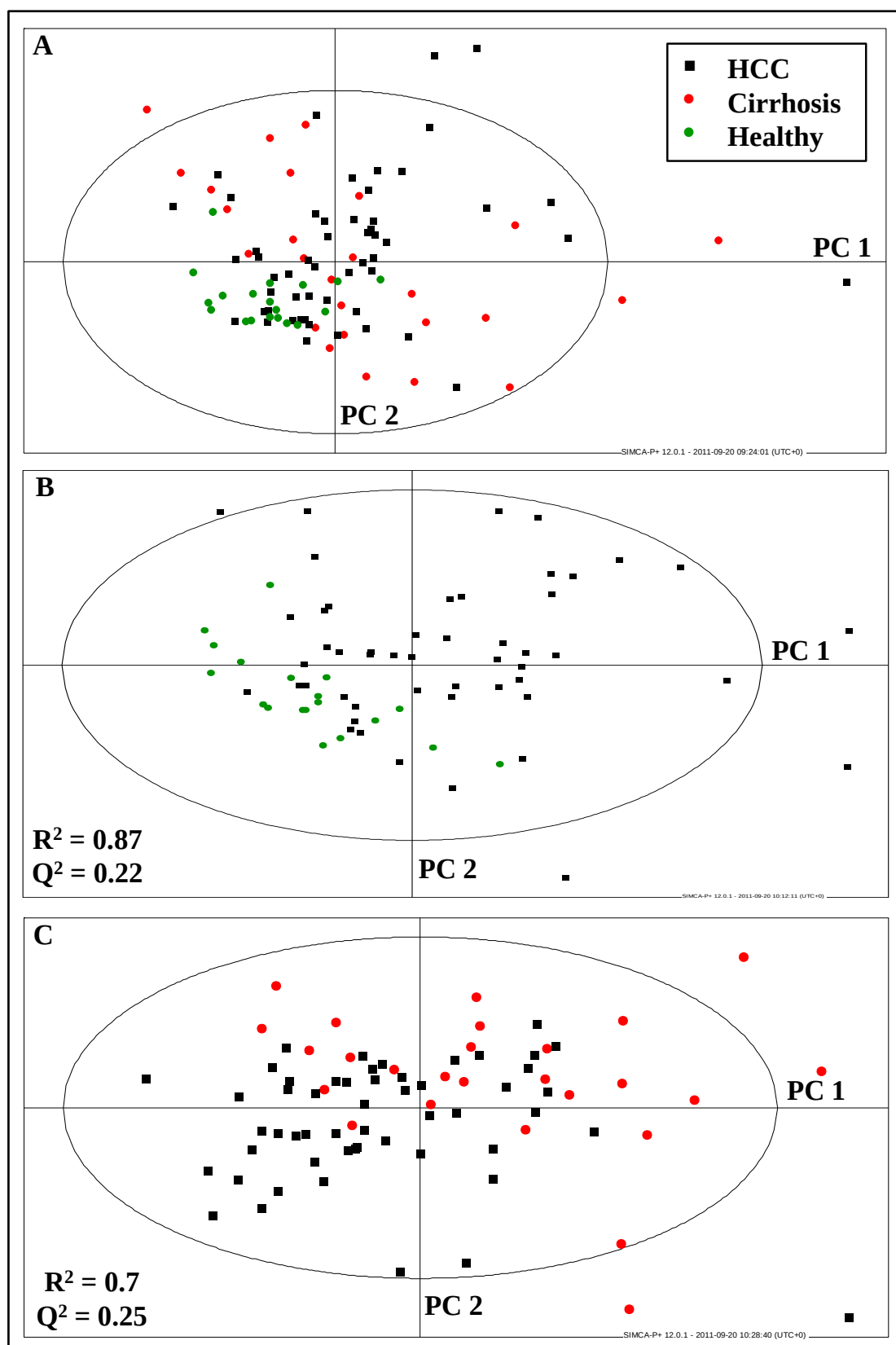


Figure 4-4. Multivariate analyses of combined Nigerian and Egyptian samples.

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.

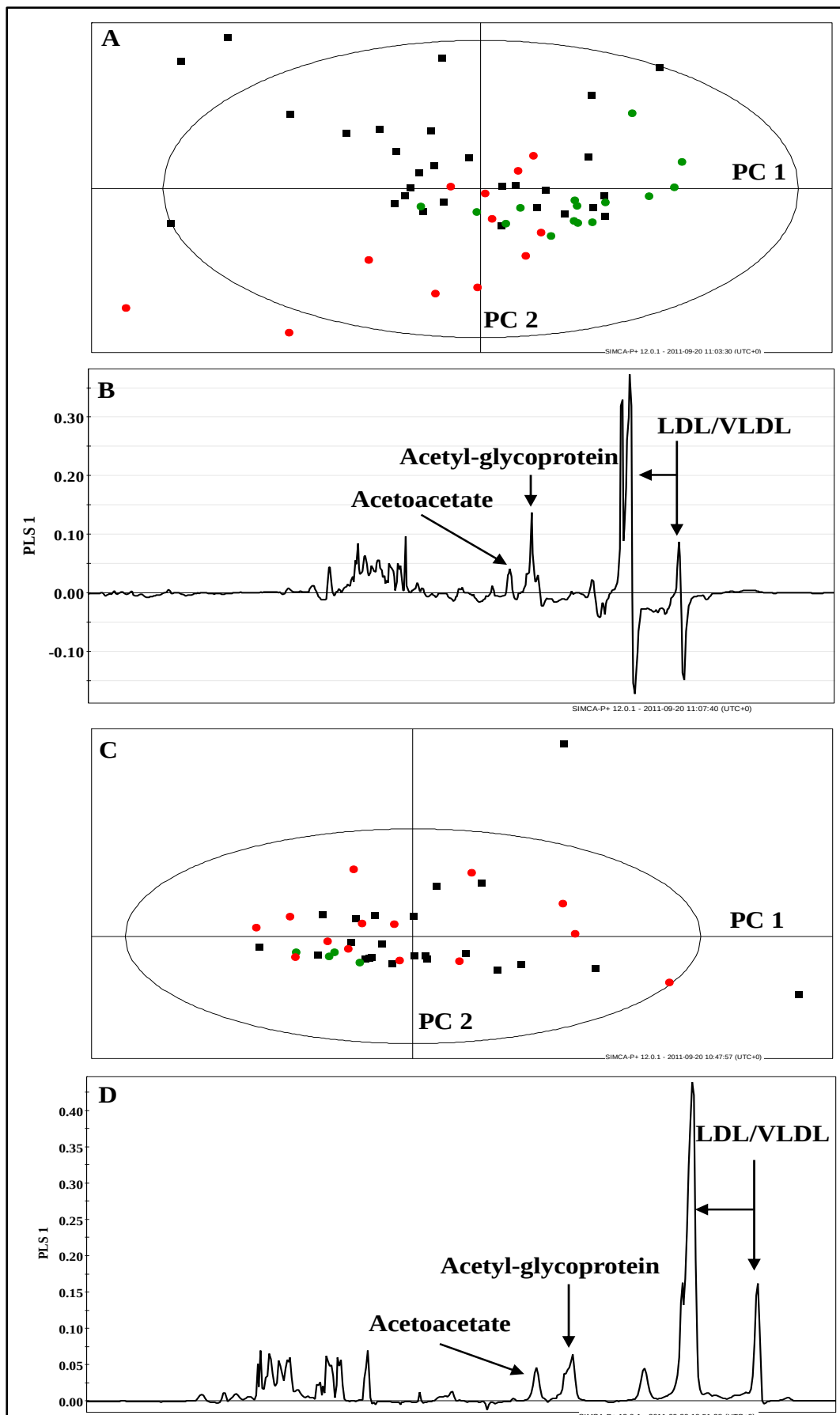


Figure 4-5. Multivariate analysis plots of Nigerian and Egyptian data.

A and B: PCA and PLS-DA loadings plot of Nigerian data; C and D: PCA and PLS-DA loadings plot of Egyptian data.

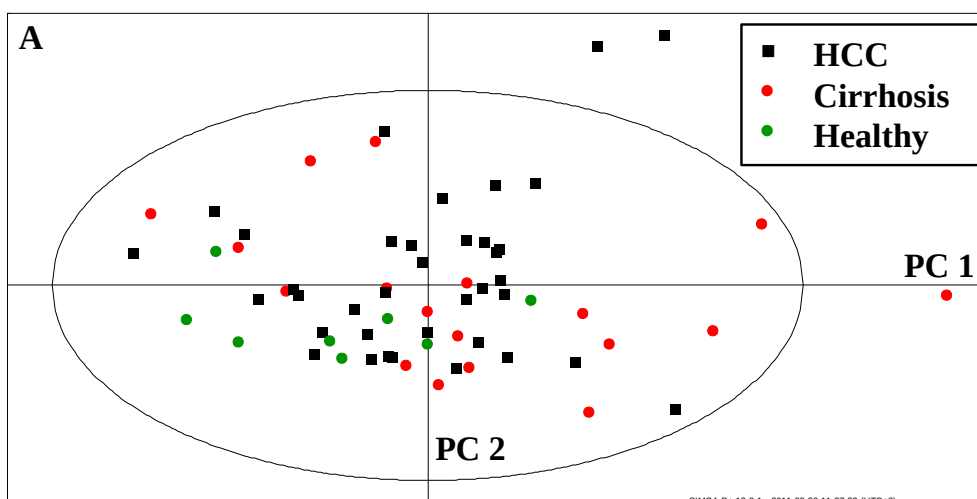


Figure 4-6. Principal components analysis of male volunteer samples.

4.4.4 Univariate statistical analysis

Univariate analyses, using the spectral integral values of one peak, which corresponds to one metabolite, were performed (**Figure 4-7 and Table 4-7**). The most prominent spectral peaks, arising from LDL and VLDL molecules, showed significant difference between the groups. Low density lipoprotein 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). Very low density lipoprotein levels were raised in patients with HCC compared to 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.

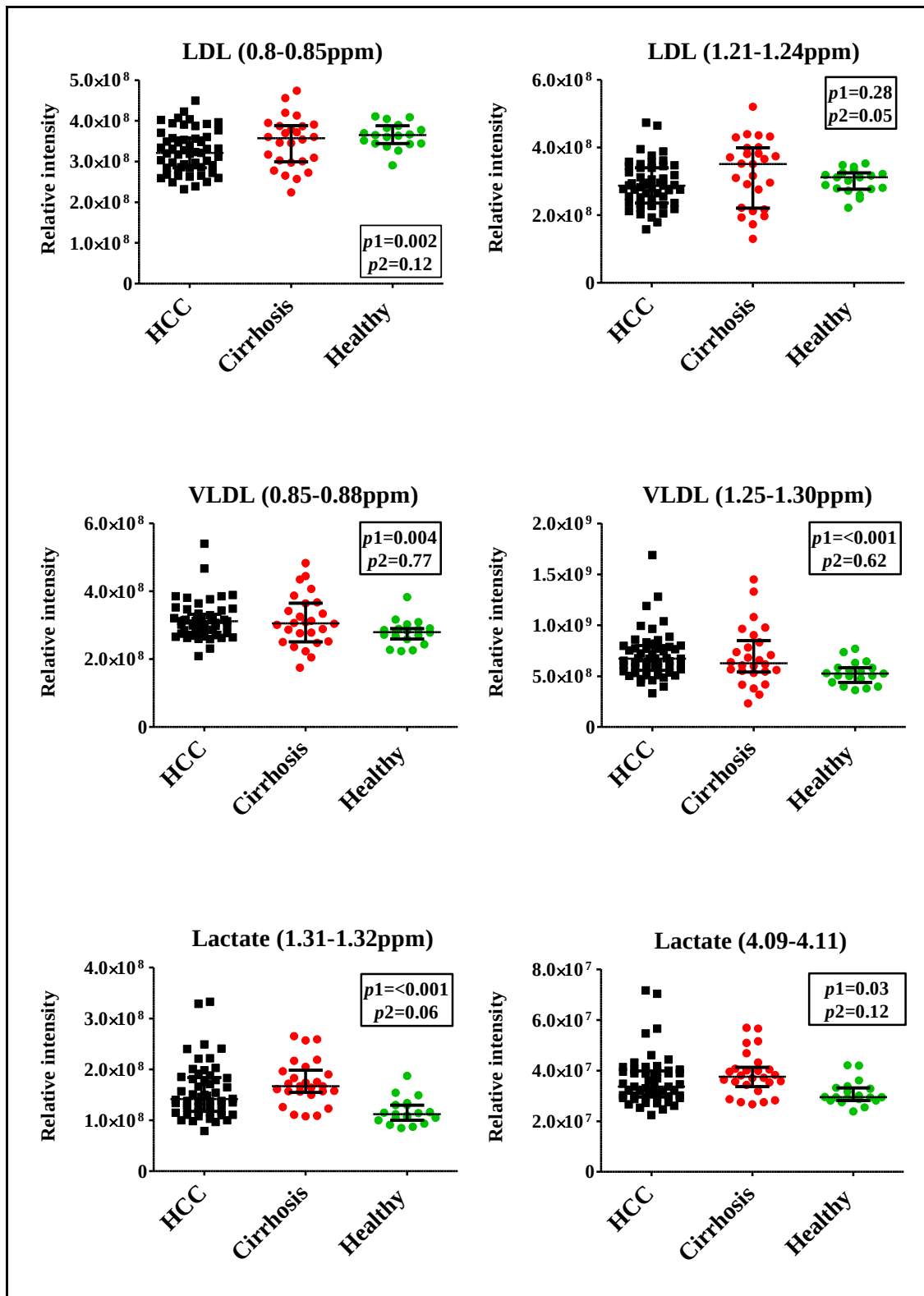


Figure 4-7. Univariate analysis of discriminatory metabolites.

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.

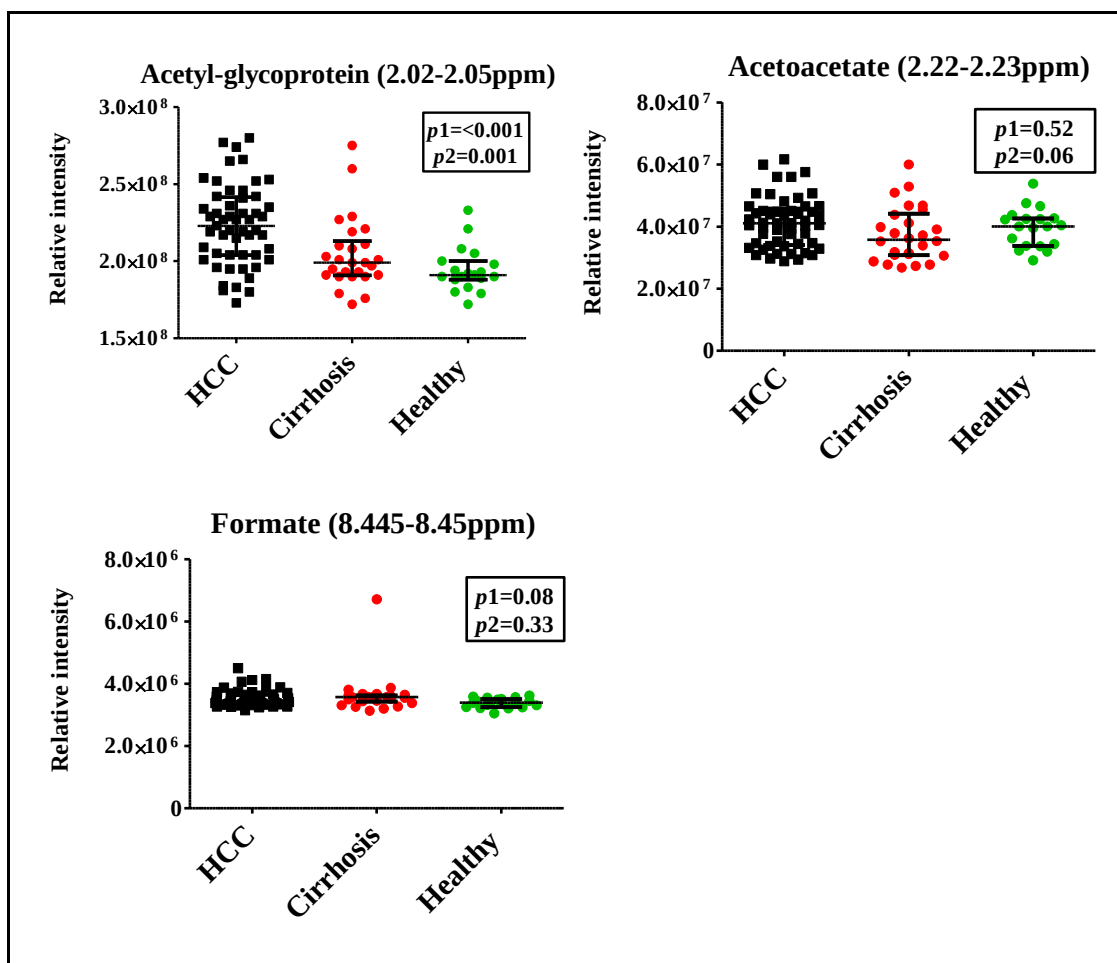


Figure 4-7 continued. Univariate analysis of discriminatory metabolites.

Key: $p1$ = p -value of HCC versus healthy control analyses; $p2$ = p -value of HCC versus cirrhosis analyses. Mann-Whitney tests of significance used for generation of p -values.

Table 4-7. 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, *indicates *p*-value <0.05.

4.5 DISCUSSION

This is the first study to characterise the metabolic changes in serum and plasma due to HCC in two diverse populations. Multivariate analysis displayed reasonable separation of disease and healthy groups and 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. To test this, it would have been revealing to compare the metabolite profiles of Nigerian patients with HCC to those of Egyptian patients with HCC, but this was not done because of the significant effect that diet, which is quite different in these two countries, would have on resultant data (Holmes *et al.*, 2008).

Gao and colleagues utilised ^1H NMR of serum from patients with HCC in comparison to patients with liver cirrhosis and healthy volunteers (Gao *et al.*, 2009), the results showing some similarities to those reported here (**Table 4-8**). The report of this study did not clarify the patient age, genders or aetiology of liver disease. The metabolites identified in the study reported here and from this 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.

Table 4-8. Metabolite alterations between groups: comparison to previous studies.

Metabolite	Moiety	Chemical shift (ppm)	HCC vs. Healthy	HCC vs. Healthy (Gao <i>et al.</i> , 2009)
LDL	CH ₃	0.8 – 0.85	↓*	↓*
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	↑*	-
Lactate	CH	4.098 – 4.108	↑*	-
N-Acetyl-glycoproteins	NHCOCH ₃	2.02 – 2.05	↑*	↑*
Acetoacetate	CH ₃	2.22 – 2.23	↑	↓*
Formate	CH	8.445 – 8.45	↑	-

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

Low density lipoprotein levels, conversely, were reduced. Acetoacetate, a by-product of fatty acid oxidation, was elevated in patients with HCC. These results, however, must be interpreted with extreme caution. Previous studies by Fossel and colleagues (Fossel *et al.*, 1986) observed strong correlation of the methyl and methylene line width resonances of lipoproteins and malignancy, originally proposed as a “blood test for cancer”. When matched for age and triglyceride levels (which affect the line width of VLDL and LDL resonances) no difference could be found on follow up studies by other groups (Bell *et al.*, 1988; Holmes *et al.*, 1988; Okunieff *et al.*, 1990; Wilding *et al.*, 1988). In the study presented here, age was higher in the cancer group compared to healthy volunteers, albeit not significantly (40 years versus 50 years, *p*=0.09). Although not significant, the *p*-value of 0.09 indicates a trend and age may certainly have influenced lipoprotein resonances and confounded results. The cirrhosis and HCC groups were well matched for age (48.5 years and 50 years, *p*=0.32), so it

is unlikely that age plays a role in explaining discrimination between lipoprotein levels in this group, although none of the univariate comparisons of levels between HCC and cirrhosis groups reached statistical significance. Unfortunately, triglyceride levels were not measured in these subjects so cannot be correlated with results. Given this background, it is unwise to attribute the changes seen in VLDL and LDL levels, which are similar to those observed by Fossel and colleagues, wholly to the presence of HCC, without exclusion of confounding by age and triglyceride levels. If, however, this is a true reflection of HCC, then it is worth exploring the possible mechanisms by which these alterations may occur.

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 (Bassendine *et al.*, 2011). 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. 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. This is likely to be the explanation for vastly reduced lipoprotein levels in the Fisher rat model of HCC described in the previous chapter (**Chapter 3**). 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 (Wurmbach *et al.*, 2007). It is possible that one of these affected pathways may affect lipid metabolism and promote the production of VLDL. A candidate may be PPAR α , a nuclear transcription factor, which, if activated, is known to decrease hepatic VLDL secretion and enhance clearance (Shah *et al.*, 2010). 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 effected through a down regulation of lipolytic enzymes such as hepatic or lipoprotein lipase, the interplay of which is highly complex in lipid metabolism (Bassendine *et al.*, 2011). The rise in acetoacetate is also difficult to explain. If peripheral lipolysis is decreased and thus tissue is taking up less triglyceride for energy or storage demands, then it would also be expected that fatty acid oxidation, of which acetoacetate is a by-product, would be reduced. In combination with raised VLDL and reduced LDL, it is not clear why acetoacetate should be elevated.

Lactate was significantly increased in patients with HCC compared to healthy controls. This finding is not unexpected given that stress induced anaerobic respiration is likely to occur in patients with cancer compared to healthy volunteers.

N-Acetylglycoproteins were increased in patients with HCC. These represent “acute phase protein” fragments of glycoproteins such as α_1 -acid glycoprotein, haptoglobin, transferrin and fibrinogen. This finding is in disagreement with a reduced level of this molecule in the Fisher rat HCC model (**Chapter 3**). Hepatocytes are known to secrete this molecule under a number of different stressful stimuli including cancer (Baumann *et al.*, 1983; Wang *et al.*, 2006). A 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 (Bell *et al.*, 1988). In the human model, it may be that hepatic function is preserved to an extent so as to secrete this molecule upon stressful stimulation. It is noteworthy that glucose levels were not a discriminatory factor between groups. This is in contrast to the rat studies where animals with HCC displayed lower blood glucose levels. It may be that the influence of liver fibrosis and cirrhosis on insulin

resistance has a moderating effect on glucose levels. None of the patients in the study displayed very low glucose levels, hypoglycaemia, known to be an occasional paraneoplastic phenomenon in the presence of HCC (Yamaguchi *et al.*, 1998).

4.6 CONCLUSIONS

This study has produced results which, if interpreted with caution, may provide insight into the altered lipid pathways induced by HCC. Follow up work would begin by more comprehensively characterising the lipidome of patients with HCC. This may be possible with ^1H NMR, as certain centres have developed an expertise in this lipid profiling method in attempt to characterise risk of coronary heart disease (Soininen *et al.*, 2009). With the method from these centres, which uses a similar pulse sequence to the study described here, VLDL, LDL, HDL and IDLs can be differentiated. It would also be helpful to analyse the samples for routine lipid profiles (which would include cholesterol and triglycerides). This, unfortunately, was not possible given the small volumes of sample remaining in storage.

Limitations of the study include the contamination of EDTA in Nigerian samples. Data recovery was reasonable despite this, but to maximise the chance of identifying biomarkers, samples should not contain EDTA or other storage molecules that display ^1H NMR resonances. It would also be essential to compare to a contemporaneously collected sample set of patients with non-liver tumours to assess whether changes are cancer related or HCC specific.

5 BLOOD LIPID PROFILING OF HEPATOCELLULAR CARCINOMA USING ULTRA-PERFORMANCE LIQUID CHROMATOGRAPHY MASS SPECTROMETRY

5.1 ABSTRACT

Introduction: The liver is a hub of lipid metabolism and previous studies have shown liver disease and HCC to be associated with altered blood lipid profiles. The primary aim of this study was to examine the lipid profile of HCC, using MS-based techniques, in an animal model and compare observations made in human populations. Traditional lipid extraction protocols involve the use of chloroform, a toxic chemical, the use of which is diminishing. The secondary aim of this study was to optimise the lipid extraction using dichloromethane instead of chloroform. **Methods:** Plasma samples were obtained from a Fisher rat model of HCC and healthy littermate controls ($n=7$ and 8). Serum and plasma samples were obtained from patients with HCC and cirrhosis from UK ($n=3$ and 4) and Nigerian ($n=5$ and 5) cohorts. All samples were analysed using UPLC-MS, optimised using dichloromethane lipid extraction protocols. Data were processed using multivariate analysis to identify lipids most discriminatory between disease groups. **Results:** In the rat model, multivariate statistical modelling was robust in classifying animals with HCC from healthy controls. In the human studies, multivariate analyses of lipid profiles were poor in distinguishing HCC from cirrhosis. Features M496 T104 and M759 T776 were identified as most contributory to multivariate models. M496 T104 is likely to represent lysophosphatidylcholine (24, 0, 0) (LPC), a major cellular membrane component. **Conclusions:** Altered global lipid profiles were robust in discriminating HCC from healthy controls in a Fisher rat model but poor in parallel human studies. Differences in LPC (24, 0, 0) were present in all studies which may indicate altered cellular turnover as a result of HCC.

5.2 INTRODUCTION

Lipids are a diverse group of molecules, ranging from fatty acids, the building block of most lipids, to glycerolipids and sphingolipids. They are defined by their insolubility in water and solubility in organic solvents. They can be variously involved in energy production, cell membrane synthesis and cell signalling cascades (Fahy *et al.*, 2009) (**Table 5-1**). The liver is a central regulator of lipid turnover, synthesising most endogenous lipids, cholesterol and apolipoproteins.

Table 5-1. Lipids (Fahy *et al.*, 2009).

Lipid	Example	Function(s)
Fatty acyls	Palmitic acid	Building blocks of major lipids
Glycerolipids	Triglyceride	Fat storage molecule in mammalian systems
Glycerophospholipids	Phosphatidylcholine, phosphaethanolamine	Cell membrane substrate, binding site for intra- and extra-cellular proteins
Sphingolipids	Sphingosine, ceramide	Cell membrane substrate, signalling cascade
Sterols	Cholesterol	Cell membrane substrate, hormone synthesis
Prenols	Caretenoid	Vitamin precursors

Cox and colleagues, using phosphorous-31 *in vitro* MRS, observed that HCC tumour tissue displayed raised phosphoethanolamine (PE) and phosphocholine (PC) and reduced glycerophosphoethanolamine (GPE) and glycerophosphocholine (GPC) levels, providing an insight into the altered metabolism of lipids in HCC (Cox *et al.*, 1992). Serum ¹H NMR spectroscopy and LC-MS studies of patients with HCC from China, described reduced levels of lysophosphatidylcholine, sphingosines, unsaturated fatty acids and low density

lipoproteins indicating that HCC-induced altered lipid metabolism could be detected in the blood (Gao *et al.*, 2009; Yin *et al.*, 2009).

In the previous chapter ^1H NMR spectroscopy of serum and plasma revealed broadly altered lipid profiles in patients with HCC. Resolution of lipid species was poor in these studies, allowing only a broad estimation of lipoproteins to be made. Liquid chromatography with MS is a validated method of analysing blood lipid profiles (Quehenberger *et al.*, 2010) and if ultra-pressure systems are employed, acquisition of high resolution data is possible.

Traditional lipid extraction protocols involve the use of chloroform (CHCl_3) and methanol two phase extraction regimens, whereby lipids are soluble and retained in the lower organic layer. These methods were introduced by Folch and colleagues in the 1950s when attempting to optimise extraction protocols for brain tissue (Folch *et al.*, 1957). Chloroform, however, is toxic and potentially carcinogenic, European regulations allowing only a 2 ppm workplace atmospheric concentration. Dichloromethane (CH_2Cl_2) is less toxic, regulations allowing a 50 ppm atmospheric concentration and has been shown, albeit in plant and seed lipid extraction experiments, to be as efficient as chloroform in extracting lipids (Cequier-Sanchez *et al.*, 2008).

The initial aim of this study was to optimise lipid extraction techniques and examine the effectiveness of dichloromethane lipid extraction. The main aim was to examine the lipid profiles of HCC in a small sample of human and rat subjects, as a proof of principal, that lipid extraction coupled with UPLC-MS could be a fruitful method of lipid profiling.

5.3 METHODS

5.3.1 Lipid extraction optimisation

Standard rat plasma was used to evaluate the extraction efficiency of dichloromethane and methanol, versus methanol alone, to establish a protocol which could be applied to all blood samples. Standard lyophilized rat plasma (Sigma-Aldrich, Poole, UK), constituted from pooled rat blood with 3.8% trisodium citrate additive as anticoagulant, was reconstituted with 1 mL H₂O. Test samples were prepared as described below and run using a high strength silica (HSS) T3 AcquityTM UPLC-MS 2.1 x 150 mm, 1.8 µm column (Waters Ltd, Elstree, UK) in a Waters AcquityTM Autosampler (Waters Ltd, Elstree, Herts, UK). Based on superior chromatographic results (**Figure 5-1**) dichloromethane and methanol extraction was utilised for all further experiments.

5.3.2 Fisher rat model of hepatocellular carcinoma

Induction of HCC, venesection and sample storage were the same as described in **Chapter 3**.

5.3.3 Patient selection

UK patients were recruited from outpatient and inpatient settings at St Mary's Hospital, Imperial College NHS Trust, Paddington, London, UK between October 2008 and March 2011. All patients gave written informed consent in accordance with local Research Ethics Committee approval. Nigerian patients were recruited from Jos University Teaching Hospital (JUTH), Jos Nigeria and all provided informed consent, signed HCCs were diagnosed in both populations by radiological measures (US, CT or MRI) and cirrhosis in the UK population diagnosed by biopsy, US, MRI or CT confirmation and in Nigeria, clinically, by the presence of portal hypertension (varices or ascites) and US or CT confirmation.

5.3.4 Blood laboratory tests

Serum ALT, total bilirubin and albumin 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). Given the low number of samples in each group, median levels were not statistically compared as results would have been prone to bias.

5.3.5 Sample collection

Random, non-fasted 5 mL blood samples were venesected into either plain serum or EDTA containing sterile tubes and placed immediately on ice or into a refrigerator at 4 °C. Samples were centrifuged within 1 – 2 hrs at 4 °C, 1000 rpm for 10 min. The supernatant was transferred as 2 mL aliquots into 2 mL microvial tubes and stored at -80 °C until analysis.

5.3.6 Sample preparation

All solvents were HPLC grade, 99.9% pure and purchased from Sigma-Aldrich, Poole, UK. All samples were thawed at room temperature and 100 µL transferred into 3.5 mL glass vials to which 267 µL methanol were added. Using a glass syringe, 1.2 mL dichloromethane were added and the sample was vortexed for 5 s to encourage mixing of sample with solvents. The mixture was centrifuged at 1,400 rpm for 5 min at room temperature which resulted in a two layer product, the bottom layer containing the lipid fraction. Using a glass 150 mm Pasteur pipette (VWR International, Pennsylvania, USA) the bottom layer was transferred into clean 3.5 mL glass vials. The solution was evaporated to dryness using a Concentrator Plus™ evaporator (Eppendorf, Hamburg, Germany) under vacuum at room temperature for 1 hr. The resultant concentrated dried lipid fraction was re-suspended in 100 µL methanol (final 1: 1 ratio of sample: methanol), placed in an ultrasonic bath for 10 min (Grant Instruments,

Cambridge, UK), vortexed for 30 s and centrifuged at 1,400 rpm for 5 min at room temperature. The supernatant was transferred into 150 μ L glass vial inserts (Waters Ltd, Elstree, UK) and capped with pre-slit polytetrafluoroethylene/silicone septated caps, ready for UPLC-MS analysis.

5.3.7 Chromatographic conditions

All chromatography was performed using a HSS T3 Acquity UPLC-MS 2.1 x 150 mm, 1.8 μ m column (Waters Ltd, Elstree, UK) in a Waters Acquity AutosamplerTM (Waters Ltd, Elstree, UK). Mobile phase solvents were made up as follows: mobile phase A consisted of 290 mL H₂O, 200 mL acetonitrile and 10 mL ammonium acetate buffer (pH 5); mobile phase B consisted of 440 mL isopropanol, 50 mL acetonitrile and 10 mL ammonium acetate buffer (pH 5). As lipids avidly adhere to this column, isopropanol was used for mobile phase B as it possesses the correct miscibility to allow lipid elution from the column. The column temperature was high, 65 °C, which also encourages lipid elution. Injection volume was 3 μ L and the flow rate set at 500 μ L min⁻¹. The mobile phase gradient (A / B) was set as 60 % / 40% for 10.5 min, 0 / 100% for 2.1 min, 60% / 40% for 4.4 min (total run time 17 min).

5.3.8 Mass spectrometry

Mass spectrometry experiments were performed on a Micromass LCT PremierTM quadrupole time of flight (Waters MS Technologies, Manchester, UK) with electrospray ionisation (ESI). Data were collected in positive ion centroided mode. The mass spectrometer was operated with a capillary voltage of 3 kV, cone voltage of 50 V, nebulizer gas flow of 800 L/h, desolvation temperature of 400 °C, and source temperature of 120 °C. The instrument was set to acquire over the mass range m/z 50-1000 with acquisition time of 5 scans s⁻¹ and an

interscan delay of 0.02 s. The analyser was run in V mode and the lock mass used was leucine enkephalin (556.2771 Da).

5.3.9 Data pre-processing

Datasets generated are complex, each sample generating chromatographic retention time and mass spectrometry data. Prior to comparative analysis it is essential that the data are aligned so that an analyte has the same mass: charge ratio (m/z) and retention time in all samples. Correction of m/z drift is straightforward with the use of a known reference standard (leucine-enkephalin, m/z 556.2771) utilised as a mass lock. Retention time drift, due to sample composition, column variability with time and environmental factors, is more difficult to correct for. For these experiments the data processing software XCMS was used (Scripps Center for Mass Spectrometry, La Jolla, California, USA) (Smith *et al.*, 2006). XCMS performs three functions resulting in comparable data information:

- i. Peak detection. The algorithm is based on cutting the data into slices a fraction of a mass unit (0.1 m/z) and operating on individual slices in the chromatographic time domain. The signal is determined by taking the maximum intensity at each time point in the slice (Smith *et al.*, 2006).
- ii. Peak matching. Overlapping fixed-interval bins of 0.25 m/z width are generated to match peaks. Because bins are overlapping, the algorithm counts the same peak twice and subsequently removes peak groups originating from overlapping bins resulting in a single peak per bin. Once peaks have been matched by mass, they are matched by retention time. This is performed by calculating the overall distribution of peaks in chromatographic time and identifying the boundaries of regions where many peaks have similar retention times. The distribution of peaks or “metapeaks” are defined in a fixed interval in which all peaks are placed into a group (Smith *et al.*, 2006).

- iii. Retention time correction. From the initial grouping, the algorithm then identifies those peak groups in which very few samples have no peaks assigned and very few samples have more than one peak assigned. The algorithm calculates the median retention time and deviation from median for every sample in that group. Consequently, a detailed, non-linear retention time deviation contour can be built for each sample and peak groups can be corrected for retention time differences (Smith *et al.*, 2006).

The resulting data matrix displays sample identification (observations) against unique mass (in Da) / retention time (“T” in sec) “features” (variables), each number corresponding to the intensity for that feature in a particular sample. The software also performs a simple univariate *t*-test between two selected groups identifying those features the levels of which are significantly different between those two groups. The features are listed in order of significance and accompanying extracted ion chromatograms (EIC) are generated to aid visualisation. UPLC with XCMS data processing has been validated for use in metabolic profiling experiments displaying excellent data preservation and high sensitivity and resolution of data (Nordstrom *et al.*, 2006).

5.3.10 Multivariate analysis

The data matrix was exported to SIMCA (Umetrics, Umea, Sweden) for PCA and PLS-DA as described in earlier chapters. All data were mean centred prior to multivariate analysis.

5.3.11 Univariate analysis

Data were exported to GraphPad Prism (La Jolla, California, USA) for univariate analysis in the form of Mann-Whitney *t*-tests comparison of medians between groups, assuming non-parametric distribution of data.

5.4 RESULTS

5.4.1 Patient and rat demographics

Patient demographics are displayed in **Tables 5-2 – 5-4**. Body mass indices were similar for patients with HCC and cirrhosis, however, all of the HCCs in the Nigerian group were AFP secreting, while those in the UK cohort were not. The aetiologies in both groups were diverse, including HCV, HBV, alcohol and NASH. The average Okuda stage was higher in the Nigerian group (2.4), compared to the UK group (2.0).

Table 5-2. Patient demographics of UK and Nigerian cohort.

Characteristic	UK group		Nigerian group	
	HCC	Cirrhosis	HCC	Cirrhosis
<i>n</i>	3	4	5	5
Median age (range) (years)	61.0	65.5	48.0	57.0
Male <i>n</i> (%)	3 (100%)	2 (50%)	4 (80%)	4 (80%)
Median body mass index (kg/m ²)	24.1	28.6	Not recorded	Not Recorded
AFP (IU mL ⁻¹)	23.9	4.3	8,877	5.97
ALT (IU L ⁻¹)	44.0	23.5	29.0	37.0
Bilirubin (µmol L ⁻¹)	10	46.5	7.1	26.4
Albumin (g L ⁻¹)	32.0	33.5	22.6	27.8

Table 5-3. Aetiologies of hepatocellular carcinoma and cirrhosis cohorts.

Aetiology	HCC		Cirrhosis	
	UK	Nigerian	UK	Nigerian
HCV	1	0	0	0
HBV	2	5	1	5
Alcohol	0	0	2	0
NASH	0	0	1	0
Total	3	5	4	5

Table 5-4. Okuda stage of patients with hepatocellular carcinoma.

Stage	UK	Nigerian
I	1	0
II	1	3
III	1	2
Mean score	2	2.4
Total	3	5

Median rat group weights at sacrifice (14 weeks) are displayed in **Table 5-5**, displaying that control rats were 103.5 g heavier than those with HCC.

Table 5-5. Fisher rat weights at time of sacrifice.

	HCC group	Control group
n	7	8
Weight at 14 weeks upon sacrifice (g)	340.5	444.0

5.4.2 Lipid extraction optimisation

Total ion chromatograms of the same standard rat plasma using different extraction methods are displayed in **Figure 5-1**. Several areas in the chromatogram are highlighted where dichloromethane with methanol extraction resulted in superior lipid recovery to methanol alone.

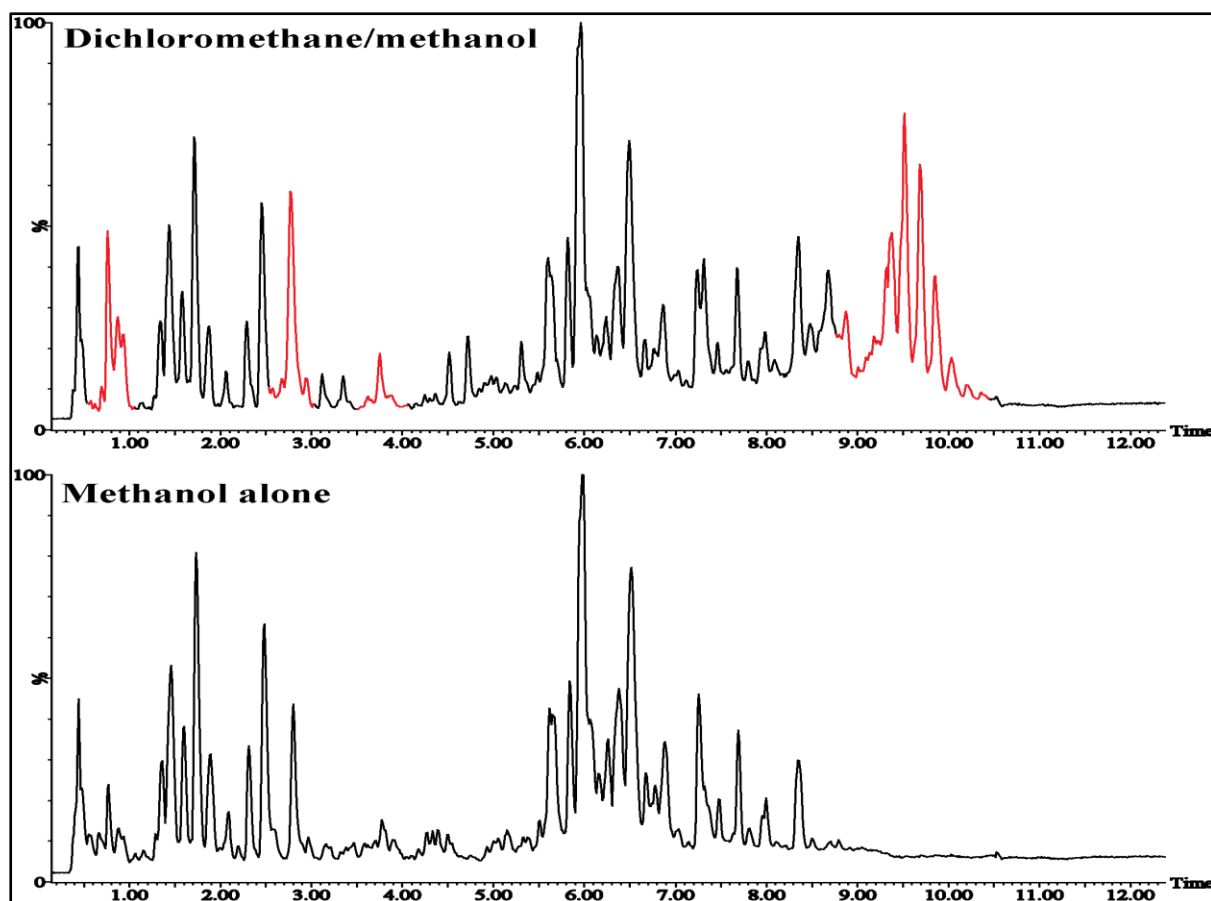


Figure 5-1. Total ion chromatograms of the rat standard plasma after dichloromethane/methanol or methanol only extraction.

Top pane: dichloromethane/methanol extraction; lower pane: methanol only extraction. Areas in red highlight improved recovery of lipid species.

5.4.3 Positive mode lipid chromatograms

Figure 5-2 displays a positive mode total ion chromatogram from a Fisher rat with HCC. In comparison, chromatograms from patients from the UK and Nigerian group are also displayed. Similarities between the chromatograms are observed suggesting similar lipid profiles overall.

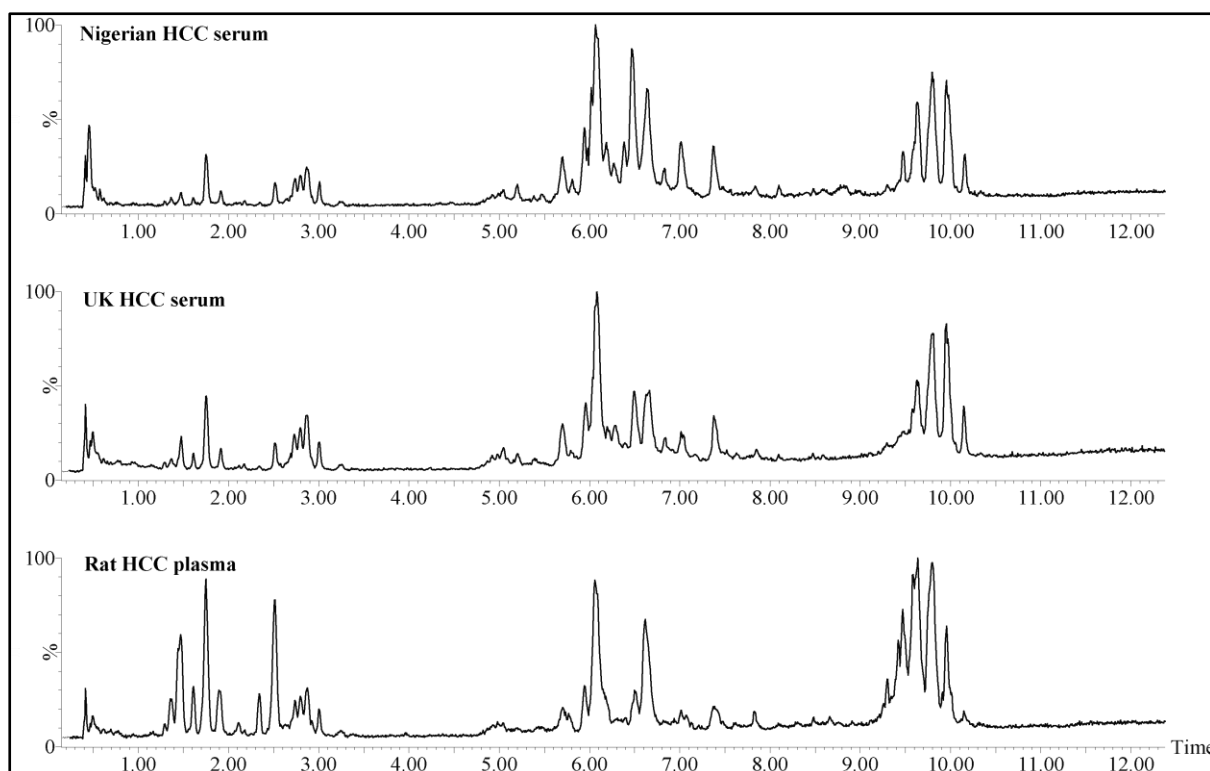


Figure 5-2. Nigerian, UK and rat serum and plasma total positive ion mode chromatograms following lipid extraction.

5.4.4 Multivariate statistical analysis

Figure 5-3 displays PCA scatter plots for rat plasma as well as those for parallel UK, Nigerian patients' serum and plasma samples, of HCC versus cirrhosis for human studies and HCC versus healthy controls for the rat studies. Clustering of groups was poor in the human studies, although in the rat studies, it was more obvious. PLS-DA of the rat data revealed a good R^2 value (0.88), or goodness of fit, and good Q^2 value (0.74), goodness of predictability. For Nigerian and UK human studies: $R^2 = 0.68$ and 0.52 , $Q^2 =$ both negative, suggesting good model fitting but poor discrimination between HCC and cirrhosis based on global lipid profiles. PLS-DA loadings plots were examined for those features that contributed most greatly to PLS components in both models. **Table 5-5** displays these features in all three groups, documenting certain features that were identified in two or more of the cohorts.

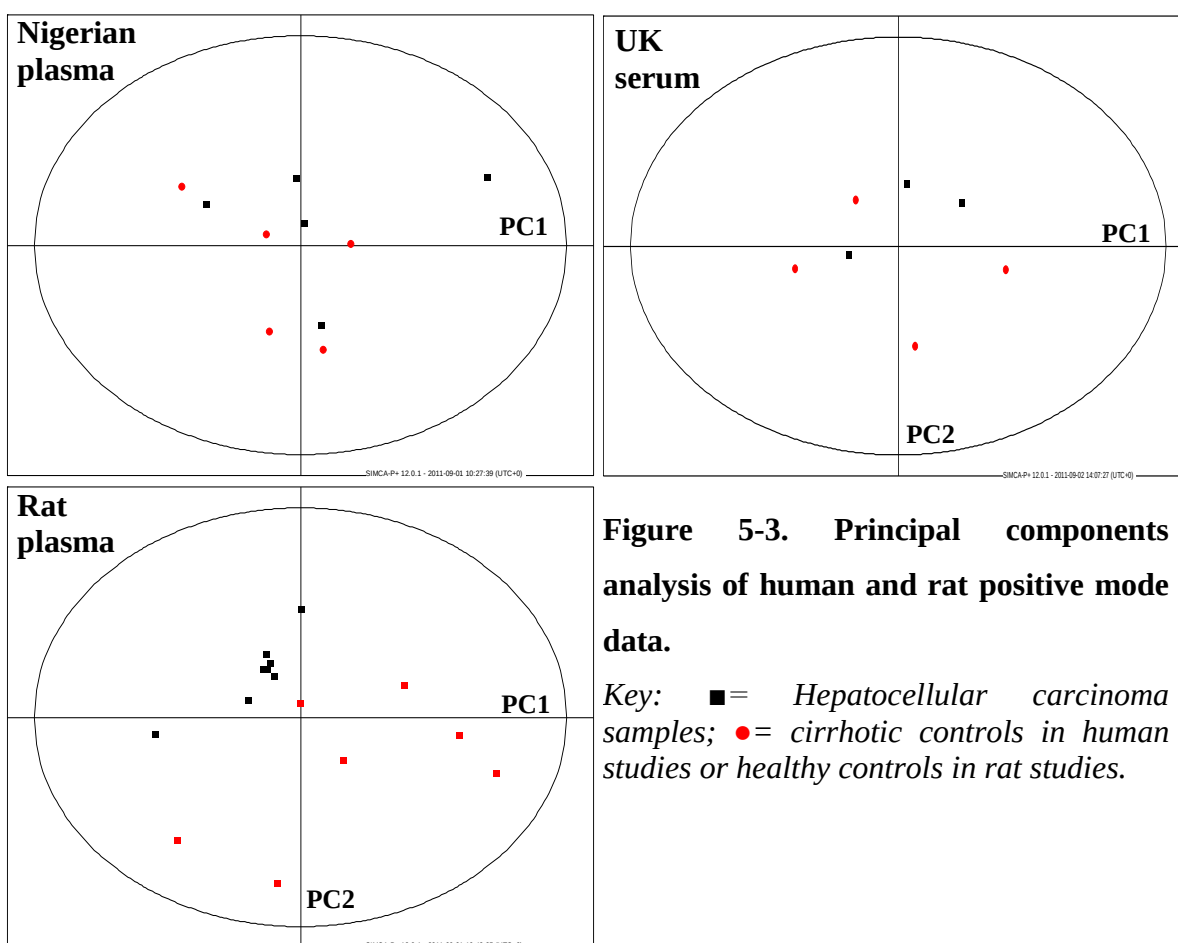


Table 5-6. Discriminatory features identified by multivariate analysis.

	Nigerian plasma and serum	UK serum	Rat plasma
Feature	M759 T366	M496 T104	M496 T104
	M849 T585	M522 T114	M269 T776
	M821 T573	M520 T87	T759 T366
	M187 T776	M187 T776	M873 T577

Text in green indicates features that were discriminatory in two or more groups.

5.4.5 Lipid feature identification

Those features that were discriminatory in two or more cohorts were selected for identification. M187 T776 was represented in all three cohorts, however, when the chromatographic data were analysed, it was apparent that at this retention time there was no

clear chromatographic peak, nor would it be likely at such a late time point (12.95 min), which is at the end of the run after the majority of ions have been eluted. XCMS had therefore erroneously labelled background noise as a peak. This feature was discounted from further analysis.

Representative extracted ion chromatograms for masses 496 Da (exact mass 496.34 Da) and 759 Da (exact mass 758.57) from the human and animal study HCC groups are displayed in **Figures 5-4 and 5-5**. These chromatographic data confirm the presence of true elution peaks for these masses. Mass spectra of these peaks from the human and animal studies are displayed in **Figure 5-6 and 5-7**. Reference to online databases (<http://metlin.scripps.edu>) identified this feature as likely to be a lysophosphatidylcholine (LPC) (24, 0, 0) ($C_{24}H_{50}NO_7P$) a lysophospholipid that can have variable fatty acid chain lengths (Wishart *et al.*, 2007) and is a major plasma lipid (**Figure 5-9**). In addition, in some spectra, a co-eluting mass at 184.07 Da is observed, which may represent a phosphocholine head group, a fragment of LPC (24, 0, 0) (**Figure 5-6**). Feature M759 T366 could not be identified from databases.

5.4.6 Univariate statistical analysis

Figures 5-8 display scatter plots of the relative intensity of each of these masses in each disease group. Feature M496 T104, likely to represent LPC (24, 0, 0), was reduced in patients with HCC compared to cirrhosis, although not to a level of significance ($p=0.4$). In the Nigerian cohort there was no significant difference ($p=0.69$). This was mirrored in the Fisher rat study where there was no significant difference between levels of M496 T104 between animals with HCC and healthy controls ($p=0.96$).

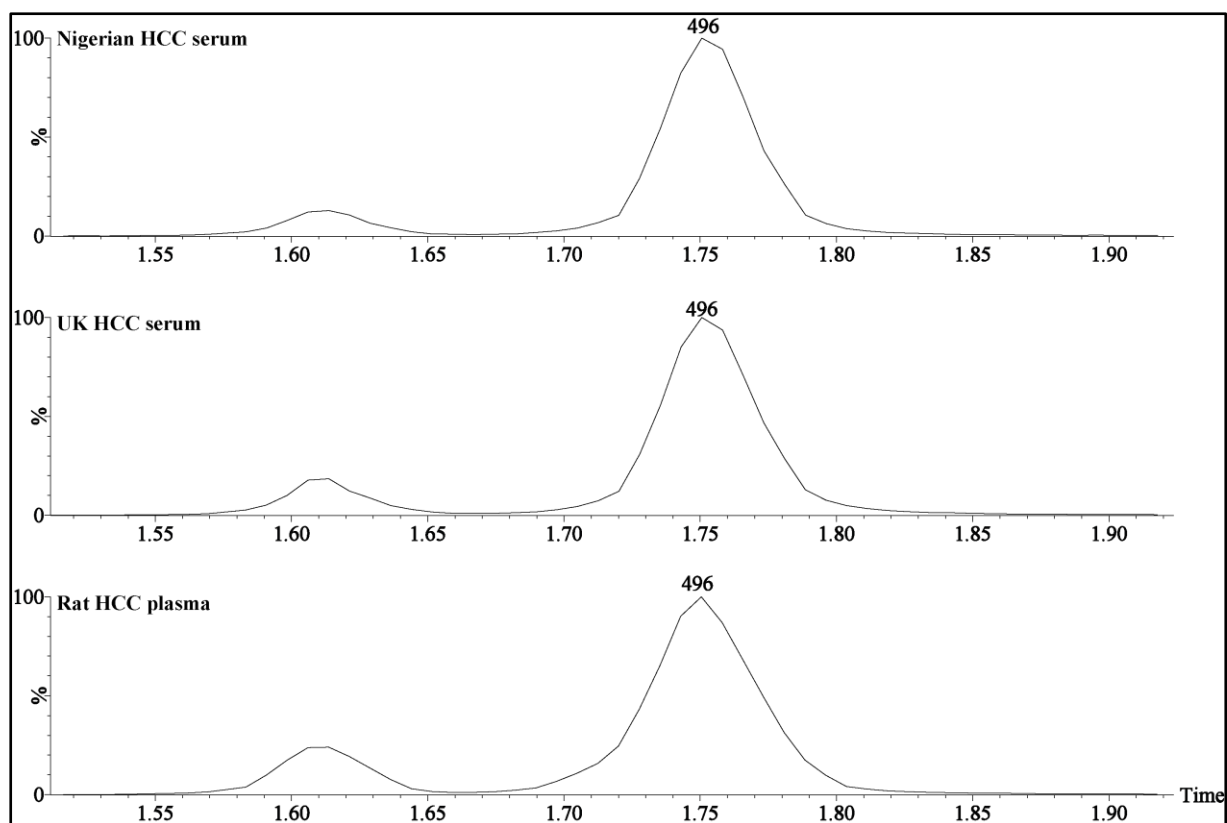


Figure 5-4. Extracted ion chromatograms for mass 496.34.

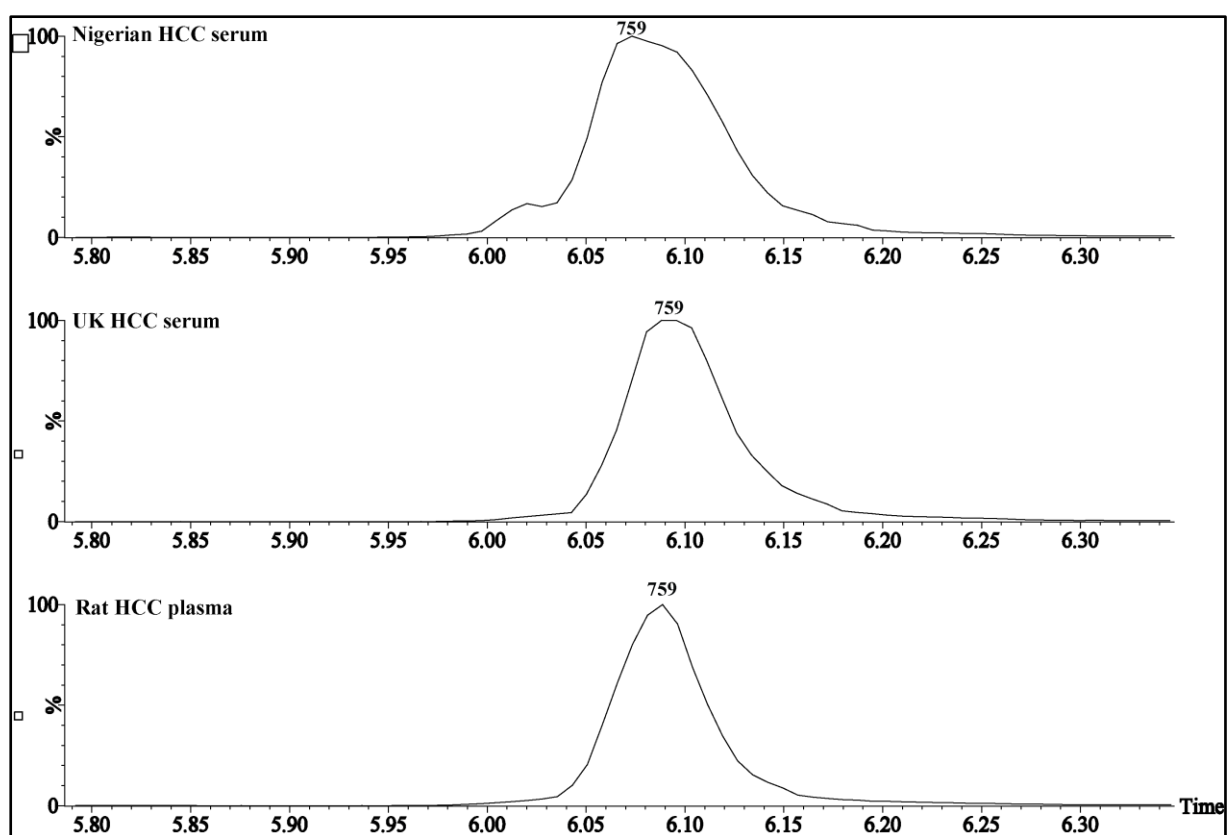


Figure 5-5. Extracted ion chromatograms for mass 758.57.

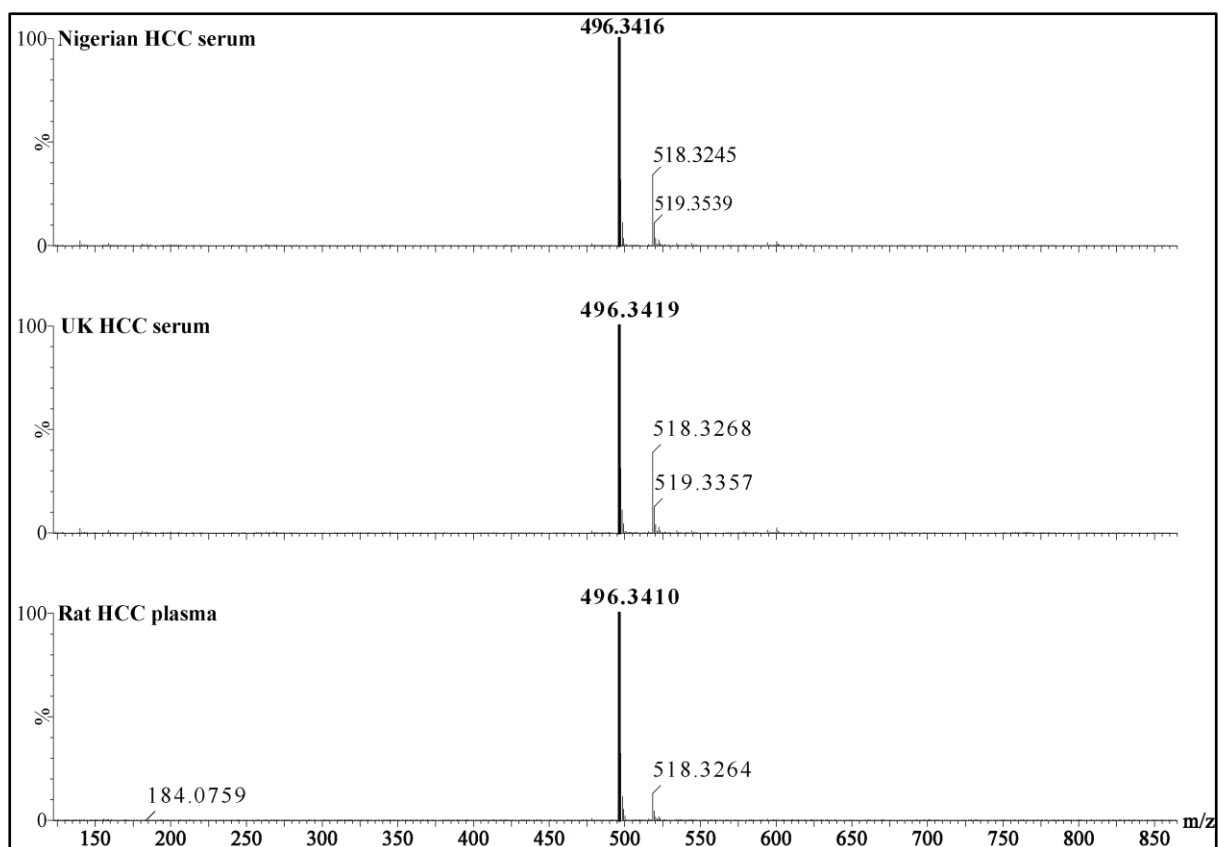


Figure 5-6. Mass spectra of feature M496 T104.

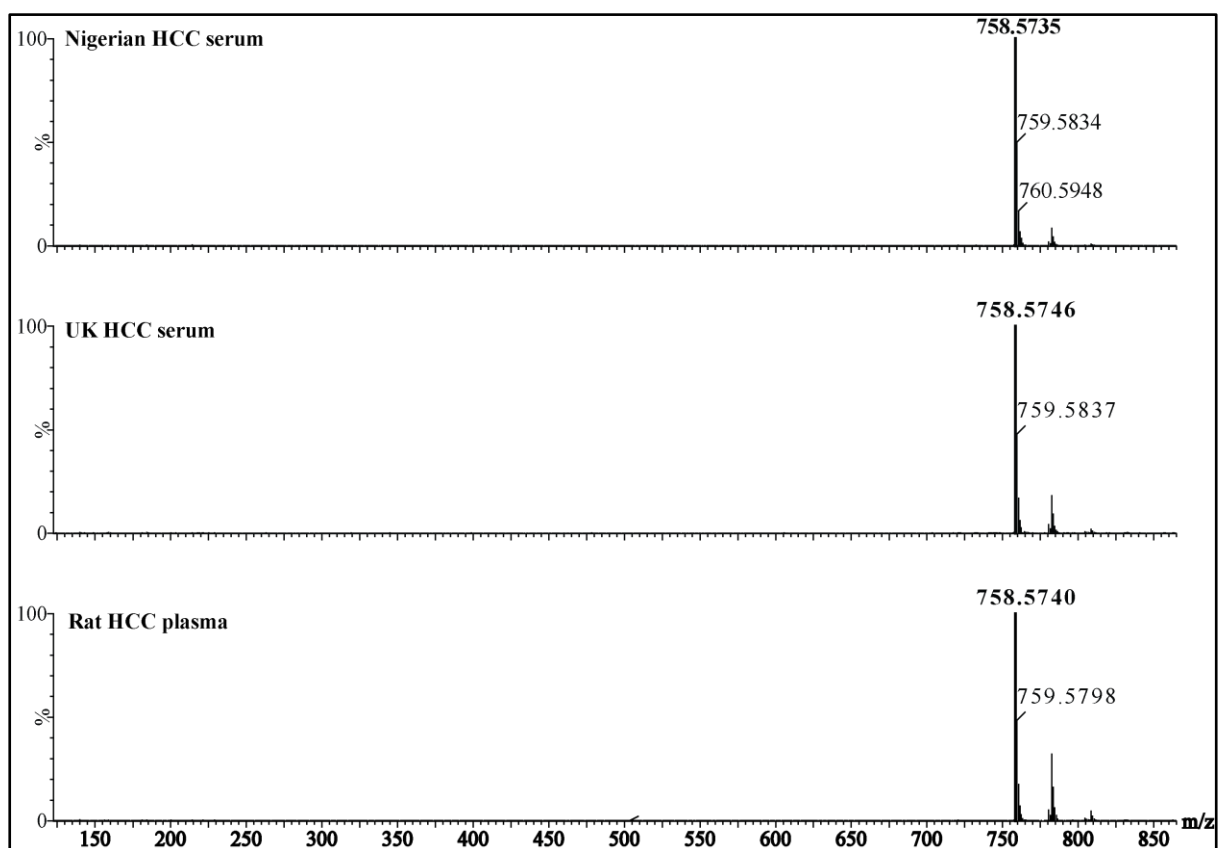


Figure 5-7. Mass spectra of feature M759 T366.

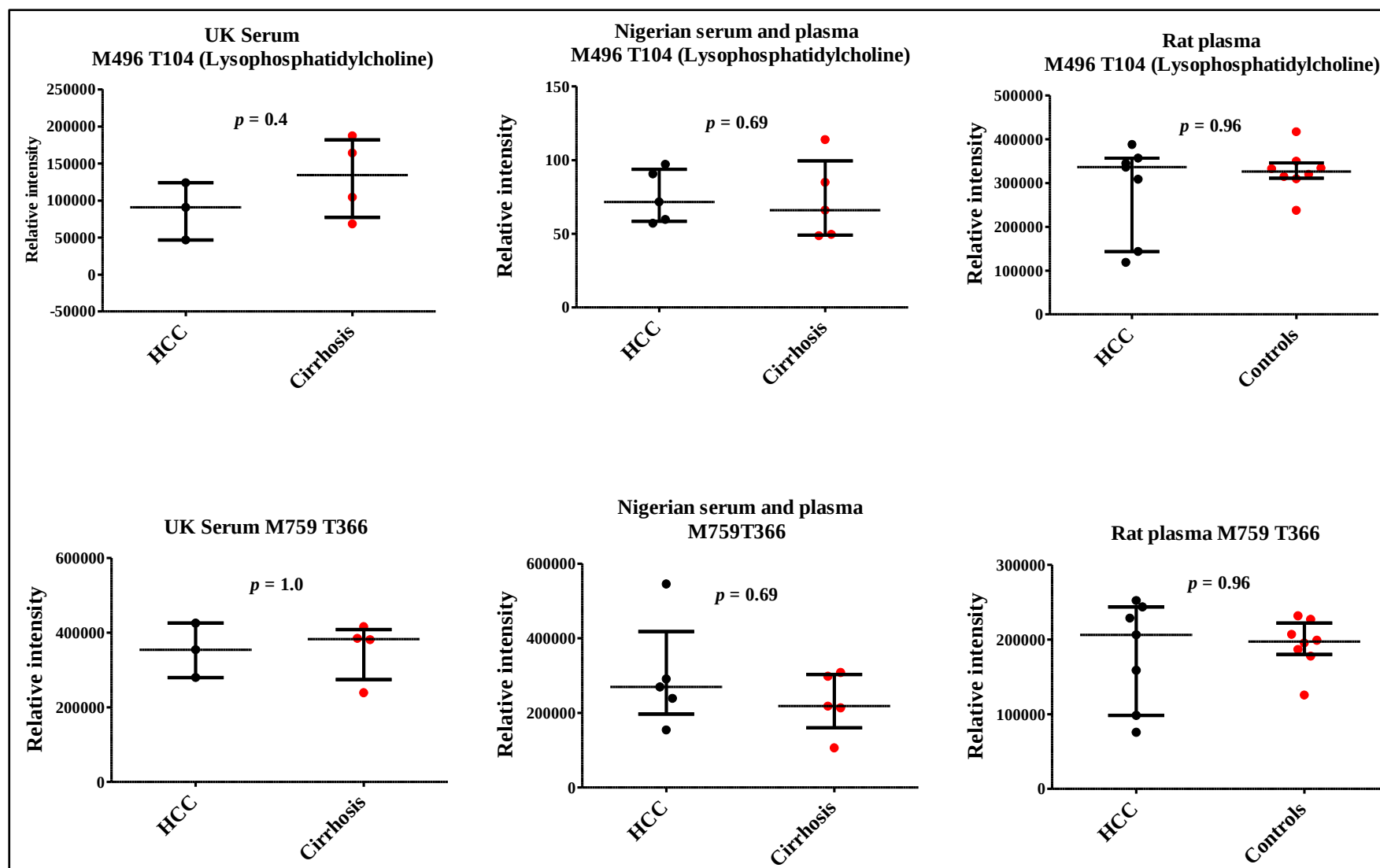


Figure 5-8. Relative intensities of features M496 T104 (likely to be lysophosphatidylcholine (24, 0, 0)) and M759 T366 in UK, Nigerian and rat cohorts. (*p* values generated from Mann-Whitney tests of median values)

5.5 DISCUSSION

Proton NMR spectroscopy studies described in the previous chapter, highlighted altered lipid profiles in animal and human studies. The aims of the study presented here were to optimise lipid extraction techniques using dichloromethane instead of chloroform and to characterise the lipid species altered in HCC. Optimisation experiments were successful in confirming that dichloromethane / methanol extraction, while lengthy, does efficiently extract lipids. Classification of disease based on lipid profiles using multivariate statistical analysis was good in Fisher rat models ($R^2=0.88$ and $Q^2=0.74$), but poor in human models ($R^2=0.52 - 0.68$ and Q^2 negative). Multivariate analysis accounts for global lipid profiles and given that there was a 100 g difference in rat weight between the controls and HCCs, it is unsurprising that lipid differences were detected in this group. The human studies were of small number, but well matched groups, both for disease severity and BMI. It is probably this reason that accounts for the similarity in global lipid profiles resulting in poor multivariate classification. Furthermore, there is a far higher degree of genetic diversity in humans which will almost certainly have an effect on lipidomic profiles. It is more illuminating, however, that certain lipids contributed more to multivariate models than others. Indeed, very little is present in the literature on the specific lipid changes in patients with HCC and with greater knowledge of the functional and signalling role of lipids, this study produced some results of note. Feature M496 T104, likely to represent lysophosphatidylcholine (24, 0, 0), was a discriminating factor in multivariate models. The rat model did not display significantly different levels of LPC between animals with tumour and healthy controls. None of the human univariate analyses reached significantly different results between subjects with HCC and controls and trends were different in both the human cohorts. In the UK population, levels of this molecule were higher, while this was not the case in the Nigerian cohort. Lysophosphatidylcholine is a major plasma glycerophospholipid which can possess variable fatty acid chain lengths, the

most common containing 16 carbons in the fatty acid chain (Wishart *et al.*, 2007). LPC transports metabolites such as fatty acids, choline and phosphatidylglycerol between tissues. It is derived from surface phosphatidylcholine of lipoproteins or cell membrane-derived phosphatidylcholine as a result of phospholipase A2 (Schmitz and Ruebsaamen, 2010). A proportion of LPC has been shown to be secreted from the liver in rats (Sekas *et al.*, 1985). The main role of LPC is thought to be as a cell membrane constituent, although it is increasingly recognised that it may have a role in cell signalling, cell proliferation, and even tumour cell invasiveness (Fukushima *et al.*, 2001). Alterations in levels of plasma LPC have also been observed in neurological diseases and carcinomas (Schmitz and Ruebsaamen, 2010). Cox and colleagues, observed increased PC and PE and decreased GPC and GPE levels in seven *in vitro* tumour extracts, three of which were from HCC, using ³¹P magnetic resonance spectroscopy. They hypothesised that this may be due to increased synthesis and decreased breakdown of phospholipids in tumours, presumably to fuel the cell membrane substrate requirements (Cox *et al.*, 1992). Yang and colleagues, in 2007, using ¹H MRS of 17 HCC tissue specimens, also detected increased choline levels, concluding that this was due to high cell turnover (Yang *et al.*, 2007). Two studies from China, in HBV infected patients with HCC, using MS and ¹H NMR techniques, observed reduced LPC levels and choline levels in patients' blood (Gao *et al.*, 2009; Yin *et al.*, 2009). It is therefore likely, that a similar explanation underlies the results observed in this UK study: lower LPC (24, 0, 0) levels in the blood indicating consumption of this molecule by the tumour to fuel its growth. Why similar results are not seen in the Nigerian cohort may be due to a number of reasons. First, biometric data are not available for the group and it may be that there is a vast difference in BMI between the HCC and cirrhosis groups. If this were the case, then, global lipid profiles would be altered, likely due to the effect of nutritional and cachexia status, rather than specific tumour-related lipid changes. Second, the tumours in the Nigerian group were more

advanced (median Okuda stage 2.4) and may not be directly comparable to the UK group (median Okuda stage 2.0). Thirdly, dietary influence may play a role in influencing lipid profiles. Unfortunately, no dietary history was recorded for the Nigerian patients, but the staple food in this area of central Nigeria consists of maize products and little meat due to its high cost. This contrasts to the protein rich Western diet which UK patients are likely to consume.

To account for these findings as being specific to HCC would be false without validation experiments in other tumours. Cox and colleagues found similar changes in PE / PC and GPE / GPC ratios in adenocarcinoma liver metastases and it is likely that any tumour may display these findings as all require building blocks to synthesis cell membranes. Caution should also be taken in interpretation of results given the small numbers in each group. This study has proved a principle that lipid profiles are altered in HCC compared to similarly matched controls; however, much larger studies are needed to confirm this. Furthermore, identification of all lipids, which display altered levels in patients with HCC, is required for full characterisation of the tumour “lipidome”. UPLC-MS, while exquisitely sensitive, does pose difficulties in species identification and future work would have to include tandem mass spectrometry fragmentation experiments and standard authentication in a larger disease group including other non-HCC tumour types as well.

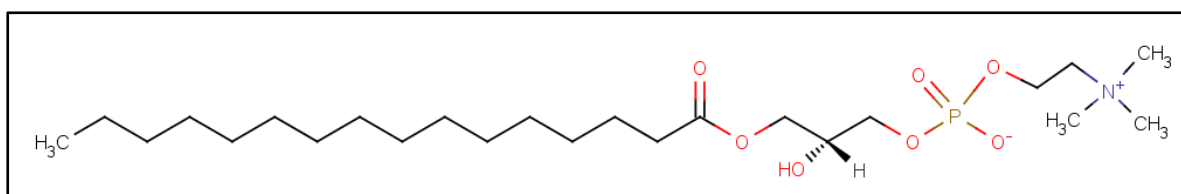


Figure 5-9. Schematic structure of lysophosphatidylcholine (C₂₄H₅₀NO₇P).

5.6 CONCLUSIONS

This study has confirmed that dichloromethane is a valid alternative to chloroform for the purposes of lipid extraction. Using this method, blood lipid profiling of HCC identified altered metabolites in both human and animal models of the disease. Altered global lipid profiles were robust in discriminating HCC from healthy controls in a Fisher rat model but poor in parallel human studies, probably due to the low sample numbers in study groups. Differences in LPC (24, 0, 0) were present in all studies which may indicate altered cellular turnover as a result of HCC.

6 THE URINARY METABOLIC PROFILING OF HEPATOCELLULAR CARCINOMA USING PROTON NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY IN A NIGERIAN PATIENT COHORT

6.1 ABSTRACT

Introduction: There have been few studies of the urine metabolic profile of HCC and none using urinary ^1H NMR spectroscopy in an African population. This study is the first to analyse the urinary ^1H NMR metabolic profiles of patients with HCC from Jos in Nigeria, in an attempt to identify metabolic changes due to the tumour. **Methods:** Using a 500 MHz NMR system, urine was analysed from three well-matched subject groups, collected at Jos University Teaching Hospital (JUTH), Nigeria. Multivariate statistical analyses were performed PCA and PLS-DA. All patients were of Nigerian descent: 18 hepatitis B surface antigen (HBsAg)-positive patients with HCC; 10 HBsAg positive patients with cirrhosis; and 14 HBsAg negative healthy Nigerian volunteers. **Results:** Urinary ^1H NMR spectral profiles from HCC patients were distinguished from healthy controls, and from patients with cirrhosis, with sensitivity: specificity of 100%: 93% and 89.5%: 88.9%, respectively. Metabolites that most strongly contributed to the multivariate models were creatinine, carnitine, creatine and acetone. Compared to patients with HCC, healthy volunteer and cirrhosis patient urine displayed reduced creatinine ($p<0.001$ and 0.06) and elevated carnitine ($p=0.04$ and <0.001) and creatine ($p=0.05$ and 0.29) levels. **Conclusions:** Urinary ^1H NMR spectroscopy with multivariate statistical analysis was able to differentiate patients with HCC from normal subjects and patients with cirrhosis permitting the notion that a urinary test for HCC is possible.

6.2 INTRODUCTION

Urine contains a wealth of information. Small metabolites (<1000 Da) are able to pass through the renal glomerulus, evade tubular re-absorption and be excreted and can form a metabolic fingerprint of the host physiology. These metabolites, which may include amino acids, peptides, nucleotides and lipid fragments can be detected using techniques such as ^1H NMR spectroscopy and mass spectrometry. Several recent studies have highlighted the value of ^1H NMR spectral urinary profiling in the diagnosis of lung cancer (Carrola *et al.*, 2011), bladder cancer (Srivastava *et al.*, 2010), pneumonia (Slupsky *et al.*, 2009) and inflammatory bowel disease (Williams *et al.*, 2009).

Hepatocellular carcinoma is prevalent in sub-Saharan Africa (Shariff *et al.*, 2009) due to the high rate of chronic HBV infection. In resource poor settings, such as rural sub-Saharan Africa, tests to diagnose HCC such as ultrasound and serum AFP are not available owing to their high cost and infrastructure requirements. A urine test for HCC would be invaluable in this setting as it would be non-invasive, robust (urine does not need immediate centrifugation as there are no erythrocytes to lyse) and cheap to collect (no phlebotomy equipment required).

There have been three studies in Oriental patient cohorts of the urinary metabolic profiles of HCC using MS-based techniques (Chen *et al.*, 2009; Chen *et al.*, 2011; Wu *et al.*, 2009). These studies have revealed a number of urinary amino acid, peptide and bile acid signatures of HCC. It is therefore clear that either directly or through changes in host metabolism, HCC can cause metabolic changes detectable in urine. This is the first urinary ^1H NMR spectroscopy study of HCC to be undertaken (Shariff *et al.*, 2010).

The aim of this study was to test the hypothesis that HCC-induced metabolic changes can be detected and categorised using ^1H NMR spectroscopy of urine in an African population. The secondary aim was to test the accuracy of detected alterations in discriminating urine between patients with HCC from that of healthy volunteers and patients with cirrhosis.

6.3 METHODS

6.3.1 Patient selection

The study protocol was in concordance with the ethical guidelines of the 1975 Declaration of Helsinki and approved by the research ethics committee of the University of Jos, Nigeria, while the metabolic profiling protocol was approved by the research ethics committee of Imperial College Healthcare NHS Trust, London, UK. All subjects provided informed, signed consent.

6.3.2 Sample collection

Samples were collected and processed for storage by Dr Nimzing G. Ladep, JUTH, Nigeria. Random, non-fasted, urine samples were collected and centrifuged at 13,000 rpm for 10 min to remove any precipitates and stored at -80°C until analysis. Samples were thawed and prepared according to standard methodology (Beckonert *et al.*, 2007; Williams *et al.*, 2009). 400 µL of urine were mixed with 200 µL of phosphate buffer solution (0.2 M Na₂HPO₄/0.2 M NaH₂PO₄, pH 7.4), and 60 µL of 3-trimethylsilyl-(2,2,3,3-²H₄)-1-propionate (TSP) / D₂O solution (final concentration of TSP = 1mM) were added. The TSP served as an internal chemical shift reference (0.00 ppm) and the D₂O provided a nucleus field lock. The buffered urine sample was left to stand for 10 min and then centrifuged at 13,000 rpm for 10 min. 550µL of supernatant were transferred into a 5mm diameter glass NMR tube (Wilmad LabGlassTM, New Jersey, USA) for ¹H NMR spectroscopy.

Blood was venesected from each subject for parallel metabolite profiling studies (**Chapters 4, 5 and 9**) and for measurement of serum urea creatinine, ALT, ALP, bilirubin and albumin levels, measured using automated techniques (AbbottTM Architect Ci16200 Analyser, UK). Serum AFP was measured using an automated SiemensTM Immulite 2500 Analyser,

(Deerfield, USA). Median and interquartile ranges (IQR) were calculated for each assay and median levels were compared using unpaired Mann-Whitney tests of significance, p -values of <0.05 were considered significant.

6.3.3 Proton magnetic resonance spectral processing

Samples were run in a randomised order over one session. Proton NMR spectra were acquired using a pulse-collect sequence with water presaturation (JEOL 500 MHz Eclipse+ NMR spectrometer). The 16 data collects were summated. A 90° pulse angle was used with a total repetition time of 18.7 s, so that fully-relaxed data were acquired. The acquisition time was 8.7 s. MR spectra were processed using the KnowItAll™ Informatics System v7.8 (Bio-Rad, Philadelphia, USA). Free induction decays were zero-filled by a factor of two and multiplied by an exponential window function with a 0.3 Hz line-broadening factor, prior to Fourier transformation. All MR spectra were phased and a baseline correction applied. All spectra were referenced to TSP (0.00 ppm) and methyl creatinine peaks were aligned to 3.05 ppm. MR spectral resonances were assigned according to literature (Holmes *et al.*, 1997; Nicholson *et al.*, 1995; Wishart *et al.*, 2007). MR spectral analysis included the range 0.20 – 10.00 ppm, excluding the region 4.50 – 6.40 ppm, to remove the residual water signal and variation in urea signal due to exchange of protons with the solvent.

6.3.4 Multivariate statistical analysis

Magnetic resonance spectra were divided into smaller regions or “buckets” of 0.02 +/- 0.01 ppm, representing specific metabolites (Psihogios *et al.*, 2008; Walsh *et al.*, 2006; Williams *et al.*, 2009) using the “intelligent bucketing” algorithm in the KnowItAll Informatics System v7.8. These regions were then integrated, normalised to the sum of the total spectral integral and the data mean-centred prior to multivariate analysis. This created a data matrix of

samples against variables (ppm bins) which was transferred to KnowItAll Informatics System v7.8 for PCA and exported to Pirouette v4.0 (Infometrix, Washington, USA) for PLS-DA. The data filtering technique of orthogonal signal correction (OSC) was used to remove variation in the spectra not directly related to the physiological condition being studied and to minimise the possible influence of inter-individual variation (Psihogios *et al.*, 2008; Westerhuis *et al.*, 2008). The discriminatory power of each model was validated using two techniques. First, leave-one-out cross validation, whereby each sample in turn was excluded from the analysis, a model created from the remaining samples and the class membership of the excluded sample predicted (Mahadevan *et al.*, 2008). Second, full external validation in which models were created from training sets (70% of the samples, selected at random) and their predictive power tested using independent “test” sets (the held-back 30% of the samples). For both techniques, the R^2 and Q^2 values and misclassification matrix of the model described the number of correctly predicted samples, and from this the sensitivity and specificity of the model could be calculated.

6.3.5 Univariate statistical analysis

The metabolites which contributed most to PCA and PLS-DA discrimination were identified through analysis of the loadings plots. Using KnowItAll Informatics System v7.8, these areas were integrated and normalised to the sum of the total spectral integral. Values were expressed as percentage index, relative to the total spectral integral. Using GraphPad Prism v5.01 (California, USA), differences between the HCC, cirrhosis and healthy control groups were analysed using the Mann-Whitney tests of significance, p -values <0.05 were considered significant.

6.4 RESULTS

6.4.1 Subject demographics

Forty-two Nigerian subjects were recruited for study. These consisted of 18 patients with ultrasound or computed tomography diagnosed HCC; 10 patients with clinically diagnosed cirrhosis with features of portal hypertension; and 15 healthy Nigerian healthy volunteers. Subject demographics are shown in **Table 6-1**. Median age of the HCC group was 46.5 years and that of the healthy and cirrhosis groups 37 years. Mann-Whitney tests of median values did not find this to be a significant difference ($p=0.47$ and 0.19). Twelve of 18 (66.7 %) patients in the HCC group were male, 8 of 10 (80 %) in the cirrhosis group and 7 of 14 (50 %) in the healthy group. Fisher's exact (chi-squared) of categorical variables of HCC versus healthy and HCC versus cirrhosis groups revealed these proportions not to be significantly different ($p=0.30$ and 0.67). Seventeen of the patients (94%) in the HCC group and all of those with cirrhosis were HBsAg positive (ELISA 3rd generation). The only HBsAg negative patient in the HCC group was also HCV serum antibody negative and therefore diagnosed as having idiopathic liver disease. HCCs were classified according to the Okuda staging system (Okuda *et al.*, 1985) (**Table 6-1**). All HCCs were reasonably advanced at stage II ($n=8$, though in 2 of these 8 subjects, stage was based only on the tumour size ($>50\%$ of liver size) and detectable ascites, as there was insufficient serum collected to measure albumin and bilirubin levels) or stage III ($n=10$). Healthy volunteers were of Nigerian descent, from the medical faculty of JUTH, and were HBsAg negative with normal hepatic function.

On direct questioning, none of the subjects admitted to taking prescribed or non-prescribed medication, nutrient or vitamin supplements, but analysis of subsequent MR spectra revealed the presence of paracetamol (acetaminophen) metabolites acetaminophen glucuronide and acetaminophen sulphate (Clayton *et al.*, 2009) (**Figure 6-1**), in a sample from a healthy 18

year old female healthy volunteer. These metabolites were not present in any other spectra. The first PCA score plot for HCC and healthy controls identified this sample as a clear outlier. As this spectrum unfairly biased the principal components it was excluded from further analysis. Paracetamol metabolites produce marked spectral resonances which heavily influence multivariate analysis. This subject was therefore excluded from all further data analyses, reducing the healthy volunteer group to 14 in total.

Table 6-1. Nigerian subject demographics.

Characteristic	HCC	Cirrhosis	Healthy	<i>p</i> -value
<i>N</i>	18	10	14	-
Median age (range) (years)	46.5 (25–85)	37 (23–62)	37 (27–80)	^a 0.47, ^b 0.19
Male <i>n</i> (%)	12/18 (66.7%)	8/10 (80%)	7/14 (50%)	^{+a} 0.30, ^{+a} 0.67
Ethnicity	Nigerian	Nigerian	Nigerian	-
HBsAg +ve	17/18 (94.4%)	10/10 (100%)	0/14	⁺ <0.0001
Okuda stage (<i>n</i>)	I=0, II=8, III=10	-	-	-

p-values: ^a and ^b denote Mann-Whitney tests of HCC versus healthy and HCC versus cirrhosis, respectively. ⁺ denotes Fisher's exact test of categorical variables.

*2 of 8 classified as Stage II on the basis of >50% tumour size and detectable ascites, though insufficient serum to obtain albumin and bilirubin levels.

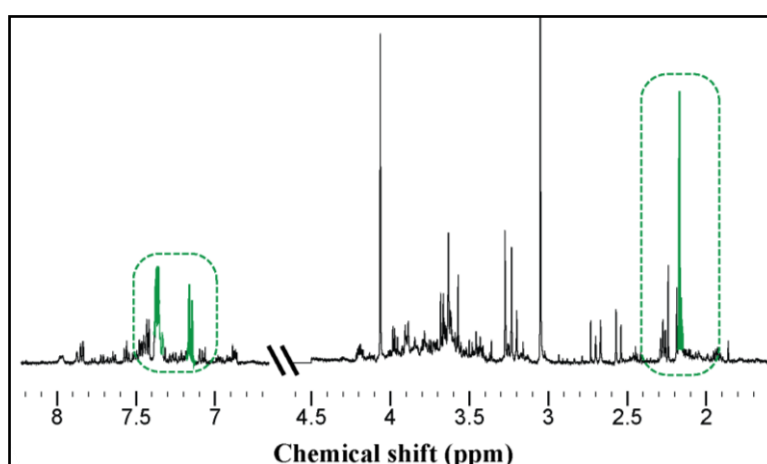


Figure 6-1. Urine magnetic resonance spectra displaying paracetamol metabolites.

6.4.2 Serum biochemical analyses

Serum AFP was significantly raised in the HCC group (median 12,474 IUL⁻¹), compared to healthy controls (median 1.66 IUL⁻¹, $p<0.001$) and patients with cirrhosis (median 5.97 IUL⁻¹, $p<0.001$). Using a serum AFP level cut-off value of 20 IUL⁻¹, 2/10 (20%) patients with cirrhosis would have produced false positive results and 2/18 (11%) patients with HCC would have been classified as false negatives. For this group of subjects, AFP, with a cut-off value of 20 IUL⁻¹ had a sensitivity: specificity of 100%: 83.3% for HCC versus healthy controls and 88.9%: 77.8% for HCC versus patients with cirrhosis. Serum urea and creatinine levels were not significantly different between groups ($p=0.27$ - 0.93). Serum ALT levels were significantly raised in the HCC group compared to healthy controls (48 IUL⁻¹ and 23 IUL⁻¹, $p=0.006$), but not compared to patients with cirrhosis and patients with cirrhosis (37 IUL⁻¹, $p=0.59$). Bilirubin and albumin levels were also significantly altered compared to healthy volunteers but not compared to patients with cirrhosis (**Table 6-2**).

Table 6-2. Serum biochemical analyses.

	HCC Median (range)	Cirrhosis Median (range)	Healthy Median(range)	<i>p</i> -values
<i>N</i>	18*	9**	9***	-
AFP IUmL ⁻¹	12474 (944-30000)	6.0 (3.3-24.9)	1.7 (1.4-3.4)	^{a,b} ≤0.001
Urea mmolL ⁻¹	4.8 (4.1-6.5)	4.3 (3.6-6)	4.9 (3.6-7.89)	^a 0.27, ^b 0.41
Creatinine mmolL ⁻¹	75.5 (44-110.3)	76 (56.5-102)	76.0 (61-101.5)	^a 0.62, ^b 0.93
ALT IU L ⁻¹	48 (30-107)	37 (25.5-69.5)	23 (17.5-26.0)	^a 0.006, ^b 0.59
ALP IU L ⁻¹	11.5 (6.6-17.7)	11.6 (9.3-270.6)	8.0 (5.85-69.6)	^a 0.77, ^b 0.42
Bilirubin μmolL ⁻¹	29.9 (22.6-87.5)	36 (25.9-52.2)	4.7 (3.3-30.3)	^a <0.001, ^b 0.98
Albumin gL ⁻¹	29.6 (20.6-40.5)	29.4 (26.5-37.4)	49.3 (46.3-52.4)	^a 0.002, ^b 0.98

Key: *HCC group- 2 samples insufficient volume for ALT, ALP, bilirubin and albumin assays, **Cirrhosis group- 1 serum sample missing, ***Healthy control group- 5 serum samples missing. *p*-values: ^a and ^b denote Mann-Whitney tests of HCC versus healthy and HCC versus cirrhosis, respectively.

6.4.3 Proton nuclear magnetic resonance spectroscopy

Representative urine ^1H NMR spectra are displayed in **Figure 6-2** and **Figure 6-3**.

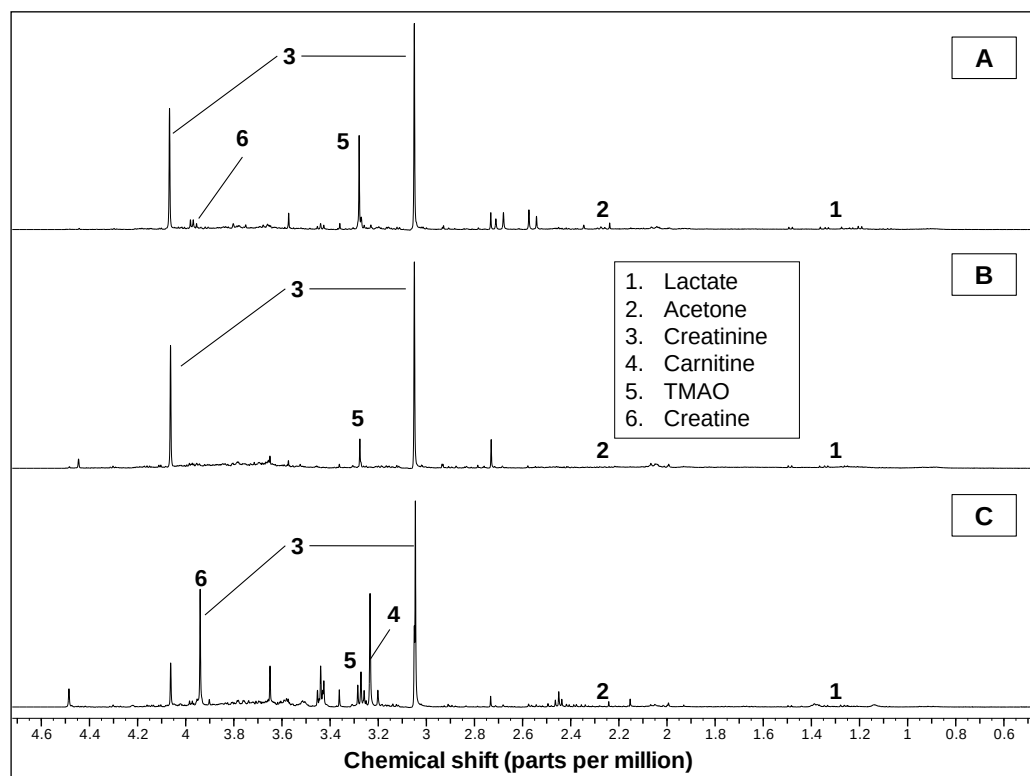


Figure 6-2. Representative magnetic resonance urine spectra.

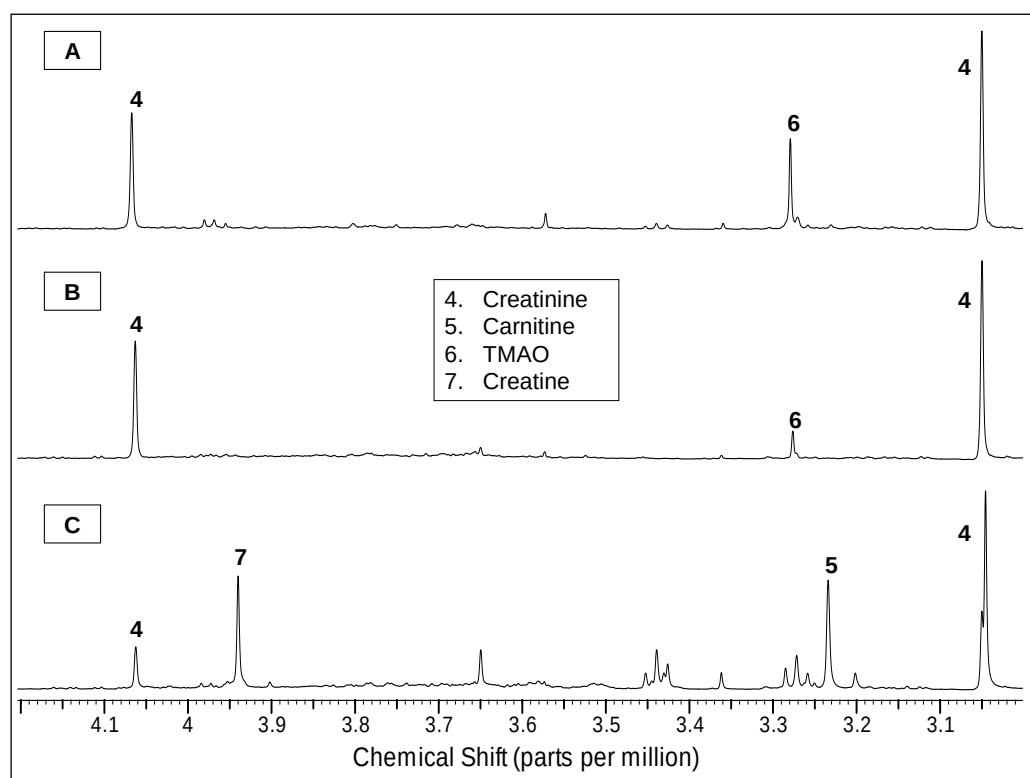


Figure 6-3. Magnetic resonance urine spectra (expanded region 3.0–4.2 ppm).
Key: A. Healthy volunteer (A), patient with cirrhosis (B) and with HCC (C).

6.4.4 Multivariate data analysis

Principal components analysis was performed following the exclusion of the outlying sample containing paracetamol metabolites, as described above. The subsequent three component PCA scores plot is displayed in **Figure 6-4** and highlights clustering of healthy controls, clearly separated from patients with HCC. Also displayed is the three component PCA scatter plot of HCC and cirrhosis samples, again displaying clustering of cirrhosis samples separated from HCC samples (**Figure 6-4**).

Partial least squares discriminant analysis models were constructed to investigate the ability of this technique to distinguish between HCC, healthy controls and patients with cirrhosis. **Table 6-3** displays the values of validity (R^2 and Q^2) and sensitivities and specificities of PLS-DA in discriminating subject cohorts using a leave-one-out paradigm (Man *et al.*, 2004). Hepatocellular carcinoma and healthy control subjects were discriminated with a sensitivity of 100% and specificity of 93% (leave-one-out cross-validation). Full external validation separated the groups with 100% sensitivity and 87.5% specificity. HCC and cirrhosis cohorts were distinguished with sensitivity: specificity of 89.5%: 88.9% and 62.5%: 100% using leave-one-out and full external validation paradigms, respectively (**Table 6-3**).

Resonances that contributed most strongly to both models of HCC and healthy volunteer and HCC and cirrhosis samples, were identified as creatinine (methyl and methylene singlets at 3.05 and 4.07 ppm) carnitine (methyl singlet at 3.23 ppm), creatine (methylene singlet at 3.05 ppm) and acetone (methyl singlet at 2.24 ppm) (Holmes *et al.*, 1997; Nicholson *et al.*, 1995; Slupsky *et al.*, 2007; Wishart *et al.*, 2007).

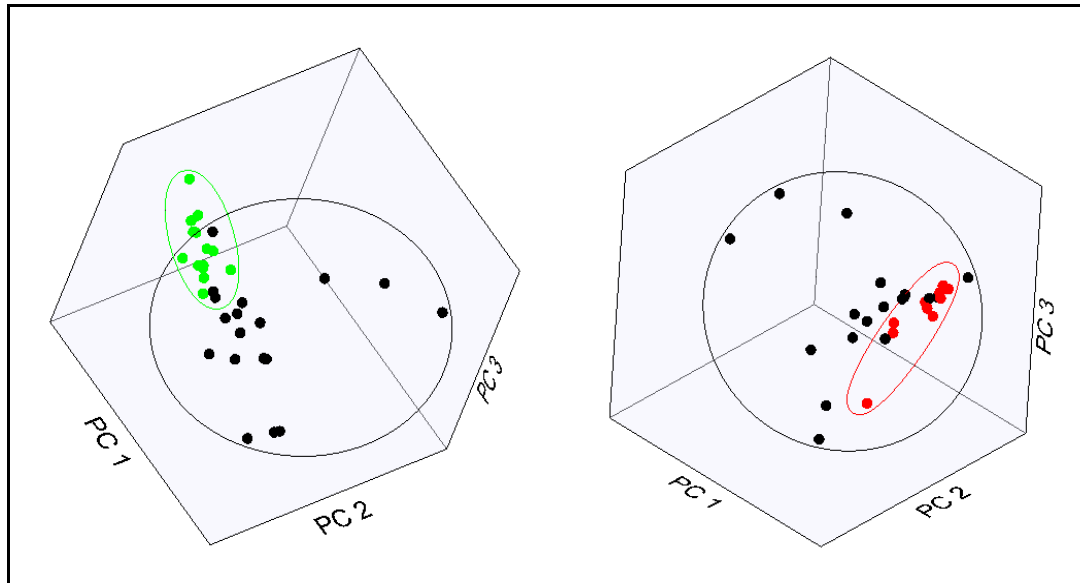


Figure 6-4. Principal components analysis scores plots.

*Key: HCC (●) versus healthy volunteer (●) and versus cirrhosis samples (●).
PC = principal component.*

Table 6-3. Validity values of partial-least squares discriminant analyses and diagnostic sensitivities and specificities.

	R^2	Q^2	Sensitivity	Specificity
HCC vs. Healthy	0.88	0.74	100 %	93 %
HCC vs. Cirrhosis	0.85	0.55	89.5 %	88.9 %

6.4.5 Univariate statistical analysis

Urinary creatinine levels (expressed as % normalized total spectral integral) were significantly reduced in the HCC cohort compared to the healthy controls ($p < 0.001$) and reduced, approaching significance compared to the cirrhosis samples ($p = 0.06$); median levels [IQR] for HCC = 6.12 [4.11 – 7.41]; for healthy = 9.81 [7.95 – 10.94] and for cirrhosis = 7.53 [6.28 – 9.62] (**Figure 6-5**). Urinary carnitine levels were significantly elevated in the HCC compared to the cirrhosis cohort ($p < 0.001$) and elevated, approaching significance compared to healthy volunteers ($p = 0.05$) median levels [IQR] for HCC = 0.86 [0.34 – 1.62]; for healthy = 0.31 [0.1 – 1.01] and for cirrhosis = 0.16 [0.07 – 0.24] (**Figure 6-5**). Urinary creatine levels were significantly raised in the HCC cohort compared to the healthy control cohort: 0.90 [0.68 – 1.39] and 0.57 [0.47 – 0.97] ($p = 0.04$); but not compared to the cirrhosis cohort: 0.72 [0.62 – 0.95] ($p = 0.29$) (**Figure 6-5**). Urinary acetone levels were not significantly different between groups: median levels for HCC = 0.35 [0.26 – 0.78]; for healthy = 0.64 [0.31–1.85] and for cirrhosis = 0.39 [0.14–1.56] ($p = 0.28$ and 0.70) (**Figure 6-5**). These results illustrate that it is the combination of metabolites, the values of which may be statistically different between groups, rather than a single metabolite that contributes to the multivariate discriminatory model.

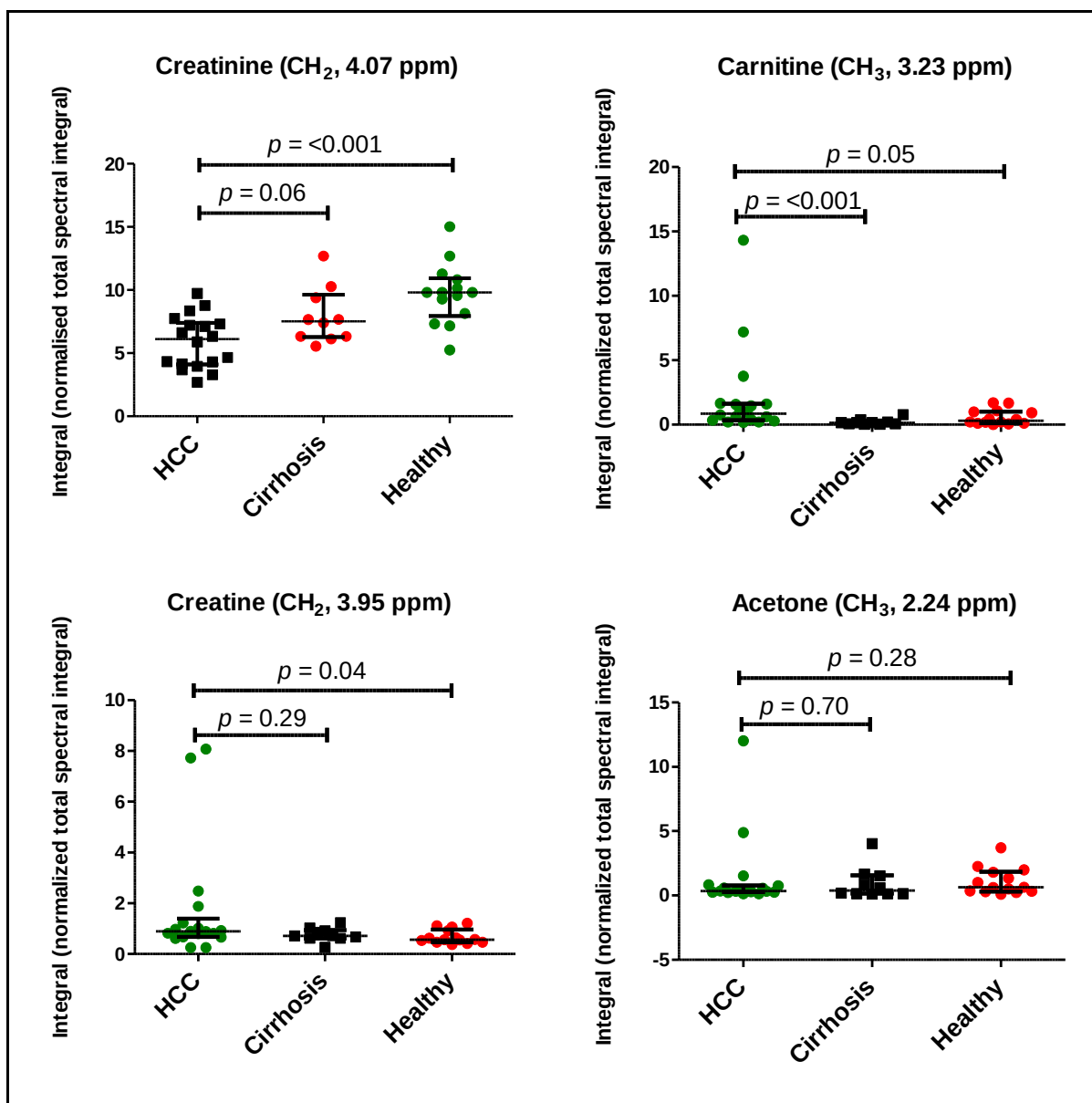


Figure 6-5. Univariate analysis of discriminatory metabolites creatinine, carnitine, creatine and acetone.

6.5 DISCUSSION

This is the first study to use ^1H NMR spectral metabolite profiling of urine to characterise patients with HCC in an African population (Shariff *et al.*, 2010). In this study, a urinary biomarker panel composed of creatinine, carnitine, creatine and acetone successfully discriminated patients with HCC from healthy controls and patients with cirrhosis. Using PLS-DA “leave-one-out” cross-validation algorithms, the sensitivity and specificity of correct group classification of HCC from healthy controls and from cirrhosis were 100%: 93.3% and 88.9%: 80%, respectively. This compares favourably to serum AFP, which for this group of subjects, using a cut-off value of 20 IUL^{-1} , displayed a sensitivity: specificity of 100%: 83.3% for HCC versus healthy controls and 88.9%: 77.8% for HCC versus patients with cirrhosis.

Urine creatinine levels were significantly reduced in patients with HCC compared healthy controls. Creatinine is a breakdown product of muscle creatine phosphate and is eliminated in urine. Renal impairment will cause a rise in serum creatinine and a concomitant rise in urine creatinine. Serum creatinine levels were similar between subject groups. A high muscle bulk and turnover may also lead to high serum and urine creatinine levels suggesting no renal impairment was present. The muscle mass of the Nigerian subjects was not recorded as owing to lack of resources, formal anthropometric assessments were not possible. It is likely that the “dry weight” of patients was reduced, as most subjects with HCC were visibly cachectic to some degree (personal communication from Dr Nimzing Ladep, JUTH). It is therefore reasonable to attribute lower urinary creatinine levels to reduced muscle mass. A previous study of urinary creatinine in the US population observed that lower muscle mass was a major contributing factor to lower urinary creatinine levels (Barr *et al.*, 2005).

Urinary carnitine levels were higher in patients with HCC compared to other groups. Carnitine is a hydrophilic molecule that plays an essential role in mitochondrial metabolic pathways. It is either absorbed from the diet or synthesised in the liver, kidney and testis and renally reabsorbed. Meat, poultry and dairy products are the main dietary sources of carnitine. Unabsorbed carnitine is mostly degraded by micro-organisms in the large intestine. In health, only a small fraction (usually <5%) of filtered carnitine is excreted (Rebouche, 2004). As healthy kidneys efficiently reabsorb carnitine, a high urinary level may correspond to excess carnitine ingestion, biosynthesis or poor reabsorption. The subjects in this study were not administered nutritional supplements, nor was their diet likely to be rich in meat products, which are sparse in this area of Nigeria. Carnitine is indispensable for energy metabolism, enabling fatty acids to enter the mitochondria for β -oxidation. Furthermore, carnitine is involved in the transfer of the products of peroxisomal β -oxidation, including acetyl-CoA, to the mitochondria for oxidation to carbon dioxide and water (Vaz and Wanders, 2002). With elevated metabolic activity and high cell-turnover, it may be hypothesised that elevated urine carnitine levels reflect tumour overproduction of carnitine to fuel mitochondrial activity and maintain rapid growth.

Urinary creatine levels were raised in subjects with HCC, significantly compared to healthy volunteers but not compared to patients with cirrhosis. Creatine is a nitrogenous organic acid, synthesised mainly in the liver from its component parts arginine, glycine and methionine and stored in muscle as phosphocreatine. It has a direct function in cellular energy transport, interacting directly with adenosine triphosphate (ATP) to produce phosphocreatine and adenosine diphosphate (ADP). It is therefore likely that creatine, an essential component of the energy transfer process, is elevated in rapidly growing cells, such as in HCC. A study by Pang and colleagues observed elevated levels of ubiquitous creatine kinase, the catalyst of the

phosphate transfer between phosphocreatine and ATP, in prostate cancer cell lines suggesting heightened metabolic states (Pang *et al.*, 2009).

There were a number of limitations to the study, but given the resources in Jos, the study was carried out with care. The number of subjects included was low and therefore multivariate analyses results, in particular PLS-DA, should be interpreted with caution as prediction can be over-optimistic when sample sizes are low (Brereton, 2006). Studies in other populations, where other disease aetiologies other than HBV predominate and with larger samples sizes are required to validate these findings. It may be that other risk factors for HCC, such as HCV, alcohol-induced cirrhosis and aflatoxin will display different patterns of metabolic derangement. Urine metabolite characteristics are also dependent on interacting factors including diet and nutrition (Holmes *et al.*, 2008), and it would therefore be crucial to attain accurate dietary histories and anthropometric measurements to exclude this as a possible confounding factor. The medical provenance of the samples is also vitally important in metabolic profiling studies. The diagnosis of cirrhosis was made on clinical and radiological grounds on the basis of the presence of signs of portal hypertension, such as ascites, gastro-oesophageal varices and portal hypertensive gastropathy. These findings correlate well with underlying cirrhosis, although the distinction would ideally have been made with histological confirmation, as patients may occasionally display signs of portal hypertension, for example with portal vein thrombosis, without the presence of cirrhosis (although this is unlikely in the context of chronic viral hepatitis).

6.6 CONCLUSIONS

This study has provided insight into the urinary changes in metabolites that may reflect the underlying effect HCC has on host physiology. It is the first to utilise urinary ^1H NMR spectroscopy with multivariate statistical analysis in a cohort of patients with HCC (Shariff *et al.*, 2010). Multivariate models were able to differentiate patients with HCC from normal subjects and patients with cirrhosis with high accuracy, comparable to that of AFP, permitting the notion that a urinary test for HCC is possible. Larger, more diverse studies are required for validation of these findings.

7 THE URINARY METABOLIC PROFILING OF HEPATOCELLULAR CARCINOMA IN AN EGYPTIAN POPULATION USING PROTON MAGNETIC RESONANCE SPECTROSCOPY

7.1 ABSTRACT

Introduction: The previous chapter identified a set of discriminatory urinary metabolites comprising creatinine, creatine, carnitine and acetone, in Nigerian, HBV-infected, patients with HCC. The study presented here, undertaken in an Egyptian, mainly HCV-infected patient group, served to validate or refute these findings. **Methods:** Urine samples were collected from Egyptian subjects with HCC (16), cirrhosis (14) and healthy volunteers (17) and metabolite profiles were generated using a ^1H NMR spectroscopy. Data were analysed using multivariate and univariate statistical analysis. **Results:** Using multivariate models, the sensitivity and specificity of the technique for distinguishing patients with tumours from healthy controls and patients with cirrhosis was 100%: 94% and 81%: 71%, respectively. Discriminatory metabolites between HCC and healthy and HCC and cirrhosis groups included creatinine ($p=0.003$ and <0.001), carnitine ($p=0.29$ and 0.30), glycine ($p=<0.001$ and 0.88), trimethylamine-*N*-oxide ($p=<0.001$ and 0.18), hippurate ($p=<0.001$ and 0.66), citrate ($p=<0.001$ and 0.12) and creatine ($p=0.12$ and 0.33). **Conclusions:** This metabolic profile bears some similarity to profiles identified in the Nigerian cohort, in the form of altered urine creatine, carnitine and creatinine levels. These metabolites may be indicative of tumour effects on physiology, energy production and aberrant chromosomal methylation.

7.2 INTRODUCTION

In the previous chapter the urinary metabolic profile of HCC was characterised in a small set of Nigerian HBV infected patients with HCC and cirrhosis and a control set healthy volunteers. Multivariate analysis was able to discriminate the ^1H NMR urine profiles of HCC from healthy and cirrhosis with high sensitivity and specificity (Shariff *et al.*, 2010). The metabolites creatinine, creatine, carnitine and acetone were found to contribute most to multivariate models and may signify an alteration in muscle mass status, energy consumption, fatty acid oxidation and respiration in patients with tumours. The study presented here, in an Egyptian, mostly HCV infected patient population, was performed to validate these findings (Shariff *et al.*, 2011).

Egypt has the highest prevalence of HCV worldwide, with population estimates ranging between 6% - 28%, largely owing to the use of unsterilised needles in a widespread schistosomiasis treatment programme in the 1950s-1970s using intravenous tartar emetic (Williams, 2006). HCV has been estimated to be the main aetiological factor of HCC in 40% - 50% of cases (Lehman and Wilson, 2009).

All previous reported studies of the metabolic profiles of HCC have been undertaken in single indigenous populations where there is usually a single aetiological pathogen of liver disease and HCC (Chen *et al.*, 2009; Chen *et al.*, 2011; Wu *et al.*, 2009). As HCC occurs on the background of multiple aetiologies, these results may not hold true for other populations or aetiologies.

The aims of this study were to 1. Identify differences in metabolic profiles between HCC, cirrhosis and healthy volunteer samples using urine ^1H NMR spectroscopy combined with

multivariate analysis, and 2. Evaluate the robustness of these models in differentiating disease groups.

7.3 METHODS

7.3.1 Subject selection

Urine and serum samples were collected from patients attending the National liver Institute, Menoufiya University, Shbeen El Kom, Egypt. Ethical approval was granted by the research ethics committees at the National Liver Institute, Menoufiya University and Imperial College London.

Subjects were recruited between 2005 and 2006 with HCC (diagnosed by two imaging techniques showing early arterial enhancement and rapid washout in portal phase or one characteristic imaging modality with serum AFP $>400 \text{ ngmL}^{-1}$); or with clinically or histologically confirmed cirrhosis, as disease controls. Healthy volunteers, with no liver disease, were recruited as healthy controls. Staging of HCC was according to the Okuda system (Okuda *et al.*, 1985), as previously described (**Chapter 4**). Principal components analysis was used to identify outlying samples for exclusion as detailed in the results section.

7.3.2 Urine sample collection

Random, non-fasted, 5 mL urine samples were collected into plain containers and stored at -80°C , 2 to 4 hrs after collection in Egypt until transportation by air on dry ice. Samples were thawed prepared according to standard methodology described in **Chapter 6**.

7.3.3 Serum biochemical tests

Serum AFP, creatinine, ALT, AST, bilirubin and albumin levels were measured at the time of urine sample collection in Egypt using a Cobas Integra 400- Autoanalyzer, (Roche, Rotkreuz, Switzerland). These data are also presented in **Chapter 4**.

7.3.4 Magnetic resonance spectral acquisition and processing

Samples were run in a random, non-grouped order. Proton NMR spectra were acquired using a pulse-collect sequence with water presaturation (JEOL 500 MHz Eclipse+ NMR spectrometer). The 16 data collects were summated. A 90° pulse angle was used with a total repetition time of 18.7 s, so that fully-relaxed data were acquired. The acquisition time was 8.7s. Data were collected into 64K points. Proton NMR spectra were processed using the KnowItAll™ Informatics System v7.8 (Bio-Rad, Philadelphia, USA). Free induction decays were zero-filled by a factor of two and multiplied by an exponential window function with a 0.3 Hz line-broadening factor, prior to Fourier transformation. All NMR spectra were phased, a baseline correction applied and referenced to TSP (0.00 ppm) and the methyl-creatinine peak was aligned to 3.05 ppm. Spectral resonances were assigned according to literature (Holmes *et al.*, 1997; Lindon *et al.*, 1999; Wishart *et al.*, 2007). The region 4.50 – 6.40 ppm was excluded to remove the residual water signal and variation in urea signal due to exchange of protons within the solvent.

7.3.5 Multivariate statistical analysis

Principal components analysis and PLS-DA were used for initial analysis. Orthogonal signal correction (**Chapter 6**) was used to remove variation in the spectra not directly related to the physiological condition being studied. The discriminatory power of each model was validated using leave one out and full external validation models as previously described (**Chapter 6**). Samples lying outside the Hotelling's distance were excluded as these unfairly influence the principal components and are not representative of the group.

7.3.6 Univariate statistical analysis

The most important discriminatory metabolite resonances, as determined by PLS-DA loadings plots were integrated and normalised to the sum of the total spectral integral. Using GraphPad Prism v5.01 (California, USA), median value differences between groups were analysed using the Mann-Whitney test for non-parametric data and p -values <0.05 were considered significant.

7.4 RESULTS

7.4.1 Subject recruitment, exclusion and demographics

Fifty-eight subjects were recruited for study between 2005 and 2006. This included 18 patients with HCC; 20 patients with cirrhosis; and 20 healthy Egyptian volunteers. Eleven samples were identified as outliers by PCA and excluded from further analysis. Outlying samples included two from the HCC cohort, one of which displayed dominant glucose resonances (probably indicating underlying undiagnosed diabetes mellitus) and the other which could not be phased adequately (**Figure 7-1**). Six spectra were excluded from the cirrhosis cohort, three of which displayed marked glucose metabolites, one which could not be phased adequately, one with dominant lactate peaks and one which displayed pronounced unidentified peaks at 1.45 ppm and 1.60 ppm which strongly affected multivariate analyses. Three spectra were excluded from the healthy control group all of which displayed dominant glucose metabolites. These samples were excluded from further analysis as detailed below. This left 16 samples in the HCC group, 14 in the cirrhosis group and 17 in the healthy control group.

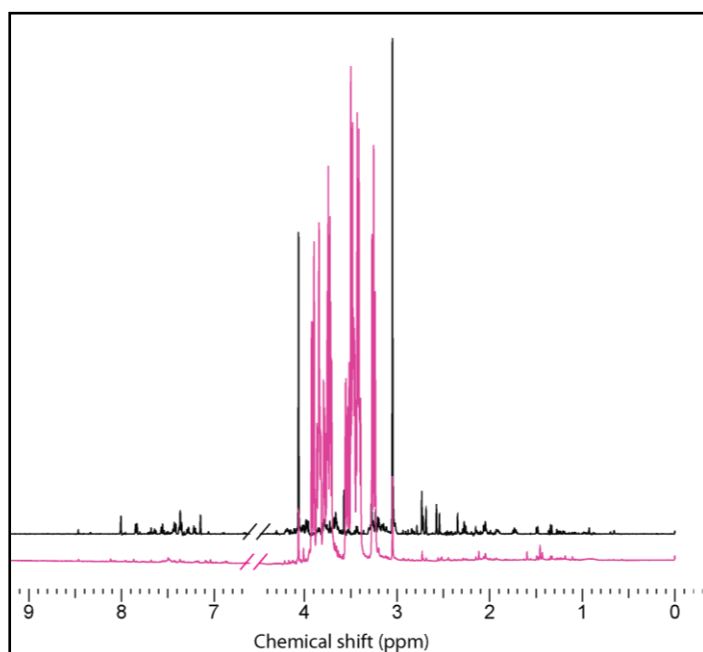


Figure 7-1. Urinary spectra of a sample containing glucose (pink) compared to normal (black).

Subject demographics are displayed in **Table 7-1**. The median age of the HCC group (51.5 years) was significantly older than that of the healthy controls (41 years) ($p=0.01$) but not when compared to that of the cirrhosis group (54 years) ($p=0.37$). There were significantly more males in the HCC group (15/16 = 94%) than the healthy control group (9/17 = 53%) ($p=0.02$), but not when compared to the cirrhosis group (11/14 = 79%) ($p=0.32$). One of the HCC subjects had stage I, 10 had stage II and 5 had stage III disease (**Table 7-1**). The majority of patients with HCC and 50% of patients with cirrhosis were HCV antibody positive: 11/16 (69%) and 7/14 (50%), respectively. The remaining patients with HCC or cirrhosis had diverse aetiologies (**Table 7-2**). All healthy controls were of Egyptian origin.

Table 7-1. Egyptian subject demographics.

Characteristic	HCC	Cirrhosis	Healthy	<i>p</i> -value
<i>n</i>	16	14	17	-
Median age (yrs)	51.5	54.0	41.0	^a 0.01, ^b 0.37
Male <i>n</i> (%)	15/16 (94%)	11/14 (79%)	9/17 (53%)	^{a+} 0.02, ^{b+} 0.32
Ethnicity	Egyptian	Egyptian	Egyptian	-
HCV Ab+	11/16 (69%)	7/14 (50%)	0	-
Okuda stg. (<i>n</i>)	I (1), II (10), III (5)	-	-	-

Key: *p*-values: ^a and ^b denote Mann-Whitney tests of HCC versus healthy and HCC versus cirrhosis, respectively. ⁺ denotes Fisher's exact test of categorical variables.

Table 7-2. Aetiology of liver disease in Egyptian patient cohorts.

Aetiology	HCC	Cirrhosis
HCV	11	7
HBV	0	1
Idiopathic cirrhosis with schistosomiasis	0	4
Budd Chiari syndrome	0	2
Idiopathic	5	0
Total	16	14

7.4.2 Serum biochemical analysis

Median values for serum AFP, creatinine, ALT, AST, bilirubin and albumin of study subjects are displayed in **Table 7-3**. Serum AFP levels were only measured in the cirrhosis and HCC groups. median serum AFP levels were significantly higher in HCC patients (745 IU mL⁻¹ versus 77 IU mL⁻¹, $p<0.001$). The lowest serum AFP in the cirrhosis group was 20 IU mL⁻¹ and using a cut-off of 20 IU mL⁻¹, all cirrhosis patients would have been falsely classified as HCC, based on serum AFP levels (sensitivity 100%, specificity 0%). If a cut-off value of 400 IU mL⁻¹ was used, specificity improved to 100% and sensitivity fell to 88%. The HCC diagnostic performance of AFP, using a cut-off value of >200 IU mL⁻¹, was therefore good in these patients. Median serum creatinine was significantly higher in the HCC group, compared to controls (106 mmol L⁻¹ versus 62 mmol L⁻¹, $p<0.001$), but not when compared to that of the cirrhosis subjects (106 mmol L⁻¹ versus 150 mmol L⁻¹, $p=0.16$). Serum ALT, AST and bilirubin levels were all significantly higher in the HCC group compared to healthy controls, but not compared to cirrhosis subjects. Serum albumin was significantly lower in the HCC group compared to healthy controls but not compared to cirrhosis subjects.

Table 7-3. Serum biochemistry profiles of subject cohorts

	HCC	Cirrhosis	Healthy	p-values
	Median (range)	Median (range)	Median (range)	
Serum Samples (n)	16	14	17	-
AFP (IU mL⁻¹)	745 (174-5000)	77 (20-174)	-	^b <0.001
Creatinine (mmol L⁻¹)	106 (44-318)	150* (97-229)	62 (44-80)	^a <0.001 and ^b 0.16
ALT (IU L⁻¹)	56 (11-164)	30 (16-119)	12 (9-18)	^a <0.001 and ^b 0.36
AST (IU L⁻¹)	79 (22-224)	51 (31-190)	15 (11-20)	^a <0.001 and ^b 0.44
Bilirubin (μmol L⁻¹)	63 (17-547)	48 (14-156)	12 (0.7-19)	^a <0.001 and ^b 0.19
Albumin (g L⁻¹)	30 (19-34)	29 (23-35)	40 (38-44)	^a <0.001 and ^b 0.79

*Key: *Two samples did not have serum creatinine levels measured. p-values: ^a and ^b denote Mann-Whitney tests of HCC versus healthy and HCC versus cirrhosis, respectively.*

7.4.3 Proton nuclear magnetic resonance spectroscopy

Representative urine ^1H NMR spectra from a healthy volunteer and patients with HCC and cirrhosis are displayed in **Figure 7-2** and resonances tabulated in **Table 7-4**.

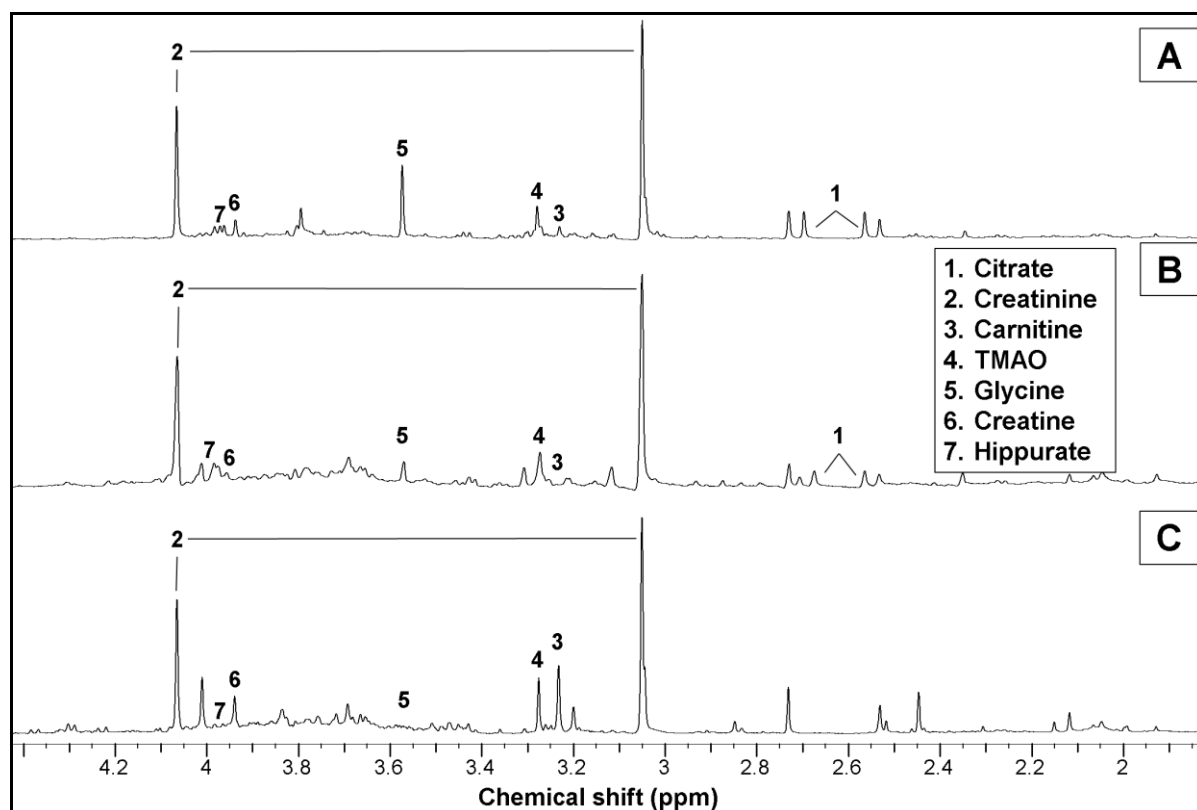


Figure 7-2. Representative Egyptian subject urine spectra

Urine MR spectra from a healthy volunteer (A), a subject with cirrhosis (B) and a subject with HCC(C). Key: TMAO, trimethylamine-N-oxide.

Table 7-4. Urine magnetic resonance metabolite resonances.

	Region (ppm)	Molecule (multiplicity)	Moeity
1	2.57	Citrate (double doublet)	CH_2
2	3.05, 4.07	Creatinine (singlets)	CH_3 , CH_2
3	3.23	Carnitine (singlet)	CH_3
4	3.27	TMAO (singlet)	CH_3
5	3.57	Glycine (singlet)	CH_2
6	3.93	Creatine (singlet)	CH_2
7	3.95	Hippurate (doublet)	CH_2

7.4.4 Multivariate statistical analysis

Principal components analysis scores plots of the resulting groups displayed clustering of HCC, healthy volunteer and cirrhosis samples (**Figure 7-3**). Analyses using OSC-PLS-DA distinguished HCC and healthy control cohorts with 100% sensitivity and 94% specificity using a leave one out algorithm ($R^2=0.8$, $Q^2=0.74$) (**Table 7-5**). Using a full external validation paradigm, discriminatory sensitivity was 100% and specificity 93%. Resonances contributing most to the PLS-DA components were glycine (3.57 ppm), trimethylamine-*N*-oxide (TMAO) (3.27 ppm), hippurate (δ 3.96 ppm), citrate (2.66 ppm), creatinine (3.05 ppm), creatine (3.93 ppm) and carnitine (3.23 ppm). Analyses using OSC-PLS-DA distinguished HCC and cirrhosis cohorts with 81% sensitivity and 71% specificity using a leave one out algorithm ($R^2=0.54$, $Q^2=0.25$) (**Table 7-5**). Using a full external validation paradigm, discriminatory sensitivity was 75% and specificity 67%. Resonances contributing most strongly to PLS-DA discrimination were TMAO (3.27 ppm), creatine (3.93 ppm) and carnitine (3.23 ppm).

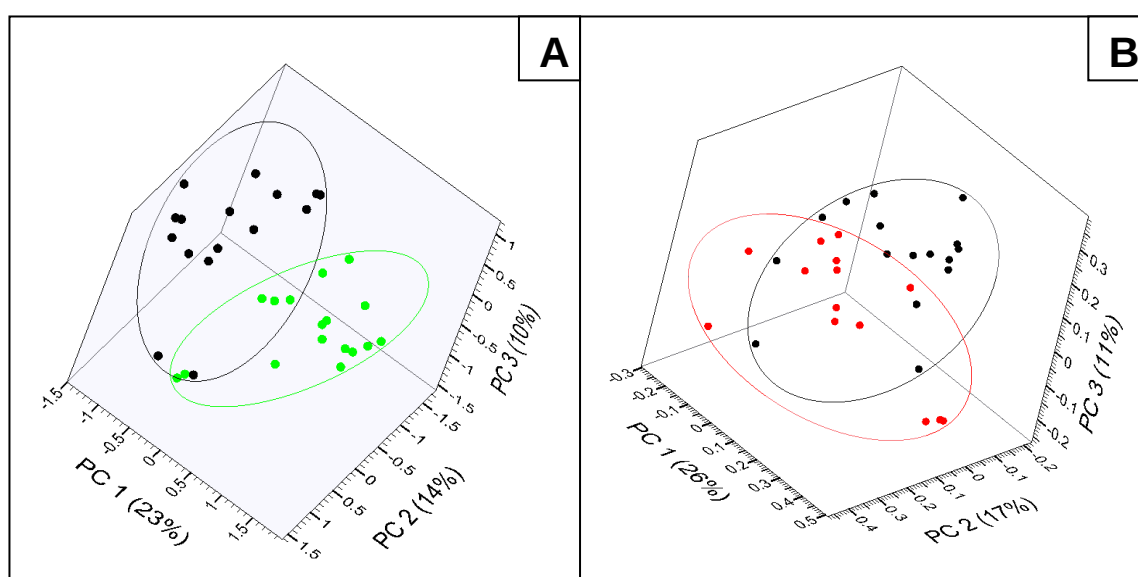


Figure 7-3. Principal components analysis scores plots Egyptian subject groups.
A. HCC subjects (●) versus healthy controls (●) and B. HCC (●) versus cirrhosis subjects (●).

Table 7-5. Validity values of partial-least squares discriminant analyses.

	R²	Q²	Sensitivity	Specificity
HCC vs. Healthy	0.8	0.74	100%	94%
HCC vs. Cirrhosis	0.54	0.25	81%	71%

Calculated using “leave one out” external validation models.

7.4.5 Male only comparisons

Male only analyses were undertaken to confirm that findings were due to the effects of HCC rather than gender variation between groups. Male HCC urine could be discriminated with 100% sensitivity and 93% specificity from healthy control urine and with 80% sensitivity and 55% specificity from urine of patients with cirrhosis. Discriminatory metabolites were similar to combined gender comparisons: glycine, TMAO, hippurate, citrate, creatinine, creatine, and carnitine.

7.4.6 Univariate statistical analysis

Univariate analyses are displayed in **Figure 7-4**. Urinary creatine levels were significantly elevated in HCC subject urine compared to healthy controls and cirrhosis subjects: 1.5 [0.92 – 3.32]; 0.54 [0.04 – 1.28] and 0.26 [0.12 -0.39] ($p=0.003$ and $p<0.001$, respectively). Urinary carnitine levels were non-significantly elevated in HCC subjects, compared to healthy controls and cirrhosis subjects: 1.16 [0.25 – 2.59]; 0.58 [0.36 – 0.86] and 0.39 [0.29 – 0.97] ($p=0.29$ and $p=0.30$). Urinary glycine levels (expressed as % normalized to total spectral integral) were significantly reduced in HCC subjects urine, compared to healthy controls: median [interquartile range] 0.46 [0.24 - 0.71] and 2.41 [1.30 - 2.95] ($p<0.001$), but were not significantly reduced compared to cirrhosis subjects: 0.53 [0.13 – 1.27] ($p=0.88$). Urinary TMAO levels were significantly reduced in HCC subject urine, compared to healthy controls: 1.17 [0.47 – 1.56] and 3.98 [2.69 – 4.67] ($p<0.001$), but non-significantly reduced

compared to cirrhosis subjects: 2.03 [0.47 – 3.07] ($p=0.18$). Urinary hippurate levels were significantly reduced in HCC subjects, compared to healthy subjects: 0.53 [0.23 – 0.98] and 1.62 [1.08 – 1.88] ($p<0.001$), but not significantly different compared to cirrhosis subjects: 0.66 [0.0 – 0.89] ($p=0.65$). Urinary citrate was significantly reduced, compared to healthy subjects: 0.04 [0.0 – 0.84] and 1.68 [1.06 – 2.74] ($p<0.001$), with a reduced trend towards significance compared to cirrhosis subjects: 0.91 [0.09 – 1.36] ($p=0.12$). Urinary creatinine levels were reduced in HCC urine, compared to healthy control and cirrhosis subjects, but not to a level of significance: 13.7 [11.59 – 18.69], 17.49 [13.82 – 24.11] and 17.71 [11.78 – 22.28] ($p=0.12$ and 0.33, respectively).

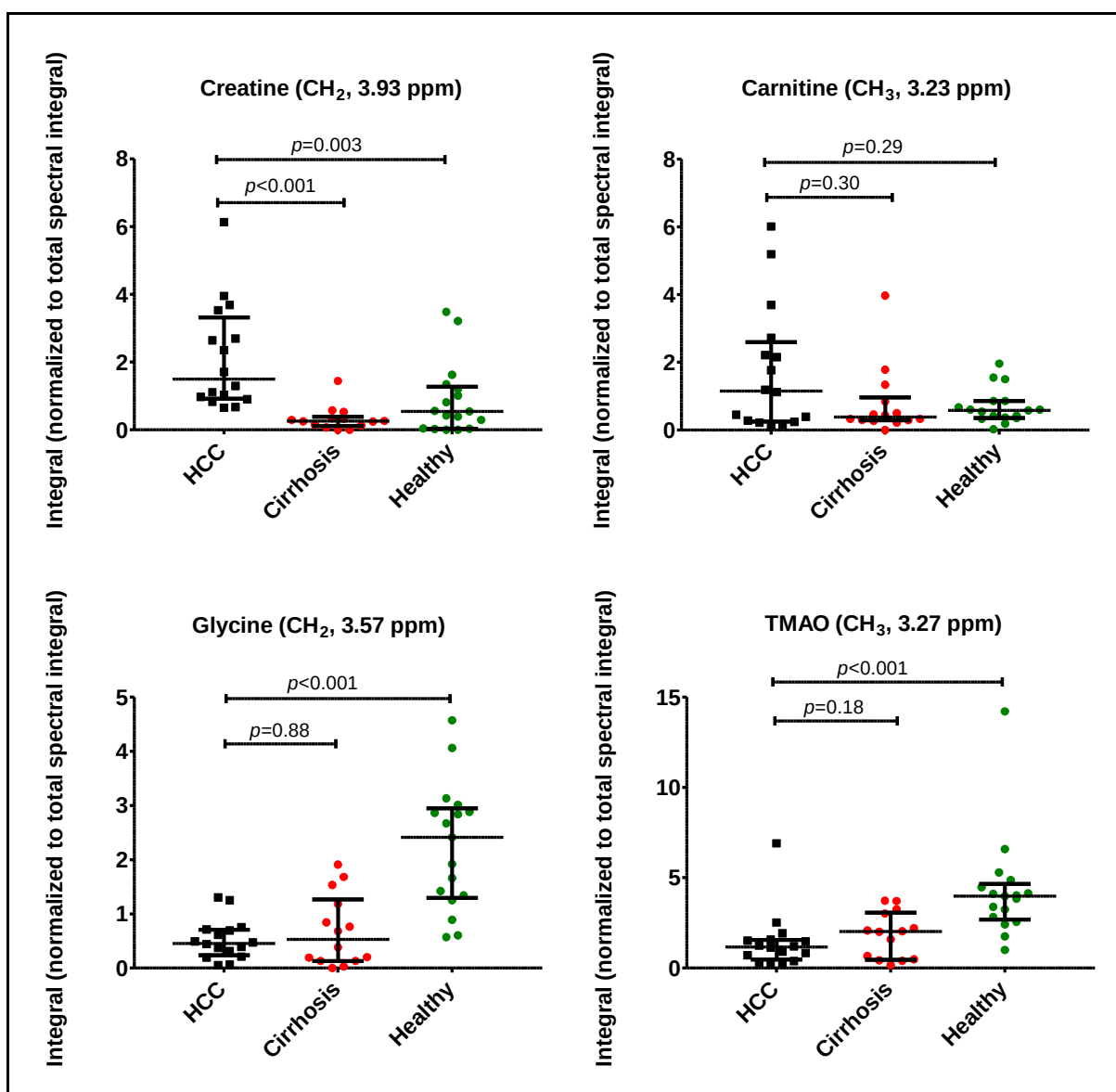


Figure 7-4. Univariate metabolite statistical analysis.

Key: p-values generated through Mann-Whitney non-parametric tests of significance.

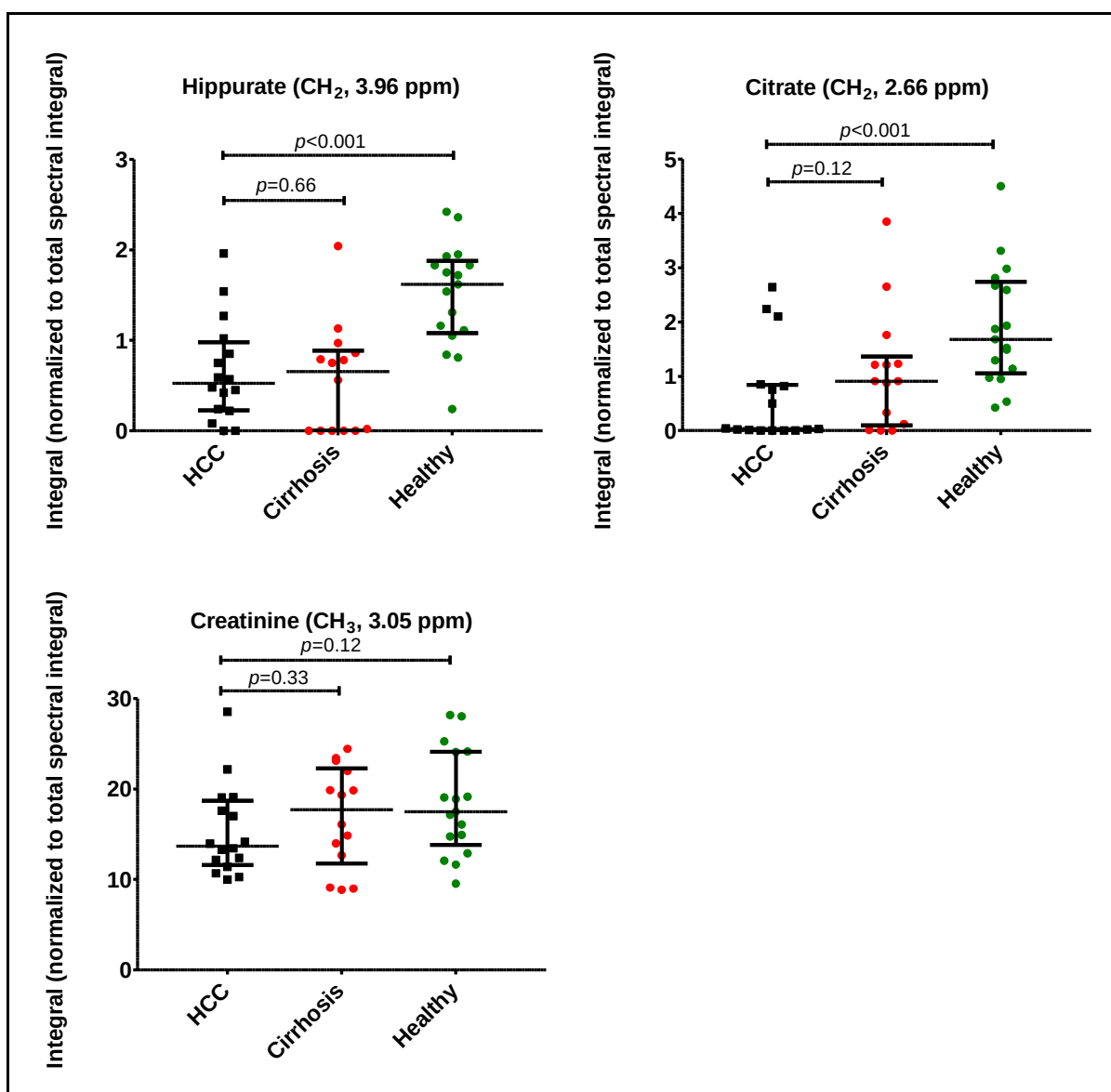


Figure 7-4. Univariate metabolite statistical analysis (continued).

Key: p-values generated through Mann-Whitney non-parametric tests of significance.

7.5 DISCUSSION

In this study of Egyptian subjects, a urinary metabolic profile was identified which distinguished patients with HCC from healthy controls and patients with cirrhosis with a sensitivity and specificity of 100%/93% and 81%/71%, respectively. Discriminatory metabolites were identified as glycine, TMAO, hippurate, citrate, creatinine, creatine and carnitine (Shariff *et al.*, 2011). In the previous chapter (**Chapter 6**) it was observed that in Nigerian subjects with HCC, the metabolites creatinine, creatine, carnitine and acetone were discriminatory between disease and healthy groups (Shariff *et al.*, 2010). These results suggest that despite differences in aetiology (HBV in Nigeria and mostly HCV in Egypt) HCC induces similar metabolite alterations in urine (**Table 7-6**). These two studies constitute the only ^1H NMR spectroscopy studies of urine conducted in two different populations identifying similar metabolite profiles.

Urinary creatine was significantly elevated in HCC subject urine, compared to healthy controls and cirrhosis subjects. Similar findings were observed in the Nigerian patient study. Creatine has a direct function in cellular energy transport, interacting directly with ATP to produce phosphocreatine and ADP. It may therefore be the case that creatine, an essential component of the energy transfer process, is elevated in rapidly growing cells, such as HCC.

Urinary carnitine levels were elevated in patients with HCC urine compared to healthy controls and cirrhosis subjects, although not to a level of significance. Chen and colleagues, using hydrophilic interaction chromatography (HILIC) and reversed phase liquid chromatography (RPLC MS), also observed elevated urinary carnitine levels in HCC subjects (Chen *et al.*, 2009). As described in previous chapters, carnitine is indispensable for energy metabolism, enabling fatty acids to enter the mitochondria for β -oxidation (Vaz and Wanders, 2002).

Nigerian urine	Egyptian urine	Table 7-6. Discriminatory metabolites from Nigerian and Egyptian urine studies. <i>Key: ▲ indicates significant univariate differences between HCC and healthy groups; ■ indicates significant univariate differences between HCC and cirrhosis groups.</i>
Creatinine▲■	Creatinine	
Creatine▲	Creatine▲■	
Carnitine▲■	Carnitine	
Acetone	Glycine▲	
	TMAO▲	
	Hippurate▲	
	Citrate▲	

The serum and plasma metabolic profiling studies presented in previous chapters (**Chapters 3 - 5**) identified alterations in lipid metabolism, perhaps as an indication of subversion of lipids for use as cellular membrane components, rather than for energy production.

Urinary creatinine levels were reduced in HCC subjects, compared to healthy controls and cirrhosis subjects, with a trend towards significance ($p=0.12$ and 0.33). Similar observations were made in the Nigerian subjects with HCC. Chen and colleagues also observed reduced urinary creatinine levels in HCC subjects (Chen *et al.*, 2009). Lower total body creatinine may be due to lower muscle mass, as previously described (**Chapter 6**) (Barr *et al.*, 2005). The muscle mass of these subjects was not formally recorded though is likely that the “dry weight” of patients was reduced, as most subjects with HCC were visibly cachectic.

Urinary glycine levels were significantly reduced in HCC subjects compared to healthy volunteers ($p<0.001$), but not compared to cirrhosis subjects ($p=0.88$). Glycine is a key component of a central methylation reaction within cells. Glycine *N*-methyltransferase (GNMT) catalyses the transfer of the methyl group of *S*-adenosylmethionine (SAME) to glycine to form *S*-adenosylhomocysteine (SAH) and sarcosine (Ogawa *et al.*, 1998). *S*-adenosylmethionine is synthesised from methionine and ATP, catalysed by the enzyme

methionine adenosyltransferase (MAT) (Lu and Mato, 2008) (**Figure 7-5**) and acts as a methyl donor, inducing the methylation of nucleic acids, proteins and phospholipids. Chromosomal hypermethylation of a number of genes, involved in proliferative, apoptotic, cell adhesion and DNA repair, has been described in HCC tissue (Tischoff and Tannapfe, 2008). Hypermethylation of CpG islands or promoter sequences has the consequence of gene silencing, which, if the genes act in a tumour suppressor role, may induce malignancy. Gene knockout animal models of MAT and GNMT are predisposed to liver pathology and HCC (Liao *et al.*, 2009; Martinez-Chantar *et al.*, 2002). In a recent study of 262 urine and plasma samples of patients with prostate carcinoma, sarcosine was found to be elevated in patients with tumours (Sreekumar *et al.*, 2009). It was observed that the addition of sarcosine to benign prostate cells induced a malignant phenotype and addition of glycine had the same effect, although to a lesser extent. In a rat model of galactosamine-induced hepatotoxicity, glycine supplementation was shown to protect against liver damage (Coen *et al.*, 2007). It therefore appears that glycine may play a part in hepatocarcinogenesis, but its exact role is as yet undefined.

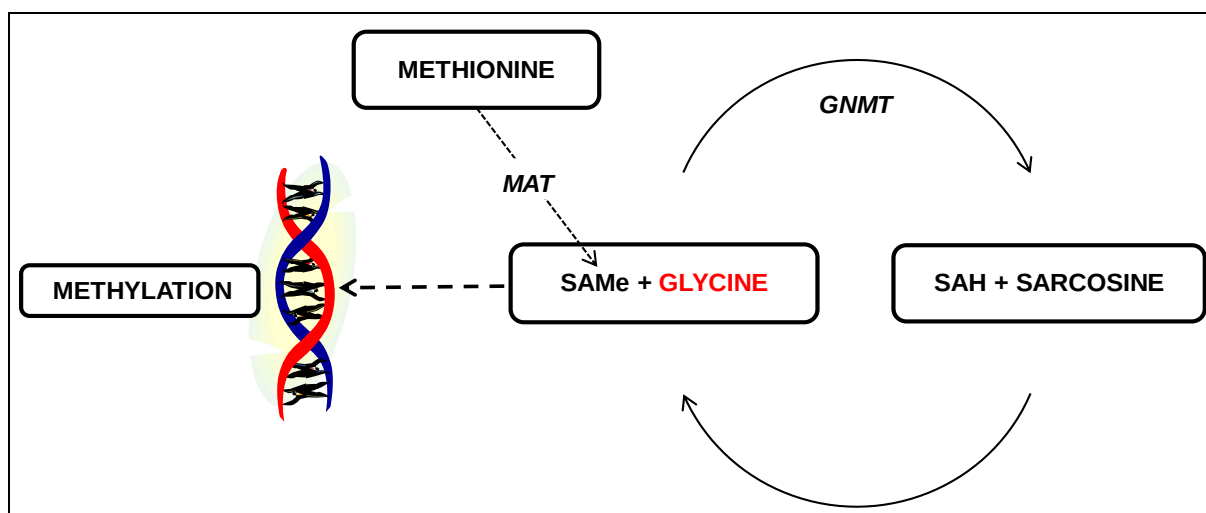


Figure 7-5. Schematic of glycine involvement in cellular methylation reactions.

Key: MAT, methionine adenosyltransferase; S-AMe, S-adenosylmethionine; GNMT, Glycine N-methyltransferase; SAH, S-adenosylhomocysteine.

Urinary TMAO levels were reduced in patients with HCC compared to healthy controls ($p<0.001$), but not compared to cirrhosis subjects ($p=0.18$). TMAO is an aliphatic amine and the oxidation product of trimethylamine (TMA) and derived from mainly dietary sources, through the action of gut microbes (al-Waiz *et al.*, 1992). It may be that the low urinary TMAO observed in our group of HCC patients reflects suppressed gut microbial activity.

Urinary hippurate levels were significantly reduced in HCC subjects, compared to healthy controls ($p<0.001$), but not compared to cirrhosis subjects ($p=0.66$). Hippurate is an acyl glycine that is formed by conjugation of benzoate with glycine in liver and kidney mitochondria (Schachter and Taggart, 1953). Benzoate is formed via the metabolism of gut microbes from dietary aromatic compounds (Nicholson *et al.*, 2005; Rechner *et al.*, 2002). It is possible that the decreased hepatic function in patients with HCC (as evidenced by lower albumin and higher bilirubin levels) translates into less efficient benzoate conjugation and subsequently lower urinary hippurate excretion levels. In this way, hippurate may be seen as a surrogate marker of liver function.

Urinary citrate levels were significantly reduced in HCC subjects compared to healthy controls ($p<0.001$) and non-significantly reduced compared to cirrhosis subjects ($p=0.12$). Citrate is a tri-carboxylic acid (TCA) cycle intermediate and reduced urinary levels may indicate altered mitochondrial aerobic respiration due to heightened physiological stress of cancer cells, which would be in agreement with Warburg's hypothesis (Warburg *et al.*, 1924). In a study of urinary metabolic changes following partial hepatectomy in male Sprague-Dawley rats, Bollard and colleagues observed initially decreased urinary citrate levels compared to control animals (Bollard *et al.*, 2010). It was hypothesised that these changes

may be due to markers of physiological stress and heightened energy demand, which may similarly occur in HCC subjects.

7.6 CONCLUSIONS

The urinary metabolic profile of HCC in this cohort of Egyptian patients (Shariff *et al.*, 2011) bears similarity to that identified in the Nigerian cohort described in **Chapter 6** (Shariff *et al.*, 2010). In both, levels of the metabolites creatine, carnitine and creatinine were altered. These changes indicate the effects of the tumour on human physiology (creatinine and creatine), tumour respiration (carnitine and citrate), gut bacterial metabolism (hippurate and TMAO) and in the form of glycine, a metabolite that may be involved with tumourogenesis itself through aberrant DNA methylation.

For validation, studies in larger populations with accurately characterised patients including earlier stage HCC (Okuda stage I) are required to confirm or refute these results. In follow up studies, anthropometric measurements would be desirable to investigate the relationship of altered metabolites, cancer cachexia and tumourogenesis.

8 URINARY METABOLIC PROFILING OF HEPATOCELLULAR CARCINOMA IN A UNITED KINGDOM COHORT USING PROTON NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

8.1 ABSTRACT

Introduction: Previous chapters have described urinary ^1H NMR studies of patients with HCC from Nigeria and Egypt. The aims of this study were to assess whether similar discriminatory urinary ^1H NMR metabolic profiles occur in a UK patient cohort where aetiologies of liver disease are of greater diversity. **Methods:** UK patients with HCC and cirrhosis were analysed using a 600 MHz ^1H NMR system. Multivariate analysis and median group MR spectra comparison allowed identification of metabolites altered between groups. Metabolite identification was achieved through literature reference and statistical total correlation spectroscopy (STOCSY). **Results:** Fifty-two patients were recruited. Following exclusions, 13 samples from HCC patients and 25 from those with cirrhosis were selected for analysis. At 200 IUml^{-1} , AFP displayed a diagnostic sensitivity of 27%. Multivariate analysis generated diagnostic models with a sensitivity/specificity of 53.6%/96%. *p*-cresol sulphate ($p=0.04$), creatinine ($p=0.03$), citrate ($p=0.21$) and hippurate ($p=0.52$) were reduced in the urine of patients with HCC. Carnitine ($p=0.31$) and formate ($p=0.44$) were elevated. **Conclusions:** Multivariate models of urine metabolic profiles were not as diagnostically sensitive as those from Nigerian and Egyptian studies. Nevertheless, they still outperformed AFP. Reduced levels of creatinine, citrate and hippurate and elevated carnitine bears similarities to findings from the Nigerian and Egyptian groups. Alterations in levels of *p*-cresol sulphate is a novel finding and may indicate an altered sulfonation capacity of the liver in patients with HCC.

8.2 INTRODUCTION

Liver disease is the fifth commonest cause of death in the UK (The British Association for the Study of the Liver and The British Society of Gastroenterology, 2009). In contrast to other chronic conditions, such as cancer and cardiovascular disease, the mortality rate of liver disease is rising. Improvements have been made in the treatment of decompensated liver disease including endoscopic variceal band ligation, primary prophylactic beta-blockade, spontaneous bacterial peritonitis antimicrobial prophylaxis, pharmacotherapy for encephalopathy, treatment of hepatorenal syndrome, alcoholic hepatitis and intensive care of liver failure. Patients with cirrhosis are therefore living longer and are more likely to develop HCC. Data from developed nations including European countries, Japan and the US, have shown that incidence of HCC in cirrhosis at 1 year is 4 – 7% when associated with HCV, 2 – 4% with HBV and 0.2 – 2% with alcohol induced liver disease (Fattovich *et al.*, 2002). Despite surveillance guidelines (Bruix and Sherman, 2005; Ryder, 2003), the 5-year mortality for HCC in the developed world is poor. From the US, the Surveillance Epidemiology and End Results (SEER) database documented a 9.9% 5-year survival for HCC, compared to over 60% for all tumours. A retrospective SEER-database based study from the US between 1994 - 2001, reported 6-monthly regular surveillance rates of only 17% in patients over 65 years with cirrhosis (Davila *et al.*, 2010). Similar rates were found in a survey of cirrhotic patients attending Imperial College NHS Healthcare trust between 2007 – 2011 (Rajasoorian, G, BSc thesis, 2010, supervised by Shariff, M). This highlights the problems patients may have in attending recurrent hospital and clinic appointments for radiological procedures and blood tests, in particular for those that live greater distances from medical centres. A simple, non-invasive urine test to gauge risk of HCC would be invaluable for this population. It would potentially allow surveillance in the community by the local General Practitioner or even the patient themselves.

In comparison to the studies reported of Nigerian and Egyptian patients (**Chapters 4, 5, 6 and 7**) (Shariff *et al.*, 2010; Shariff *et al.*, 2011), the aetiological variation of patients in the UK with HCC is greater, including viral hepatitis, alcohol and NASH. The collection of samples in the UK also allowed detailed patient and disease demographics to be collated. It was therefore possible to accurately stage and grade liver disease. In this respect, the study was designed to limit confounding factors such as the presence of cirrhosis, age and gender, to allow focus on cancer-specific urinary biomarkers to be isolated.

Based on this, the main aims of this study were to:

1. Identify tumour specific urinary metabolite changes in well matched patients with HCC on the background of cirrhosis and those with cirrhosis alone.
2. Compare findings to previous similar studies in Nigerian and Egyptian cohorts.

8.3 METHODS

8.3.1 Patient Selection

Patients were recruited from outpatient and inpatient settings at St Mary's Hospital, Imperial College NHS Trust, London, UK between October 2008 and March 2011. All subjects gave written and informed consent, in accordance with local Research Ethics Committee approval. Hepatocellular carcinoma was diagnosed with two confirmatory imaging modalities and cirrhosis with either histological or radiological confirmation. Tumour staging was according to the BCLC staging system (Bruix and Sherman, 2005) which is based upon tumour size and multiplicity, patient performance status, the presence or absence portal vein invasion, the presence or absence of portal hypertension and Child-Pugh scores of liver function (Child and Turcotte, 1964; Pugh *et al.*, 1973) (**Figure 8-1**). Patients with cirrhosis were graded according to the Child-Pugh score. At the time of sample collection, participant height, weight, medical, drug, smoking and alcohol intake history were recorded. A 24-hour dietary history was also recorded paying attention to foodstuffs known to produce prominent NMR signals, such as vanilla (vanillin) and fish (acetic acid) (**Figure 8-2**). Exclusion criteria included those patients not meeting the diagnostic criteria cited above for HCC and cirrhosis, those patients with HCC who had undergone curative resection or transplant, patients co-infected with HIV and those samples identified as outliers on principal component analysis.

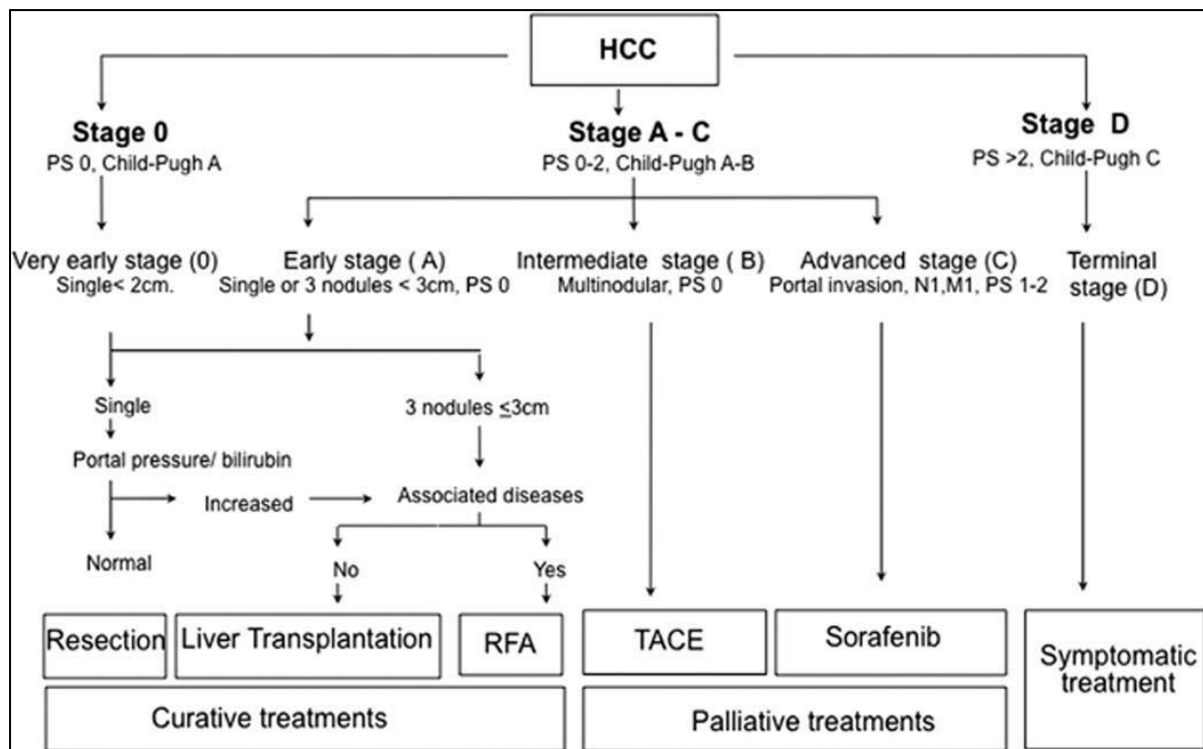


Figure 8-1. Barcelona Clinic Liver Cancer algorithm (Bruix and Sherman, 2005).

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BILIARY MALIGNANCY PROTEOMICS AND METABONOMICS STUDY - CRF

Please complete as fully as possible.

CASE NUMBER: _____

DEMOGRAPHICS

Patient initials: _____ Hospital number: _____ Sex: M / F DoB: / /

Ethnic origin: _____ Units of alcohol/day: _____ Cigarettes/day: _____

Occupation: _____ Rec drugs/betel nut: _____ LMP & cycle length: _____

Weight (kg): _____ Height (m): _____ BMI: _____

Exercise in last 24hrs: none / mild / moderate / hard

MEDICAL HISTORY

Principal current hepatobiliary diagnosis (circle all that apply):

CC / HCC / Liver Mets / PSC / Pancreatic Ca / Cirrhotic / Healthy control / Other.....

Diagnostic certainty:

☐ Histologically proven ☐ MDT consensus, fully investigated ☐ Diagnosis likely, ix pending

Location of disease, if known:

☐ Diffuse process ☐ Metastatic, extrahepatic disease

Previous therapy (tick all that apply, with date of last session)

☐ Resection / / ☐ Ablation / / ☐ Chemotherapy / / ☐ Radiotherapy / /

☐ Biliary stent / / ☐ TACE / / ☐ PDT / / ☐ Other..... / /

OTHER CURRENT MEDICAL PROBLEMS

List active diagnoses (esp malignant, inflammatory or infective processes):

☐ Diabetes ☐ Cholangitis ☐ Systemic sepsis, any source

DRUG HISTORY

Please list all current medications (including herbal remedies and paracetamol/acetaminophen):

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DIETARY HISTORY

Time last ate ANYTHING _____

Details of food and drink taken in the last 6 hours:

List of other food and drink taken in last 24 hrs:

Checklist of specific foods and drink taken in last 72hrs:

Food	✓	Details or comments	Food	✓	Details or comments
Vegetarian		Vegan/non-vegan	Nutrition drinks		
Meat		Red/white	Cheese		
Fish		Type:	Grapofruit		
Fizzy drinks			Berries		
Tea		Normal/herbal	Liquorice		
Milk			Walnuts		
Cherries			Vanilla		
Yoghurt					

SAMPLE COLLECTION

Pt. information sheet issued? Y / N

Informed consent obtained? Y / N

Samples taken:

☐ 1 x EDTA (Purple FBC tube)

☐ 1 x plain serum (Bright red cap tube)

☐ 1 x lithium heparin (Green cap tube)

☐ 1 x SST plasma (Yellow cap tube)

☐ 15 ml urine ☐ MSU ☐ CSU

☐ Bile, uncontaminated

☐ Bile, with some saline

☐ Bile, contaminated with contrast

Samples placed on ice Y / N

Light excluded? Y / N

Samples processed by:

Samples stored in which freezer?: _____

Blood samples taken at: _____

Plain serum sample centrifuged at: _____

☐ All sections completed esp. time last ate, time of blood sample, time of centrifuge

OTHER INVESTIGATIONS

Most recent bloods on ___ / ___ / ___ ALT: _____ ALP: _____ Bili: _____ Alb: _____ INR: _____

AFP: _____

Summary of ERCP findings (if performed):

Summary of most recent axial imaging findings (if performed):

Please direct any queries about this CRF to Dr Chris Wadsworth or Dr Mo Shariff

Email: c.wadsworth@imperial.ac.uk or m.shariff@imperial.ac.uk

Figure 8-2. UK patient demographics and dietary questionnaire.

8.3.2 Sample collection

15 mL of random, non-fasted urine were collected into preservative-free universal containers and placed immediately on ice or into a refrigerator at 4 °C. Five to 10 mL of urine was transferred to 15 mL falcon tubes and centrifuged within 1 – 2 hrs 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 until analysis.

8.3.3 Sample preparation

Urine samples were thawed at room temperature and 400 µL were transferred to 1.5 mL microvial tube to which 200 µL of phosphate buffer solution (pH 7.4), containing 1 mM of TSP, sodium azide 3 mM (as a bacterial biocidal agent to inhibit bacterial growth and contamination) and D₂O 20%. Samples were centrifuged for 5 min at 13,000 rpm and 550 µL of supernatant were transferred to 5 mm NMR tubes (Norell, Landisville, New Jersey, USA).

8.3.4 Proton nuclear magnetic resonance spectroscopy

Samples were run in a random non-grouped order under automation on a Bruker DRX-600 NMR system operating at 600.44 Hz ¹H frequency (Bruker Biospin, Rheinstetten, Germany). at the department of Biomolecular Medicine, Imperial College London. The system was tuned, matched and frequency locked onto deuterated hydrogen 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. Pulse programme and acquisition parameters were set according to optimised protocols for urine from the department of Biomolecular Medicine (Beckonert *et al.*, 2007). Spectra were acquired at 300 K using a one-dimensional (1-D) noesypr1d 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 FIDs were collected into 32, 000 data points with a spectral width of 20 ppm. A line broadening function of 0.3 Hz was applied prior to Fourier transformation. Spectra were manually phased, baseline corrected and referenced to TSP at 0 ppm using TOPSPIN v2.0 (Bruker Biospin, Rheinstetten, Germany). Spectral peaks were assigned with reference to the literature (Clayton *et al.*, 2009; Holmes *et al.*, 1997; Wishart *et al.*, 2007).

8.3.5 Spectral processing

Proton NMR spectra were exported to MATLAB R2010 (MathWorks, Natick, Massachusetts, U.S.A) and to avoid influence on analyses from water suppression aberration, the water region from 4.5 ppm – 6 ppm was excluded. Spectra were aligned to negate the effect of pH dependent variation in metabolite resonances which may occur despite sample buffering. To remove the effect of differential urine concentrations, data were normalised using median fold-change normalisation. Median spectra for both clinical groups were generated to allow direct visual comparison of average spectra and allow the selection of regions that were visually divergent, in addition to those identified by multivariate analysis, for peak integration.

8.3.6 Statistical Total Correlation Spectroscopy (STOCSY)

Using in-house software for use in MATLAB, developed at the department of Biomolecular Medicine, Imperial College London, the spectral database could be interrogated for correlated peaks across the whole spectrum. Peaks that co-varied with high correlation could be identified by input of one peak region of interest. Correlation factors could be varied from 0 –

1. This method was utilised to identify unknown peaks to extract the total spectrum for an individual metabolite.

8.3.7 Multivariate and univariate statistical analysis

Full resolution data were used for analyses. Data matrices were generated in MATLAB containing ppm variables as columns and sample identities as rows. After water exclusion, this amounted to a matrix of 28,238 variables and 43 observations. This matrix was exported to SIMCAP-12 (Umetrics, Umea, Sweden) for multivariate analysis and variables from 0.2 – 10.0 ppm were used for analysis, thus removing any influence from variation in TSP concentration between samples. Data were mean-centred and PCA was performed first to model variation and identify outliers. Pareto scaled data and unit variance data were also generated, but both tended to model noise rather than signal. Therefore, only mean-centred data were used for further analysis. After outliers were identified and excluded, PLS-DA was performed to identify the discriminant strength of the model and to generate a loadings plot from which metabolites could be identified which most greatly contributed to differences between the groups. In SIMCAP-12, PLS-DA models are generated through seven-fold cross validation. In this method, every 7th sample is 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 is repeated for all the samples (grouping the 2nd, 9th, 16th and 3rd, 10th, 17th and so on) until all the samples have been excluded once. The results are averaged to produce a model that is externally cross-validated, similar to the cross validation algorithms described in previous chapters.

Spectral peaks that were most contributory to PLS-DA models and those peaks that appeared divergent on comparison of median spectra were selected for peak integration. Comparison of

integrals was performed using GraphPad Prism (La Jolla, California, USA) using Mann-Whitney non-parametric tests of significance, as normal Gaussian distribution could not be assumed, p -values of <0.05 were considered significant.

8.4 RESULTS

8.4.1 Subject recruitment, exclusion and demographics

A total of 51 patients were recruited: 19 with HCC and 32 with cirrhosis. From the HCC cohort, two patients were excluded as they had undergone curative orthotopic liver transplant or curative hepatic resection prior to sample collection; one patient was excluded as they were co-infected with HIV and a further three samples were excluded following PCA, the details of which are given in the results section. The total number of HCC samples after exclusions was therefore 13. From the cirrhosis cohort, four patients were excluded owing to insufficient radiological or biopsy evidence of cirrhosis status and two samples were excluded following PCA, leaving a total of 25. Median ages were similar in both groups, 61 yrs for HCC and 58 yrs for cirrhosis patients. There were proportionally more males in the HCC group (85% versus 56%) but this was not statistically significant ($p=0.26$). Body mass indices were comparable, 25.9 kg/m² in the HCC group and 27.3 kg/m² in the cirrhosis group, again the difference not reaching significance ($p = 0.23$) (**Table 8-1**).

Table 8-1. UK patient demographics.

Characteristic	HCC	Cirrhosis	<i>p</i> -value
<i>N</i>	13	25	
Median age (range) (years)	61 (29 – 82)	58 (28 – 79)	0.55 ^a
Male n (%)	11 (85%)	14 (56%)	0.26 ^b
Median body mass index (kg/m ²)	25.9	27.3	0.23 ^a
Child-Pugh A	4 (40%)	17 (74%)	
Child-Pugh B	3 (30%)	4 (17%)	
Child-Pugh C	1 (10%)	2 (9%)	
Non-cirrhotic	2 (20%)	0	
Unknown due to insufficient data	3	2	

Key: ^aMann-Whitney test for non-parametric data, ^bFisher's exact test for categorical data.

There were greater numbers of Child's-Pugh grade A patients in the cirrhosis group (74% versus 40%), but cumulatively, grade A + B patients accounted for the majority of patients in both groups: 70% in the HCC cohort and 91% in the cirrhosis cohort. Aetiologies of liver disease are displayed in **Table 8-2**. The majority of patients had either chronic HCV, HBV or alcohol related disease.

Disease stages of HCC are displayed in **Table 8-3**. Three tumours were BCLC stage A, five grade C and two grade D. Three tumours could not be staged owing to insufficient data.

Table 8-2. Hepatocellular carcinoma and cirrhosis aetiological factors.

Aetiology	HCC	Cirrhosis
HCV	6	6
HBV	3	3
Alcohol	2	9
NASH	0	2
Haemochromatosis	1	1
HCV + Alcohol	1	2
HBV + Alcohol	0	0
NASH + Alcohol	0	1
Idiopathic	0	1
Total	13	25

Table 8-3. Barcelona Clinic Liver Cancer stages of hepatocellular carcinoma.

Stage	Number
A	3 (30%)
B	0
C	5 (50%)
D	2 (20%)
Unknown due to insufficient data	3 (30%)
Total	13

8.4.2 Serum biochemical analysis

Median AFP levels were significantly higher in the HCC group (18.1 IU mL⁻¹ versus 4.6 IU mL⁻¹, Table x3.5). Using an AFP cut off level of 20 IU mL⁻¹, sensitivity and specificity were 45% and 95%, respectively and at 200 IU mL⁻¹, were 27% and 100%, confirming the poor sensitivity of AFP for HCC in this cohort (**Table 8-4**). **Table 8-5** displays the median serum biochemistry profiles of the cohorts, which for serum creatinine, ALT, bilirubin and INR are similar, with the exception of albumin which was lower in the HCC group compared to the patients with cirrhosis (30 gL⁻¹ and 35 gL⁻¹, respectively).

Table 8-4. Alpha fetoprotein diagnostic sensitivity and specificity.

	Sensitivity	Specificity
AFP cut off 20 (IU mL ⁻¹)	45%	95%
AFP cut off 200 (IU mL ⁻¹)	27%	100%
AFP cut off 400 (IU mL ⁻¹))	27%	100%

Table 8-5. Serum biochemistry profiles of subject cohorts.

	HCC Median (range)	Cirrhosis Median (range)	<i>p</i> -values
Serum Samples (<i>n</i>)	13	25	
AFP (IU mL ⁻¹)	18.1 (1.2-1230) ⁺	4.6 (1.8-29.2) ⁺⁺⁺	<0.01
Creatinine (mmol L ⁻¹)	79 (59 – 96) ⁺⁺	71 (48 – 132) ⁺⁺	0.26
ALT (IU L ⁻¹)	43.5 (7-147) ⁺⁺	37 (8-193) ⁺	0.7
Bilirubin (μmol L ⁻¹)	10 (5-79) ⁺⁺	17 (6-87) ⁺	0.08
Albumin (g L ⁻¹)	30 (22-40) ⁺⁺	35 (19-48) ⁺⁺	0.02
INR (IU)	1.1 (1-1.5) ⁺⁺⁺	1.2 (1-3.1) ⁺⁺⁺	1

Key: *p*-values calculated using Mann-Whitney tests of significance. ⁺2 measurements missing, ⁺⁺3 measurements missing, ⁺⁺⁺4 measurements missing.

8.4.3 Median spectra

Median overlaid spectra from HCC and cirrhosis groups are shown in **Figure 8-3**, displaying high colinearity.

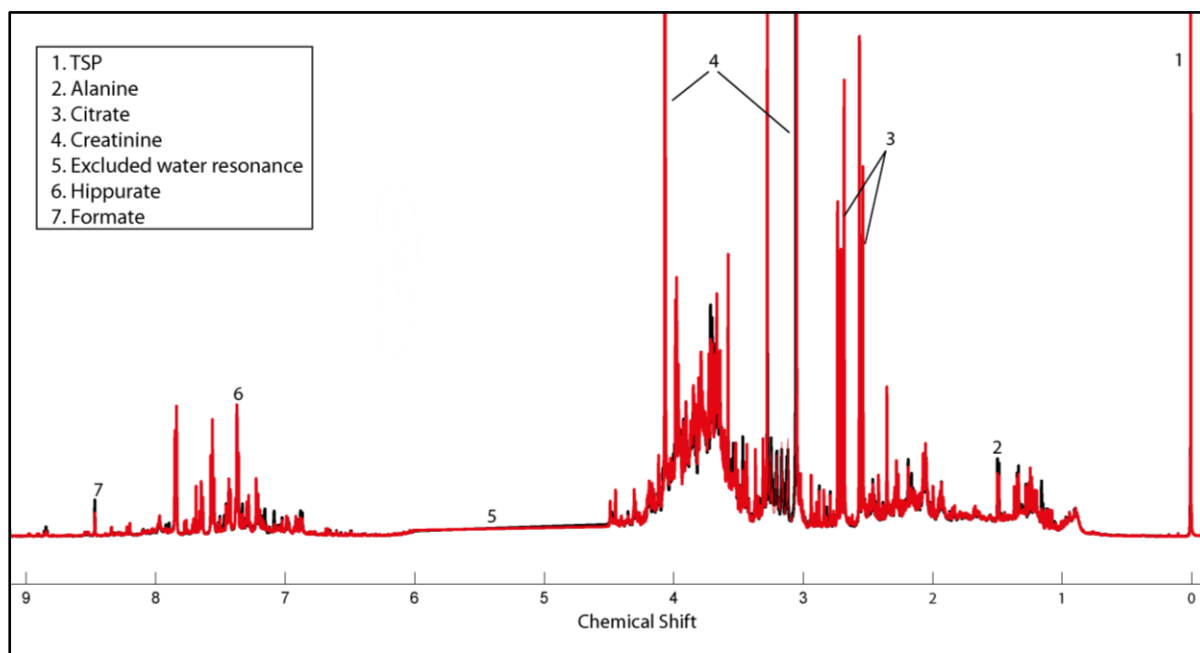


Figure 8-3. Median proton nuclear magnetic resonance urine spectra.

Key: HCC (black) and cirrhosis (red).

8.4.4 Multivariate statistical analysis

The initial PCA scatter plot is displayed in **Figure 8-4** clearly demonstrating two HCC and one cirrhosis outlying samples, positioned outside the Hotelling's T^2 limit. Examination of the MR spectra of these samples identified glycosuria in one HCC sample, with dominant peaks from 3.2 ppm to 4.0 ppm (**Figure 8-5**) which were the major contributor to PC1, in addition to a triplet at 1.24 ppm. Principal component 2 was heavily weighted by resonances at 1.18 ppm (triplet), 3.65 ppm (quadruplet) and 1.14 ppm (doublet). The cirrhosis and remaining HCC outlying samples displayed dominant resonances at 1.18 ppm (triplet) and 3.65 ppm (quadruplet), which correspond to ethanol resonances (**Figure 8-6**). Of note, the

patient from whom one of these samples was from admitted to consumption of five cans of Guinness in the 24 hrs prior to sampling (equivalent to 11.5 units or 115 g of ethanol).

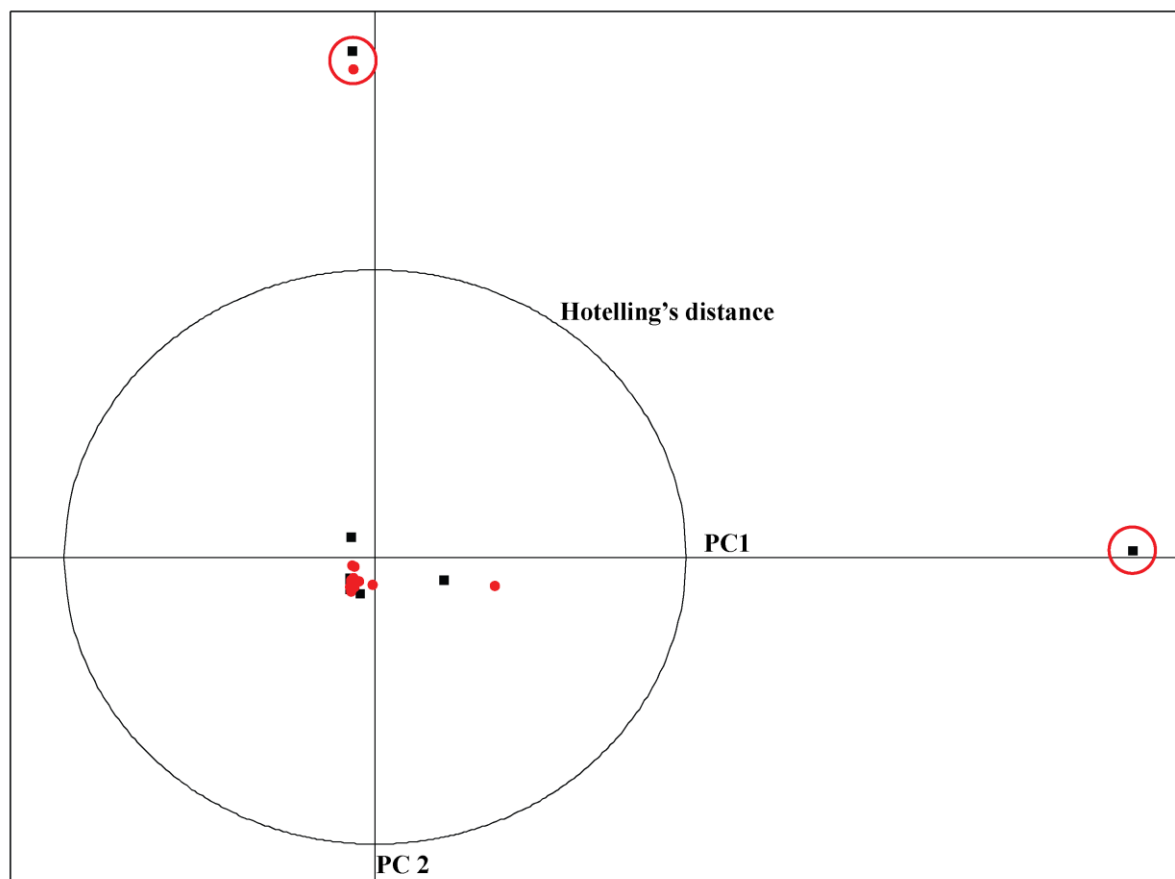
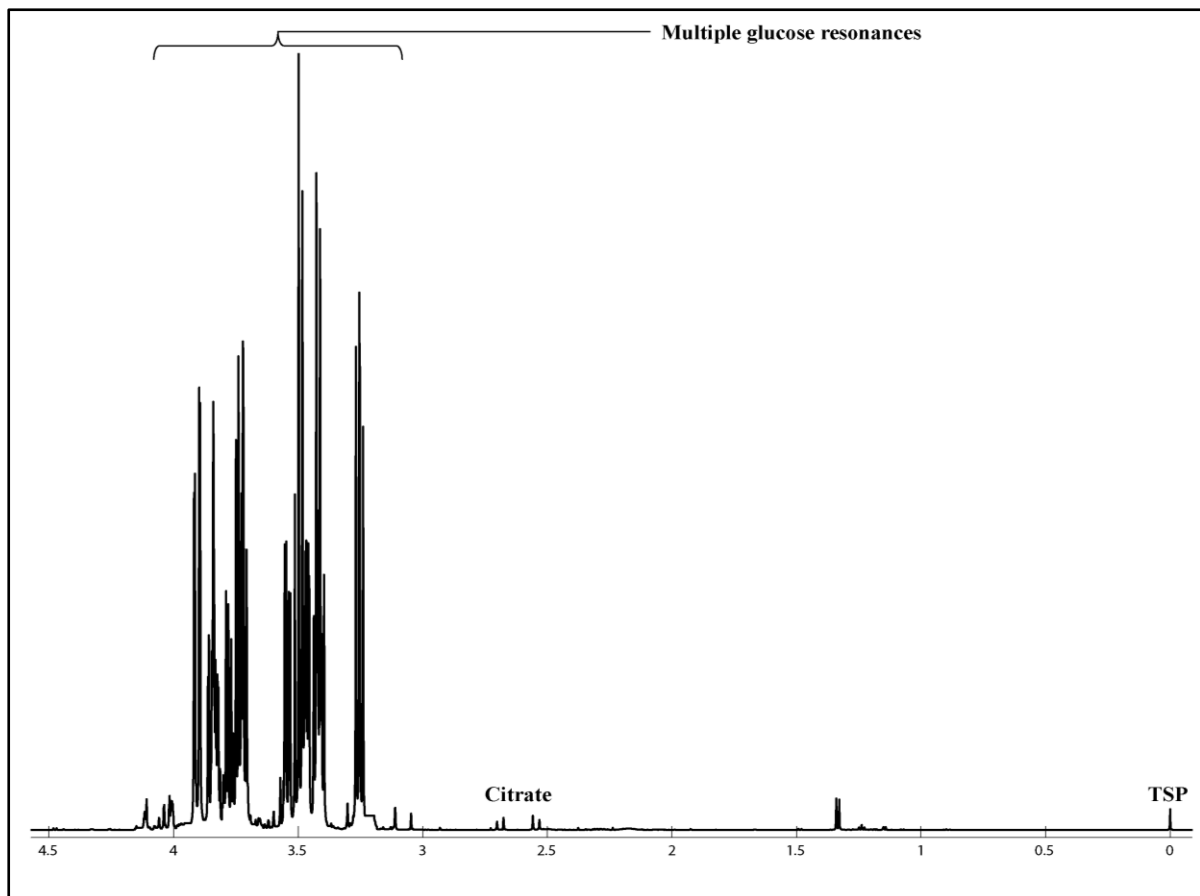


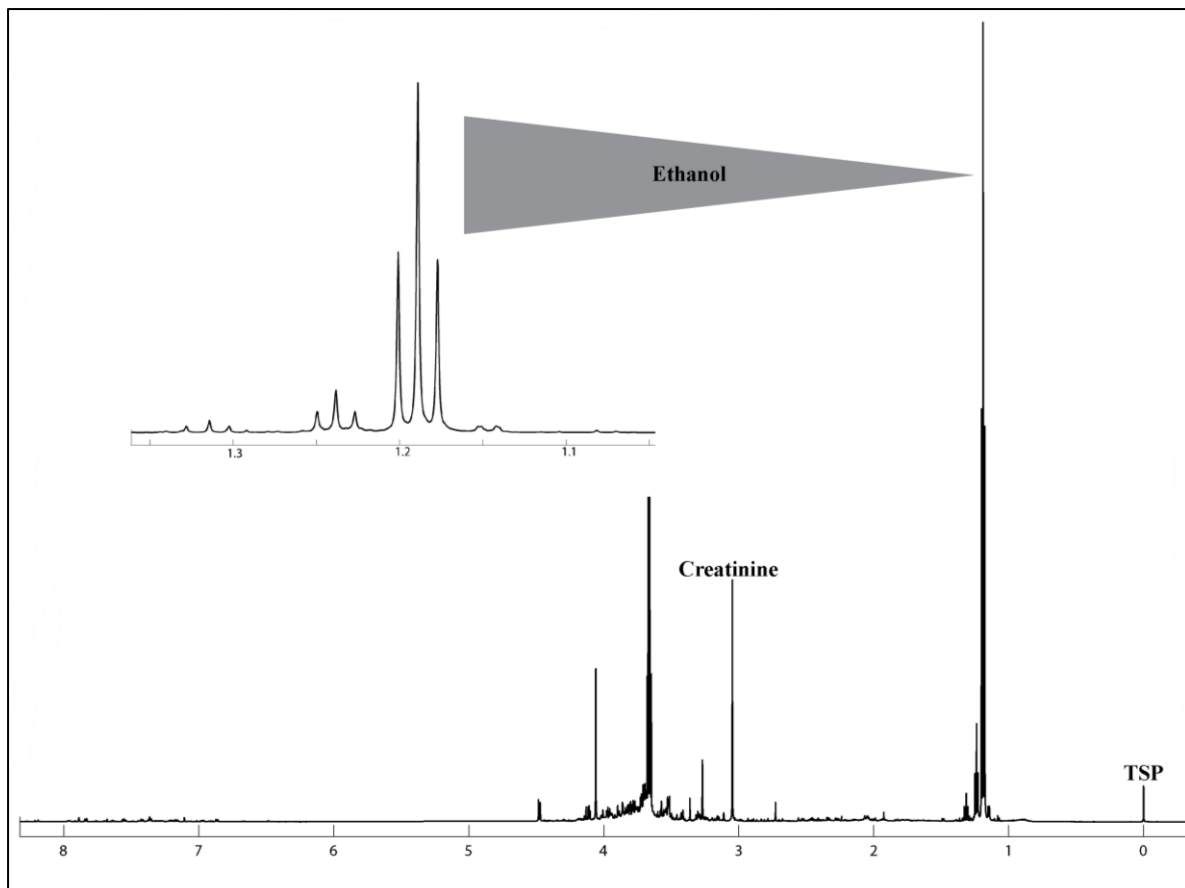
Figure 8-4. Principal components analysis all UK urine samples.

Key: HCC samples (■); cirrhosis samples (●). Outliers indicated in red circles.

Principal components analysis, following exclusion of outlying samples, identified further significantly outlying samples. Two outliers in this analysis revealed heavy influence from glucose metabolites. One of these patients, although not formally diagnosed as diabetic, had a high capillary glucose level (18.2 mmolL^{-1}) at the time of sampling. The other had no documented diagnosis of diabetes. These samples were excluded and a third PCA scatter plot was generated with R^2 and Q^2 values of 0.51 and 0.31, respectively (**Figure 8-7**).



Figures 8-5 and 8-6. Urine magnetic resonance spectra displaying marked glucose and ethanol resonances.



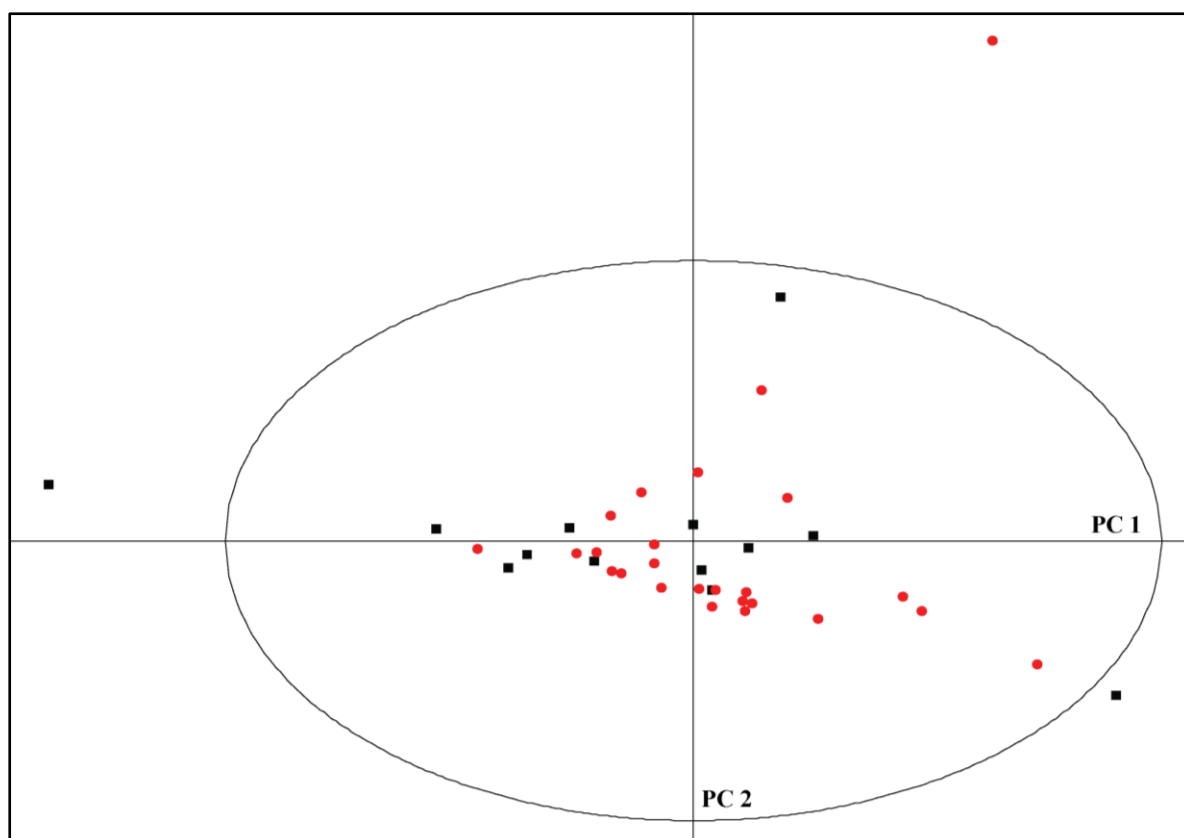


Figure 8-7. Principal components analysis following exclusions.

Key: HCC samples (■); cirrhosis samples (●). ($R^2 = 0.51$ and $Q^2 = 0.31$).

To identify discriminatory metabolites between the two groups, PLS-DA was performed. In a two component model, model fitting was poor ($R^2 = 0.48$) with negative Q^2 values, suggesting that, as a predictive tool, the model would be poorly predictive (**Figure 8-8**). The misclassification matrix generated from these analyses is displayed in **Table 8-6**, and results in a diagnostic sensitivity of HCC of 53.6% and specificity of 96%, highlighting the model's poor predictability. The most influential variables, from PLS-DA loadings plot and visual comparison of median spectra are summarised in **Table 8-7**.

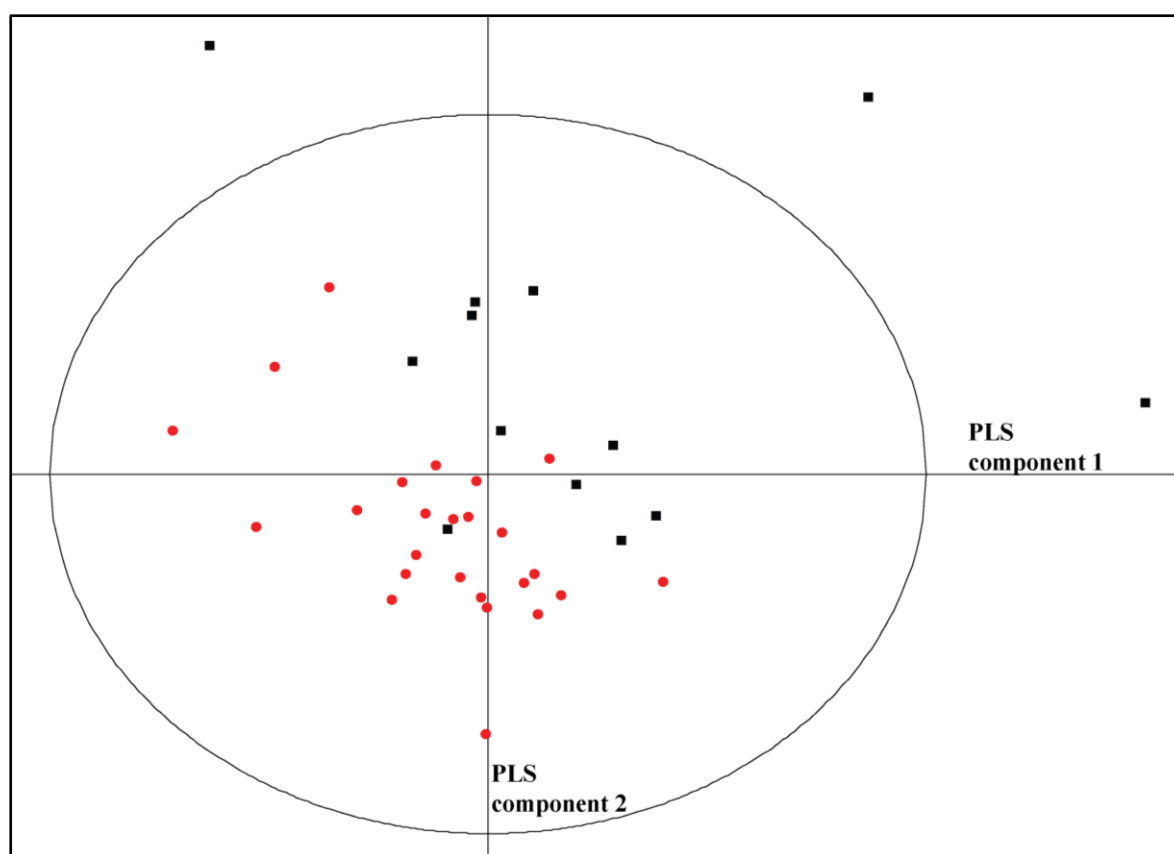


Figure 8-8. Partial least squared discriminant analysis of UK urine samples.

Key: HCC samples (■); cirrhosis samples (●). ($R^2 = 0.48$, Q^2 negative).

Table 8-6. Misclassification matrix from partial least squared discriminant analysis.

	Predicted as HCC	Predicted as Cirrhosis	Total
HCC	7	6	13
Cirrhosis	1	24	25

Table 8-7. Discriminatory metabolites comparison between subject cohorts.

Discriminatory metabolite	HCC median (arbitrary units)	Cirrhosis median (arbitrary units)	<i>p</i> -values
↑ Carnitine (3.24 ppm)	1.16×10^7	9.37×10^6	0.31
↑ Formate (8.5 ppm)	6.36×10^6	3.13×10^6	0.44
↓ Citrate doublet (2.56 ppm)	4.65×10^7	7.33×10^7	0.21
↓ Citrate doublet (2.68 ppm)	5.39×10^7	7.20×10^7	0.25
↓ Hippurate (3.97 ppm)	4.25×10^7	4.68×10^7	0.52
↓ Hippurate (7.56 ppm)	3.94×10^6	4.45×10^6	0.69
↓ <i>p</i> -cresol sulphate (2.35 ppm)	1.15×10^7	2.61×10^7	0.06*
↓ <i>p</i> -cresol sulphate (7.21 ppm)	6.68×10^6	1.32×10^7	0.04*
↓ Creatinine methyl (3.05 ppm)	4.50×10^8	5.01×10^8	0.26
↓ Creatinine methylene (4.06 ppm)	2.83×10^8	3.62×10^8	0.03*

p-values calculated using Mann-Whitney tests of significance.

8.4.5 Univariate statistical analysis

Influential metabolite peak integrals are displayed in **Table 8-7** and **Figure 8-9**. Metabolites elevated in the urine of patients with HCC were carnitine (3.24 ppm, $p=0.31$) and formate (8.5 ppm, $p=0.44$), although neither result reached significance. Urinary metabolites reduced in patients with HCC included citrate double doublet (2.56 ppm and 2.68 ppm, $p=0.21$ and $p=0.25$, respectively) and both the aliphatic and aromatic resonances of hippurate (3.97 ppm, $p=0.52$ and 7.56 ppm, $p=0.69$), neither of these results reaching significance. Peaks at 2.35 ppm ($p=0.06$) and 7.21 ppm ($p=0.04$) were significantly reduced in patients with HCC and were identified as arising from the same metabolite when a STOCYSY analysis was performed (**Figure 8-10**). This metabolite was identified as *p*-cresol sulphate from the literature (Clayton *et al.*, 2009), the resonance at 2.35 representing the methyl signal and that at 7.21, the aromatic signal. A comparison of the median spectral integrals of the 7.21 ppm region is displayed in **Figure 8-11**. Finally, urinary creatinine was found to be reduced in patients with HCC, at both the creatinine methyl singlet at 3.05 ppm ($p=0.26$) and significantly, at the methylene singlet at 4.06 ppm ($p=0.03$) (**Figure 8-12**).

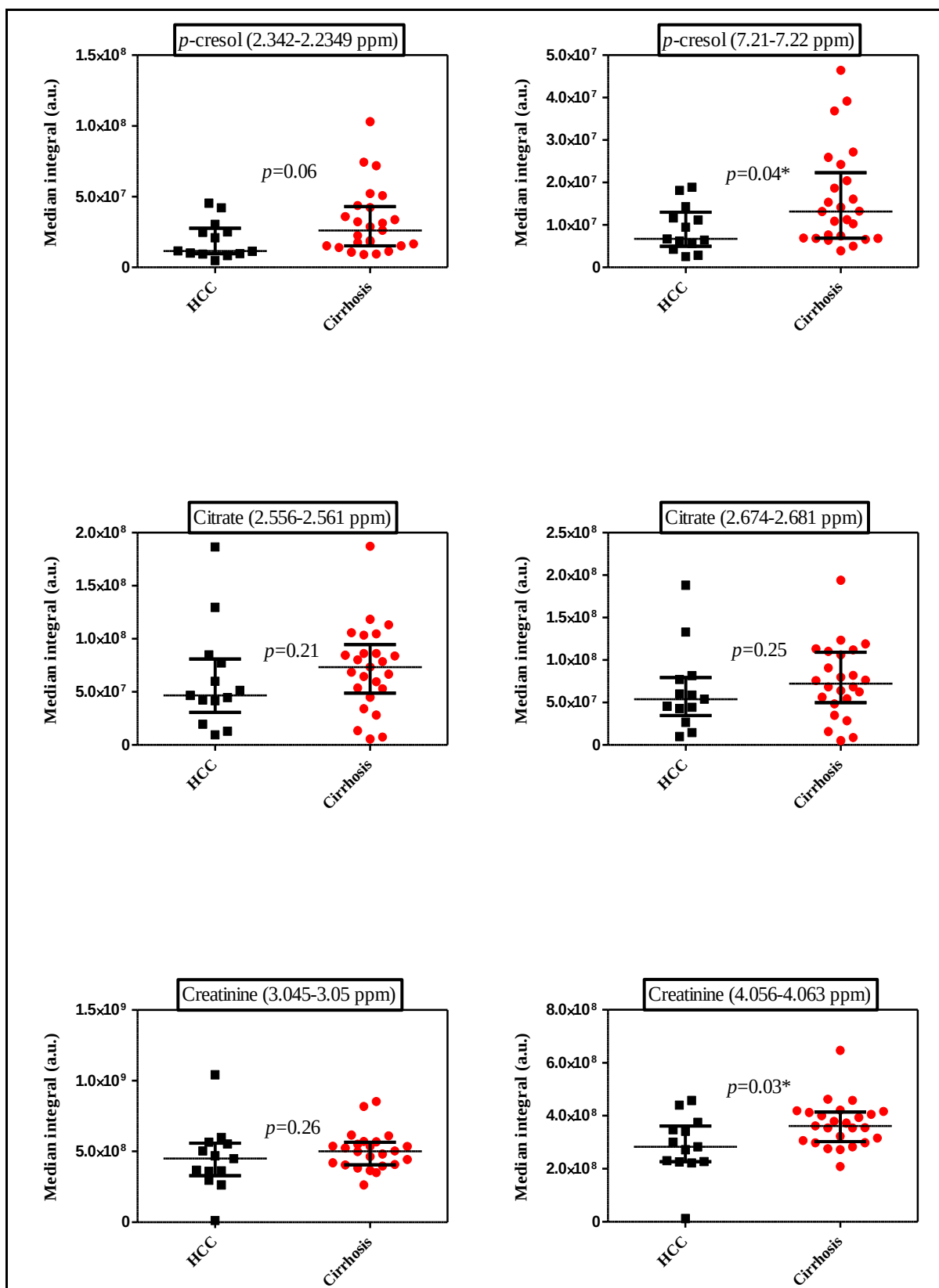


Figure 8-9. Median value scatter plots of discriminatory variables.

p-values calculated using Mann-Whitney tests of significance.

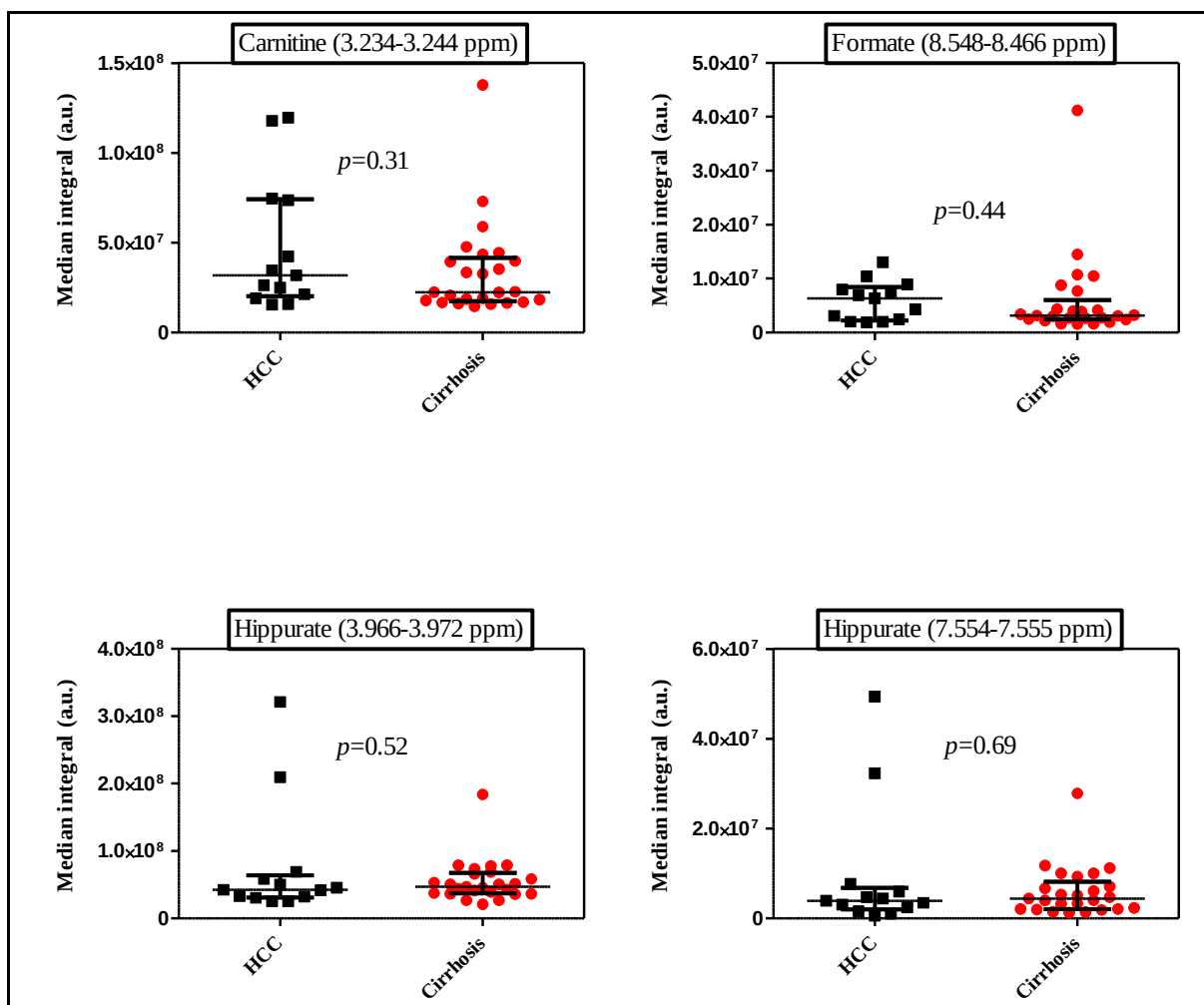


Figure 8-9. Median value scatter plots of discriminatory variables.
p-values calculated using Mann-Whitney tests of significance.

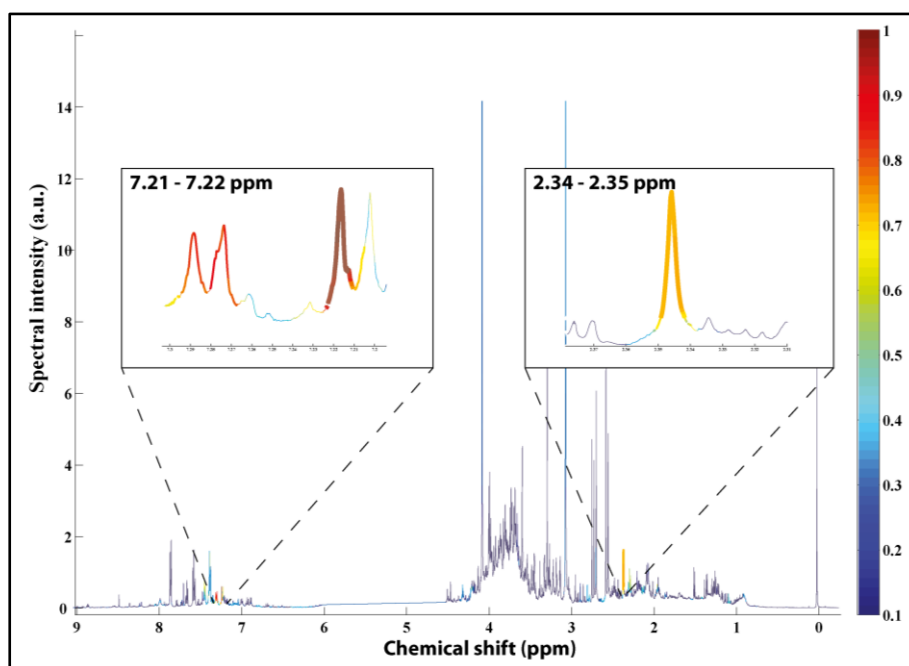


Figure 8-10.
 STOCSY analysis of region at 2.35 ppm, revealing correlation with double doublet at 7.21 ppm.

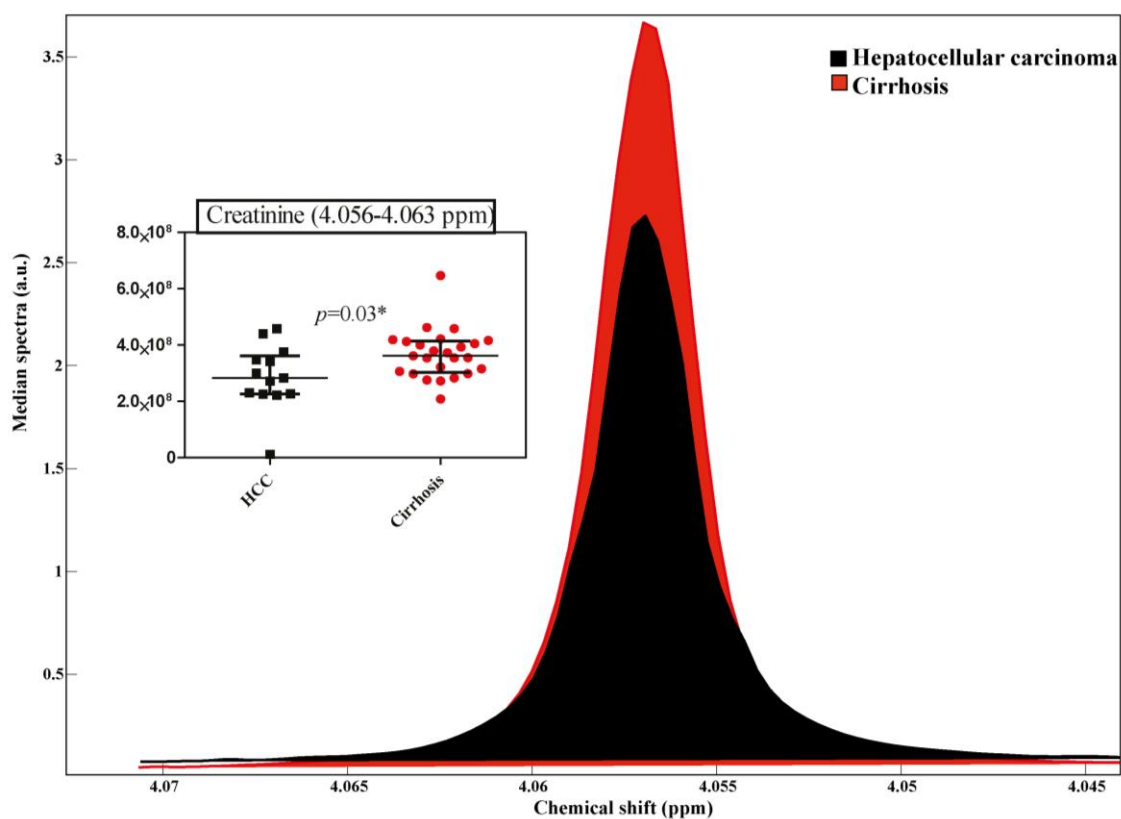


Figure 8-11. Comparison of median spectra of creatinine (4.06 ppm).

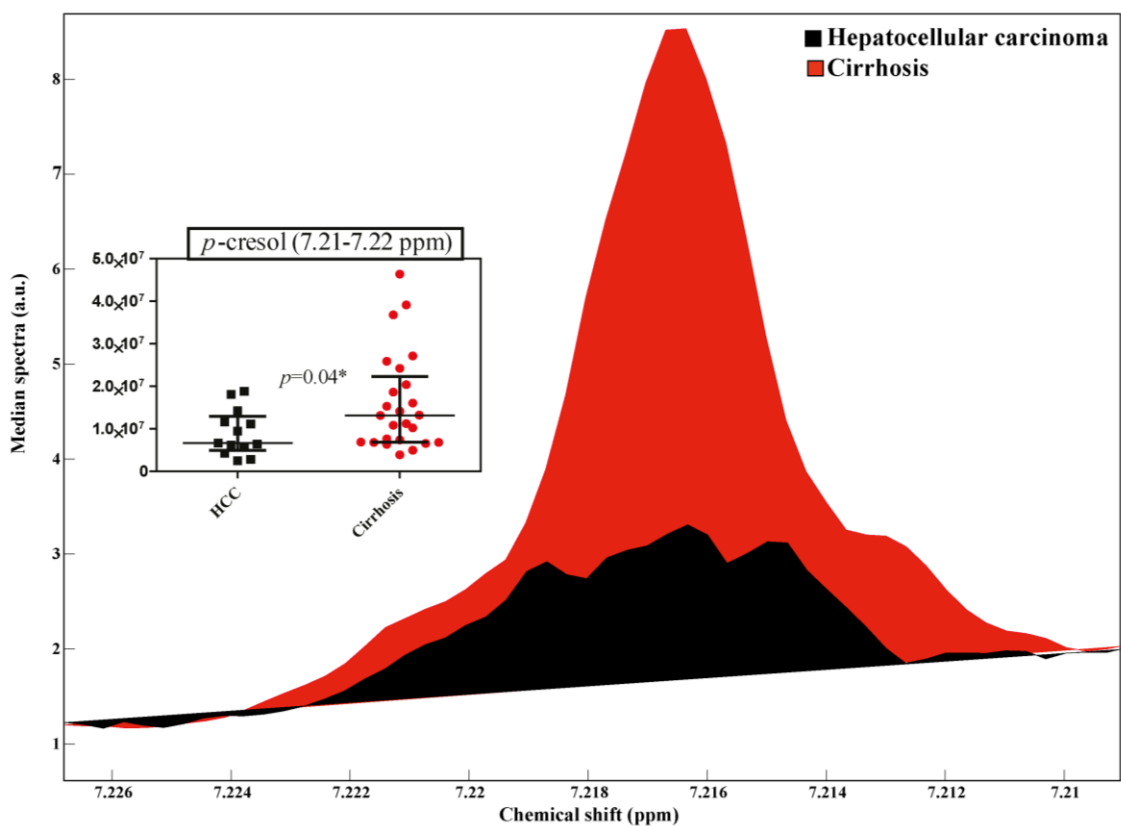


Figure 8-12. Comparison of median spectra of *p*-cresol sulphate (7.21 ppm).

8.5 DISCUSSION

This is the first reported study to identify HCC specific urinary ^1H NMR markers in a closely matched group of patients with HCC. Similar to the studies of Nigerian and Egyptian patient cohorts, reduced urinary creatinine, citrate and hippurate and elevated carnitine levels were found in patients with HCC in comparison to those with cirrhosis, albeit not to levels of statistical significance. The study presented here also identified significant reductions in urinary *p*-cresol sulphate, a metabolite which was not discriminatory in previous studies.

Study patients were well matched, the majority being Child-Pugh stage A and B. In these patients, serum AFP, the most widely used marker for HCC, displayed poor diagnostic sensitivity (27%) at a cut off of 200 IU mL⁻¹, while urinary multivariate analysis, despite displaying poor fitting and predictive parameters, produced a superior sensitivity (53.6%). Diagnostic specificity was similar for both tests (100% for AFP and 96% for multivariate models). These results compare poorly to both the Nigerian and Egyptian urinary ^1H NMR studies, which displayed multivariate model sensitivities and specificities approaching >90%. The reasons for poorer discrimination in this UK cohort may be due to a number of factors. Firstly, the cohorts for this study were intentionally selected to be well matched, both for demographic and physical status and for aetiology of liver disease. No healthy control group was included as the metabolic profiles of healthy patients have been shown to be very different to those of diseased groups, which may be due to global metabolic and hepatic function rather than tumour specific changes. Furthermore, the stage of the tumours tended to be higher in the Nigerian and Egyptian cohort, compared to the UK cohort. It is therefore possible that the close matching of cirrhosis and HCC patients, with $\geq 70\%$ in both groups being Child-Pugh stage A or B, resulted in higher similarity of spectra. Certainly, BMI was similar in both groups, so it is unlikely that cancer cachexia, which may have been a

prominent factor in causing altered spectral profiles in the Nigerian and Egyptian group, had a great influence on discriminatory profiles in this study. Nevertheless, PLS-DA and median MR spectral comparison identified a number of spectral regions elevated or reduced in the HCC compared to the cirrhosis cohorts.

Urinary *p*-cresol sulphate, identified through STOCSY analysis, was significantly reduced in the HCC cohort at 7.21 ppm ($p=0.04$). This corresponded to the aromatic resonances of the molecule. The methyl group, on the benzene ring, resonated at 2.35 ppm, and comparative median values approached significance ($p=0.06$). This finding was not evident in the Nigerian and Egyptian groups. *p*-cresol is a product of colonic bacterial metabolism of tyrosine. Certain bacteria, such as *Clostridium difficile*, are particularly high producers. Sulfonation to *p*-cresol sulphate occurs in the liver. Clayton and colleagues in 2008, observed that healthy human subjects with high pre-test urinary *p*-cresol sulphate levels sulfonated acetaminophen (paracetamol) to the less toxic acetaminophen glucuronide to a lesser extent than those with lower pre-test *p*-cresol sulphate levels (Clayton *et al.*, 2009). This was proposed to be due to the competitive inhibition of *p*-cresol sulfonation in the presence of paracetamol. This study was performed in healthy patients, so it is not possible to surmise whether this would translate in higher likelihood of drug induced liver toxicity, although sulfonation of hepatically metabolised drugs is a common occurrence and it is possible that *p*-cresol sulphate is a surrogate marker of liver function, lower levels reflecting reduced sulfonation in those patients with HCC and worse liver function.

Urinary creatinine was reduced in patients with HCC, the methylene (CH₂) singlet at 4.06 ppm significantly reduced ($p=0.03$), while the methyl singlet (CH₃) at 3.05 ppm, non-significantly reduced ($p=0.26$). Similar findings were present in both the Nigerian and

Egyptian cohorts of patients. Chen and colleagues, using HILIC and RPLC MS, also observed reduced urinary creatinine levels in HCC subjects (Chen *et al.*, 2009). Creatinine is a breakdown product of creatine phosphate in muscle and is eliminated from the body by glomerular filtration and partial tubular excretion. Renal impairment will cause a rise in serum creatinine and a concomitant rise in urine creatinine. Serum creatinine levels were similar in both groups, so is unlikely to have contributed to urinary differences. Lower total body creatinine may be due to reduced muscle mass. Although the BMI of each group was non-significantly different (HCC = 25.9 kg/m² and cirrhosis = 27.25 kg/m², $p=0.23$) it may be that the slight difference in weight is due to reduced muscle mass in HCC patients, which is reflected in lower urinary creatinine levels. A study of urinary creatinine in a US population observed that lower muscle mass was a major contributing factor to lower urinary creatinine levels (Barr *et al.*, 2005) .

Formate (8.5 ppm, $p=0.44$) was non-significantly elevated in the HCC group and was not identified as a discriminatory metabolite in the Nigerian or Egyptian groups. Formate is a small 46 Da carboxylic acid that is endogenously produced as the product of 1-carbon metabolism and from dietary fibre digestion by the gut microbiome (Holmes *et al.*, 2008). As with glycine, which was found to be reduced in patients with HCC in the Egyptian cohort, formate, through the 1-carbon metabolism pathway, may be involved in the methylation status of the cell. In an international study of urinary metabolic profiles and blood pressure, Holmes and colleagues described a positive correlation of blood pressure and urinary formate, proposing that formate affects blood pressure through aberration of sodium chloride reabsorption in the renal tubules. Blood pressure data was not collected on the subjects in this study, although cirrhosis tends to induce a relatively low blood pressure state owing to

decreased systemic vascular resistance, and higher portal pressures causing shunting of fluid into the third space.

Urinary citrate levels (2.56 ppm and 2.68 ppm, $p=0.21$ and $p=0.25$, respectively) were reduced in the HCC cohort, although not to a level of significance. This finding is similar to that of the Egyptian cohort and may represent a repression of oxidative phosphorylation in the mitochondria, as predicted by the Warburg hypothesis (Warburg *et al.*, 1924; Warburg, 1958). In addition, a previous study in Sprague-Dawley rats by Bollard and colleagues observed reduced urinary citrate after partial hepatectomy, proposed to be a marker of heightened energy demand and cellular stress, as would be the case in patients with **HCC** (Bollard *et al.*, 2010).

Urinary hippurate levels (3.97 ppm, $p=0.52$ and 7.56ppm, $p=0.69$), an aromatic acyl glycine, that is formed by conjugation of benzoate with glycine in liver and kidney mitochondria (Schachter and Taggart, 1953), was non-significantly reduced in the urine of patients with HCC. Hippurate was similarly reduced in the urine of the Egyptian cohort of HCC patients. Benzoate is formed via the metabolism of gut microbes from dietary aromatic compounds (Nicholson *et al.*, 2005; Rechner *et al.*, 2002), but it is possible that the decreased hepatic function, as evidenced by higher Child-Pugh scores in patients with HCC translates into less efficient benzoate conjugation and subsequently lower urinary hippurate excretion levels. In this way, hippurate may be seen as a surrogate marker of hepatic function, however, alterations in the urinary level of this metabolite have also been reported in inflammatory bowel disease (Williams *et al.*, 2009) and pneumonia (Slupsky *et al.*, 2009) questioning its specificity for a particular disease.

There were several areas where the study could have been improved. Firstly, the number of patients was low and it is therefore difficult to draw firm conclusions from the data. Although Imperial College NHS Healthcare Trust treats many patients with HCC, it was not possible to sample all of them owing to low recruitment rates. This was improved by contacting patients by mail and telephone to arrange suitable dates for sampling. Despite this, many patients declined to partake owing to their frail physical state and the difficulty in attending hospital. Based on the study presented here, however, recruitment of HCC patients for urinary metabolic profiling is underway as a UK Clinical Research Network listed study (UKCRN ID 8457, [http://public.ukcrn.org.uk/Search/StudyDetail.aspx Study 8357](http://public.ukcrn.org.uk/Search/StudyDetail.aspx?StudyID=8357)). This will provide a much larger cohort of subjects allowing validation of the results described here. Secondly, although tumour stages were less advanced than that of the Nigerian and Egyptian cohorts, it would have been preferable if more very early tumours could have been recruited for study. This would not have been possible in the time-frame of this study as early tumours remain rarely detected owing to the poor sensitivity of current surveillance techniques of US and AFP. As part of the UKCRN Nationwide study, patients with cirrhosis are being recruited and followed up. A small proportion of these patients will develop HCC and it will be possible to compare pre- and post-HCC urinary profiles to isolate markers of early disease. Finally, as in the Egyptian and Nigerian study, many samples had to be excluded owing to certain metabolites overwhelming the spectral profiles resulting in bias of multivariate analysis. In this study, ethanol and glucose (glycosuria) were the main factors for exclusion. For future studies it would, therefore, be important to ensure that diabetes is not present in subjects, and if it is, then serum glucose levels are recorded prior to sampling. Excessive alcohol consumption would also be an exclusion criteria.

8.6 CONCLUSIONS

This is the first urinary ^1H NMR spectroscopy metabolic profiling study of HCC to be performed in a UK population. Overall, the findings provide further insight into the metabolic pathogenesis of liver disease and HCC and corroborate some of the findings highlighted in earlier studies in Nigerian and Egyptian cohorts. Similarities include reduced urinary creatinine, citrate and hippurate and raised carnitine, albeit not to significant level. This study has also identified *p*-cresol sulphate as a discriminatory urinary metabolite in this UK cohort, significantly reduced in patients with HCC compared to those with cirrhosis. The explanation for this is not clear but may be due to altered liver function.

Larger cohort numbers are required for validation of these data. Based on the study presented here, recruitment of patients with HCC for urinary metabolic profiling is now underway as part of the UKCRN portfolio of studies. This will hopefully confirm the alterations described by this study and prove that urine does indeed possess diagnostic capabilities.

9 THE URINARY METABOLIC PROFILING OF HEPATOCELLULAR CARCINOMA IN A NIGERIAN PATIENT COHORT USING ULTRA HIGH PERFORMANCE LIQUID CHROMATOGRAPHY MASS SPECTROMETRY

9.1 ABSTRACT

Introduction: Mass spectrometry has been utilised for the identification of urinary biomarkers of HCC in several previously published studies. The study presented here utilises UPLC-MS for metabolic profiling of urine samples from the same Nigerian cohort of subjects recruited for ^1H NMR studies. The aims were to examine whether the metabolite profiles detected by UPLC-MS could also distinguish these patients, provide validity data and identify metabolites altered in the urine of patients with HCC. **Methods:** Liquid chromatography was performed using a 1.7 μm high strength silica column and mass spectrometry run in positive and negative ion mode using a quadrupole time of flight system. Univariate and multivariate analysis identified discriminatory ions and tandem fragmentation MS was employed for metabolite identification. **Results:** A number of discriminatory ions were identified, however, it was only possible to identify acetylcarnitine (204 Da) and an ion weighing 229 Da, eluting at 1.14 mins, likely representing leucylproline. Acetylcarnitine levels were raised in the urine of patients with HCC compared to cirrhosis ($p < 0.001$) and healthy volunteers ($p = 0.07$). **Conclusions:** These data complement the matched ^1H NMR spectroscopy study in confirming elevation in urinary carnitine molecules, in this case, acetylcarnitine. This result provides insight into the altered energy metabolism processes of HCC. In particular it may suggest a subjugation of fatty acid metabolism to provide lipid building blocks for rapidly dividing tumour cells.

9.2 INTRODUCTION

The use of ultra performance liquid chromatography mass spectrometry (UPLC-MS) has been validated in cancer biomarker identification and metabolic profiling (Antoniello *et al.*, 1998; Jeng *et al.*, 2009; Motyl *et al.*, 1993; Psychogios *et al.*, 2011; Tamura *et al.*, 1986; Want *et al.*, 2010). It is complimentary to ^1H NMR spectroscopy displaying higher sensitivity (picomolar versus micro/nanomolar range) and has the ability to detect metabolites that ^1H NMR spectroscopy may not (Psychogios *et al.*, 2011).

Liquid and gas chromatography MS have been utilised in the identification of urinary biomarkers of HCC since the 1980s, although the majority of these studies had limitations. In 1986, Tamura and colleagues detected elevated levels of urinary pseudouridine using high performance (HP) LC in patients with HCC. Pseudouridine is a C-glycoside isomer of the nucleoside uridine and it is the most prevalent of the over one hundred different modified nucleosides found in RNA. In this study, in combination with serum AFP, urine pseudouridine levels displayed an 83% sensitivity for HCC diagnosis (Tamura *et al.*, 1986). Urinary pseudouridine levels have also been shown to be elevated in other malignancies, such as non-Hodgkin's lymphoma (Motyl *et al.*, 1993), probably as an indicator of overall cellular proliferation of tumour growth and not a specific attribute to HCC.

In 2009, based on a further HPLC-based study in Taiwanese subjects, the nucleosides, adenosine, cytidine and inosine were proposed as urinary markers of HCC (Jeng *et al.*, 2009). When combined with serum AFP, sensitivity for tumour diagnosis was 80%, however, the study was only conducted with one healthy subject group as controls and no patients with cirrhosis, substantially reducing the validity of the findings.

In 1998, using RPLC, Antonella and colleagues reported increased urinary levels of free and acetylated polyamines in patients with HCC compared to healthy controls and patients with cirrhosis (Antoniello *et al.*, 1998). These polyamines were identified as putrescine, spermine and spermidine which are required for cellular proliferation, although their exact role is unclear. Putrescine acts on SAMe to produce spermine which in turn acts on further SAMe molecules to produce spermidine (Wishart *et al.*, 2007). Unfortunately, as with other markers, polyamines were found not to be sensitive enough for accurate early tumour detection and may not be specific to HCC.

There have been only two LC-MS studies reporting global urine metabolic profiling of hepatocellular carcinoma. The first was performed by Chen and colleagues in 2008 using UPLC-MS coupled with both RPLC and HILIC, on urine samples from 21 patients with HCC compared to 24 from healthy controls (Chen *et al.*, 2009). In this study, HILIC was found to be complimentary to RPLC as polar compounds, such as creatinine, were superiorly retained on the hydrophilic HILIC chromatography column. Multivariate analyses, in the form of PCA and PLS-DA, were utilised for biomarker identification. Twenty four metabolites (16 from HILIC and eight from RPLC) were identified as having greatest influence on PLS-DA loadings plots. Of these, four were authenticated with standards and MS-MS fragmentation studies: creatinine and *N*-methyl nicotinic acid, both of which were reduced in patients with HCC; and carnitine and acetylcarnitine, which were elevated in patients with HCC. The remaining influential metabolites, which were not authenticated, included nine carnitine derivatives: hypoxanthine, proline betaine, leuacylproline, phenylacetylglutamine and four unidentified analytes. The alteration in carnitines and acetylcarnitine were attributed to a decrease in mitochondrial β -oxidation of fatty acids. Decreased urinary creatinine was attributed to disordered hepatic metabolism of arginine, glycine and methionine. Lower *N*-

methyl nicotinic acid urine levels were hypothesised to be caused by nicotinamide and nicotinic acid hepatic metabolism alterations. This study, although one of the first to describe urinary metabolic profiling in HCC, provided no demographic features of the participants. It is accepted that age, sex and nutritional status may heavily confound metabolic profiling studies. Furthermore, the stage and grade of HCC were not reported. Patients with advanced HCC may be more successfully discriminated from those with early disease owing to the global physiological effects advanced tumours will have on the body. The second study, also performed in China in 2009, profiled 20 urine samples from male patients with HCC in comparison to 20 samples from healthy male controls using GC-MS (Wu *et al.*, 2009). This modality has the advantage of being highly sensitive to even very small polar metabolites (Pasikanti *et al.*, 2008) and the disadvantage of requiring lengthy derivitisation steps to form volatile analytes. Identification of metabolites was performed with reference to the National Institute of Standards and Technology database and confirmed with comprehensive standard authentication, identifying 103 metabolites. Principal components analysis was utilised to identify discriminatory metabolites. Octanedioic acid, glycine, tyrosine, threonine and butanedioic acid were reported to be elevated in patients with HCC. Other metabolites, including primidine, xylitol and hypoxanthine, were reduced. A diagnostic model using 18 of the identified metabolites, using cross validated Y-values, was subjected to area under the curve analysis and generated an area of 0.93. Metabolite alterations were attributed to heightened cellular proliferation (hypoxanthine), glycolysis (glycine and xylitol) and nucleic acid turnover (primidine). Efforts were made to control for gender and age, however, there was no description of tumour stage or grade and an adequate control group of patients with cirrhosis was not included. Furthermore, PCA may not be the most appropriate multivariate tool for this purpose. It is an unsupervised model that does not seek to discriminate between groups but describes the maximum variation across all the samples, plotted in orthogonal

components. Certainly, from the scores plots displayed in this study, the first component does not split the groups. If the loadings were used from this component then the metabolites identified may be those of greatest variation in all the samples, rather than those different between the two groups. Nonetheless, these two studies were the first to identify that metabolic profiling of urine using LC or GC MS can potentially identify discriminatory patterns.

In **Chapter 6**, it was shown that in a cohort of Nigerian subjects, ^1H NMR spectroscopy of urine can provide discriminatory metabolite profiles of patients with HCC (Shariff *et al.*, 2010). The aims of the study presented here were to examine whether the metabolite profiles detected by UPLC-MS could also distinguish these patients, provide validation data and identify any other metabolites altered in the urine of patients with HCC, compared to well matched healthy and cirrhosis control subjects.

9.3 METHODS

9.3.1 Patient selection

The study protocol was in concordance with the ethical guidelines of the 1975 Declaration of Helsinki and approved by the research ethics committee of the University of Jos, Nigeria. The metabolic profiling protocol was approved by the research ethics committee of Imperial College London, UK. Subjects were recruited as previously described (**Chapters 4 and 5**).

9.3.2 Serum biochemical tests

Serum AFP, urea, creatinine, ALT, ALP, total bilirubin and albumin levels were measured at St Mary's Hospital, Paddington as previously described (**Chapter 8**). Median levels were compared using unpaired Mann-Whitney tests of significance.

9.3.3 Urine sample collection

Urine samples were collected, transported and stored as previously described (**Chapter 6**).

9.3.4 Sample preparation

150 μ L aliquot of each urine sample was added to 150 μ L of sterile H₂O. The samples were centrifuged at 13 000 g for 10 mins to remove particulates. 250 μ L of the resultant diluted urine was transferred into 96-well plates for analysis. 5 μ L of each urine sample were pooled to constitute three quality control (QC) samples which were run at the beginning (the 10 first runs), after every 10th sample and at the end of the experiment, in both positive and negative ion modes. The QCs served as measures of retention time drift and subsequent reliability of the data as interpreted by initial PCA. Samples were run in random, non-grouped order over a total of 35 hrs.

9.3.5 Chromatographic conditions

Chromatographic separations were performed on a Waters 2.1mm x 100mm ACQUITYTM 1.7 μ m HSS T3 column (Waters, Elstree, UK) maintained at 40 °C in an ACQUITYTM UPLC system (Waters MS Technologies, Manchester, UK) based at Imperial College London.

Samples were maintained at 4 °C prior to injection of 5 μ L onto the column. A 12 min gradient was employed with mobile phase A of 0.1a% formic acid (FA) in water and mobile phase B 0.1% FA in acetonitrile. The composition was increased linearly from 0 to 15% B over 1 min, then to 50% B over the next 2 min, 95% B over the next 3 mins, remained 95% B until 9.9 mins then returned to 100% A over 0.2 min, and then re-equilibrated over the next 1.9 min prior to injection of the next sample. Analytes elute 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 RPLC-MS and to reduce the pH of the mobile phase, to suppress the ionisation of weak organic acids and thereby improve retention.

9.3.6 Mass spectrometry

Mass spectrometry experiments were performed on a Micromass LCT PremierTM (Waters MS Technologies, Manchester, UK) with electrospray ionisation (ESI) operated in both positive and negative modes. The mass spectrometer was calibrated using a solution of sodium formate. All analyses were acquired using the lock spray to ensure accuracy and reproducibility; 200 pg mL^{-1} leucine-enkephalin (m/z 556.2771) in $\text{H}_2\text{O}:\text{ACN}$ (50:50) was used as the lock mass with a flow rate of 30 mL^{-1} . Data were collected in positive and negative ion centroid mode, the lockspray frequency was set at 200 sec, and data were averaged over 10 scans. The mass spectrometer was operated with a capillary voltage of

2.4 kV for negative mode and 3.2 kV for positive ion mode, cone voltage of 35 V, nebulizer gas flow of 900 L/h, desolvation temperature of 350 °C, and source temperature of 120 °C. The instrument was set to acquire over the mass range m/z 50-1000 with acquisition time of 5 scans sec^{-1} and an interscan delay of 100 ms. These settings have been validated for the analysis of urine using UPLC-MS (Want *et al.*, 2010).

9.3.7 Tandem mass spectrometry (MS-MS)

Fragmentation experiments were therefore undertaken to identify the most discriminatory variables. Tandem mass spectrometry (MS-MS) was performed using a quadrupole TOF PremiereTM instrument (Waters MS Technologies, Manchester, UK). Chromatographic conditions were the same as preceding MS experiments and survey scans were performed for masses identified through multivariate and XCMS analysis. A collision ramp was set for the argon filled collision cells from 5 – 30 eV to ensure passage and fragmentation of the parent ion selected by the quadrupole. Fragmentation patterns and retention times were then interrogated to identify candidate molecules using online databases for identification (<http://www.hmdb.ca/> and http://metlin.scripps.edu/metabolites_list.php) (Wishart *et al.*, 2007). Acetylcarnitine standard was purchased from Sigma-Aldrich (Gillingham, UK), and made up to 1g mL^{-1} concentration for authentication experiments.

9.3.8 Data pre-processing

Data underwent peak-picking, retention time correction and grouping using XCMS (Scripps Center for Mass Spectrometry, La Jolla, California, USA) (Smith *et al.*, 2006) as previously described (**Chapter 5**).

9.3.9 Multivariate analysis

The data matrix was exported to SIMCA (Umetrics, Umea, Sweden) for PCA and PLS-DA as described in earlier chapters. All data were mean centred prior to multivariate analysis and outliers were excluded on the basis of causing heavy bias to the loadings components.

9.3.10 Univariate analysis

Data were exported to GraphPad Prism (La Jolla, California, USA) for univariate analysis in the form of Mann-Whitney t-tests comparison of medians between groups, assuming non-parametric distribution of data.

9.4 RESULTS

9.4.1 Subject recruitment, exclusion and demographics

A total of 47 Nigerian subjects, in three cohorts, were recruited for study: 18 patients with HCC; 12 patients with cirrhosis; and 17 healthy Nigerian controls. The majority of subjects recruited for this study were the same as the parallel ^1H NMR urine study (Chapter X). As a method of comparison, **Table 9-1** outlines the baseline demographics of each subject group. Multivariate analysis identified a number of outlying samples which were excluded based on unrepresentative metabolite profiles as described in the following sections. Different samples were outlying in the two ion modes, leading to their exclusion. Two demographic data tables are therefore displayed.

Three HCC and one healthy control samples were excluded from positive ion mode studies following multivariate analysis in the form of PCA, leaving 15 HCC, 12 cirrhosis and 16 healthy control samples. Subject demographics are shown in **Table 9-2**. All patients in the HCC and cirrhosis groups were HBsAg positive (ELISA, 3rd generation). All control subjects were HBsAg negative with normal hepatic function.

In negative ion mode, one sample from a patient with HCC and one healthy control sample were excluded following PCA, leaving 17 HCC, 12 cirrhosis and 16 healthy control samples (**Table 9-4**). Of the remaining samples, all the cirrhosis subjects and 16/17 (94%) were HBsAg positive. The single HCC patient negative for HBsAg had idiopathic liver disease. All subjects denied taking prescribed or non-prescribed medication, nutrient or vitamin supplements to direct questioning.

These data are directly comparable to multivariate analysis data as samples that were excluded following PCA, on the basis of outlying and biased influence upon factors, were also excluded from the baseline demographics.

In both ion modes, patients with HCC were older than those with cirrhosis and healthy controls, albeit, not significantly. Patients with HCC and cirrhosis had deranged hepatic biochemistry compared to healthy controls, as would be expected. All of the patients with HCC had vastly elevated serum AFP levels. In both ion modes, serum creatinine was not significantly different between the groups (**Tables 9-3 and 9-5**).

Table 9-1. Comparison of subject demographics in ^1H NMR and UPLC MS studies.

	HCC		Cirrhosis		Healthy	
	^1H NMR	UPLC MS	^1H NMR	UPLC MS	^1H NMR	UPLC MS
<i>N</i>	18	18	10	12	14	17
Median age (yrs)	46.5	47	37	40	37	45
Male <i>n</i>	12	12	8	9	7	8
Ethnicity	Nigerian	Nigerian	Nigerian	Nigerian	Nigerian	Nigerian
HB sAg+	17/18	17/18	10/10	12/12	0	0
Median AFP	12474	19422	6	6	1.7	1.6

Table 9-2. Demographic profiles for samples used in positive ion mode.

Characteristic	HCC	Cirrhosis	Healthy	p-value
<i>N</i>	15	12	16	-
Median age (range) (years)	45 (25 – 85)	40 (23 – 65)	37 (18 – 80)	^a 0.74 and ^b 0.79
Male <i>n</i> (%)	9/15 (60%)	9/12 (75%)	7/16 (44%)	-
Ethnicity	Nigerian	Nigerian	Nigerian	-
HB sAg+	15 (100%)	12 (100%)	0	-

Key: *p*-values: ^a and ^b denote Mann-Whitney tests of HCC versus healthy and HCC versus cirrhosis, respectively.

Table 9-3. Serum biochemistry profiles for samples used in positive ion mode.

	HCC	Cirrhosis	Healthy	p-values
	Median	Median	Median	
	(range)	(range)	(range)	
Serum Samples (<i>n</i>)	***15	**11	*11	
AFP (IU mL⁻¹)	15,522 (2.1–30,000)	6.0 (2.9 – 1,825)	1.7 (1.0 – 6.9)	^a and ^b <0.001
Creatinine (mmol L⁻¹)	74 (18 – 166)	76 (51 - 143)	74 (29 – 203)	^a 0.90 and ^b 0.80
ALT (IU L⁻¹)	42.5 (11 – 476)	37 (15 – 132)	22 (7 – 41)	^a 0.006 and ^b 0.83
ALP (IU L⁻¹)	11.5 (5.8 – 24.9)	10.5 (5.9 – 980)	8.1 (5.3 – 92.2)	^a 0.60 and ^b 0.78
Bilirubin (μmol L⁻¹)	47.8 (14.4 – 318.4)	28.8 (9.1 – 67.1)	6.5 (2.8 – 30.3)	^a <0.001 and ^b 0.27
Albumin (g L⁻¹)	28.7 (15.7 – 59.3)	27.8 (18 – 39.5)	49.3 (29.3 – 79.2)	^a 0.005 and ^b 0.81

Key: *Five serum samples missing; **one serum sample missing; ***three HCC samples insufficient for ALT, ALT, bilirubin and albumin measurements. ^a and ^b denote Mann-Whitney tests of HCC versus healthy and HCC versus cirrhosis, respectively.

Table 9-4. Demographic profiles for samples used in negative ion mode.

Characteristic	HCC	Cirrhosis	Healthy	p-value
<i>N</i>	17	12	16	
Median age (range) (years)	45.0	40.0	37.0	^a 0.42 and ^b 0.52
Male n (%)	11/17 (64.7%)	9/12 (75%)	8/16 (50%)	-
Ethnicity	Nigerian	Nigerian	Nigerian	-
HB sAg+	16/17 (94%)	12 (100%)	0	-

Key: p-values: ^a and ^b denote Mann-Whitney tests of HCC versus healthy and HCC versus cirrhosis, respectively.

Table 9-5. Serum biochemistry profiles for samples used in negative ion mode.

	HCC	Cirrhosis	Healthy	p-values
	Median	Median	Median	
	(range)	(range)	(range)	
Serum Samples (n)	17***	11**	11*	
AFP (IU mL⁻¹)	15,522 (2.1-30,000)	6.0 (2.9-1825)	1.4 (1.0-6.9)-	^a and ^b <0.001
Creatinine (mmolL⁻¹)	77 (18-166)	76 (51-143)	76 (29-615)	^a 0.80 and ^b 0.96
ALT (IU L⁻¹)	46 (11-476)	37 (15-132)	22 (8-41)	^a 0.003 and ^b 0.60
ALP (IU L⁻¹)	11.5 (5.8-24.9)	10.5 (5.9- 980.4)	6.4 (5-92.2)	^a 0.27 and ^b 0.88
Bilirubin (μmol L⁻¹)	30.7 (14.4-318.4)	28.8 (9.1-67.1)	4.7 (2.8-30.3)	^a <0.001 and ^b 0.44
Albumin (g L⁻¹)	30 (15.7-59.3)	27.8 (18-39.5)	48 (29.3-79.2)	^a 0.006 and ^b 0.51

Key: *Five serum samples missing; **one serum sample missing; ***three HCC samples insufficient for ALT, ALT, bilirubin and albumin measurements. ^a and ^b denote Mann-Whitney tests of HCC versus healthy and HCC versus cirrhosis, respectively.

9.4.2 Positive ion mode data

Positive and negative ion mode chromatographs are displayed in **Figure 9-1**, highlighting that complementary data that can be generated from the same sample.

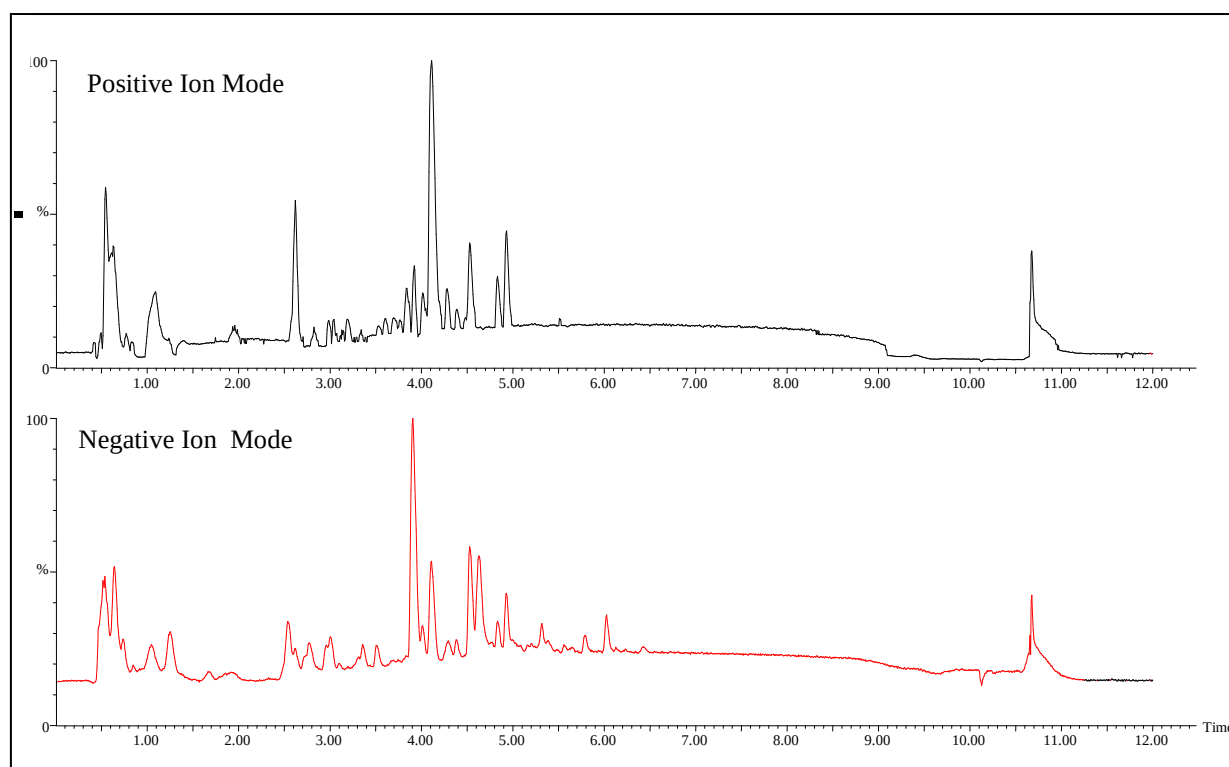


Figure 9-1. Ultra-performance liquid chromatography mass spectrometry total ion chromatograms from a patient with hepatocellular carcinoma.

9.4.2.1 Multivariate statistical analysis

XCMS processing of initial data results in the output of a matrix of variables (2,915 m/z (M) :RT (T) readings, or “analytes”). Principal components analysis of all three groups and QCs displayed group clustering with four outlying samples (**Figure 9-2**). Quality control samples were well clustered. The “drift” in the first few QC samples is to be expected as the column equilibrates to the experimental run. Tight QC clustering is observed thereafter indicating minimal retention time drift. The experimental data can, therefore, be reliably attributed to sample metabolic characteristics rather than retention time aberrations. Outliers in this PCA

correspond to one healthy control and three HCC samples. Examination of the loadings plots for these samples identified large contributions from variables M332-T249, M229-T94, M481-T258, M437-T250 and M525-T264. Examinations of the loadings plots of the outlying healthy control and HCC samples revealed markedly raised levels of these variables, confirming the disproportional influence from the presence of these samples in the model. These four samples were therefore excluded from the dataset. The identity of these analytes was not investigated further so it is not possible to comment on the reasons these samples displayed such altered levels. It is noteworthy that these outliers were different to those identified in the Nigerian urine ^1H NMR spectroscopy study, which was attributed to paracetamol metabolites.

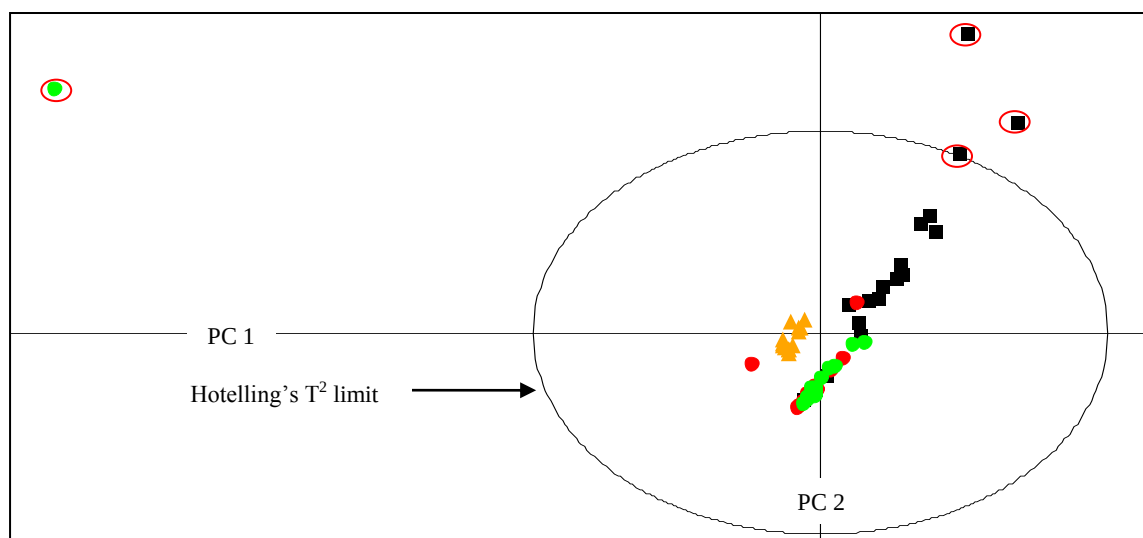


Figure 9-2. Positive ion mode: principal components analysis.

Key: Hepatocellular carcinoma (■), cirrhosis (●), healthy controls (●) and quality controls (▲). Outlying samples are circled in red. PC = principal component.

The PCA scores plot displayed in **Figure 9-3A** represents healthy controls and HCC samples following exclusion of outlying samples. Group clustering is observed. The cumulative R^2 , or the explained variance, with a two principal component model was 0.57, suggesting the

model is well fitted. Two component PLS-DA displayed clear separation of cohorts with good model fitting ($R^2 = 0.66$) and predictability ($Q^2 = 0.53$). Analysis of the loadings plot and the variable importance list (regression vectors), identified the most influential analytes in the multivariate model (**Table 9-6**).

Principal components analysis of patients with HCC and patients with cirrhosis, following outlying sample exclusion, also displayed clustering of disease groups (**Figure 9-3B**). PLS-DA analysis revealed good separation of the samples with an R^2 value of 0.64 and Q^2 of 0.43. The variable importance list of the most influential variables in this model is displayed in **Table 9-6**. It is noteworthy that there was marked similarity between variables in both models.

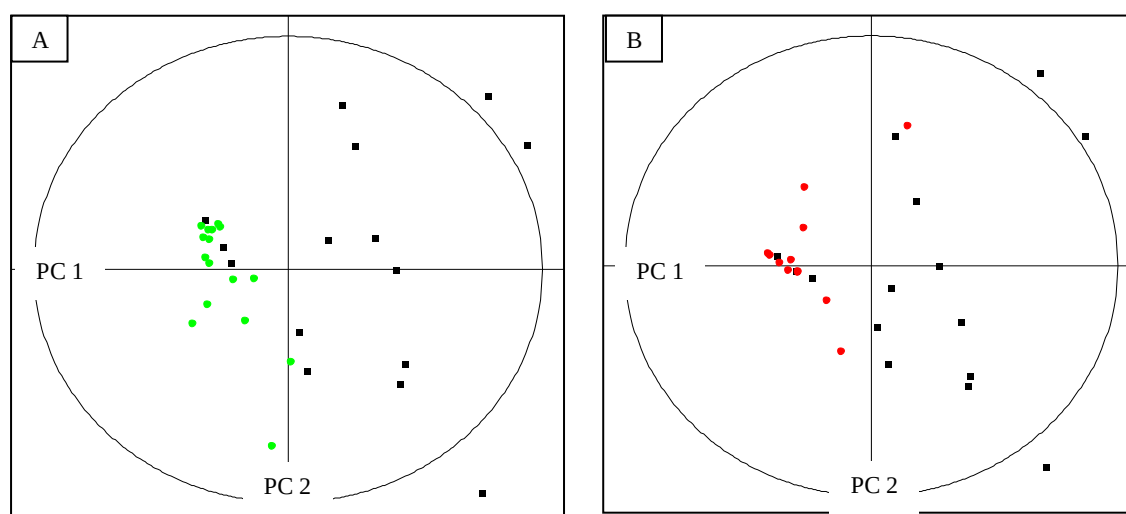


Figure 9-3. Positive ion mode: principal components analysis following exclusions.

Key: Hepatocellular carcinoma (■), healthy controls (●), cirrhosis (●). PC = principal component.

Table 9-6. Positive ion mode discriminatory variables.

HCC versus Healthy Controls	HCC versus Cirrhosis
M229-T94	M204-T67
M204-T67	M229-T94
M302-T208	M302-T208
M332-T249	M332-T249
M478-T199	M478-T199

9.4.2.2 XCMS generated univariate statistical analyses

The data matrix produced by XCMS is ordered in terms of statistical significance, by means of a student's *t*-test, of variables most altered between two designated groups. The five most significant variables are displayed in **Table 9-7**.

Table 9-7. Positive ion mode discriminatory variables comparisons generated by XCMS driven univariate analysis.

HCC versus Healthy Controls	<i>p</i> -value	HCC versus Cirrhosis	<i>p</i> -value
M565-T29	<0.001	M180-T77	<0.001
M577-T27	<0.001	M335-T41	<0.001
M597-T27	<0.001	M457-T193	<0.001
M429-T29	<0.001	M875-T194	<0.001
M595-T27	<0.001	M913-T194	<0.001

p-values calculated using *student's *t*-test.

9.4.3 Negative ion mode

9.4.3.1 Multivariate statistical analysis

Figure 9-4 displays all group PCA, including QC samples, displaying some grouping of samples and two outlying samples based on Hotelling's T^2 index. Analysis of the loadings plots of the all group PCA and the contribution plots from the outlying samples identified heavy bias from variables M230-T167 and M187-T258, which were markedly raised in the two outlying samples. These samples were therefore excluded from any further analyses.

Subsequent PCA of HCC and healthy control samples (**Figure 9-5A**) revealed some clustering, albeit not as marked as compared to positive mode data. The R^2 , or cumulative variance of this model was 0.46, reflecting poorer model definition. PLS-DA of these two groups produced a less robust model compared to positive mode data, with R^2 and Q^2 values of 0.40 and 0.22. The variable importance list of the most influential variables in this model is displayed in **Table 9-8**.

Principal components analysis of HCC and cirrhosis samples were less well clustered, displaying an R^2 of 0.47 and PLS-DA displayed R^2 and Q^2 levels of 0.47 and -0.06, respectively, suggesting poorer predictability of the model (**Figure 9-5B**). The variable importance list of the most influential variables in this model is displayed in **Table 9-8**. Similar to positive mode data, three of the influential variables were the same in both analyses.

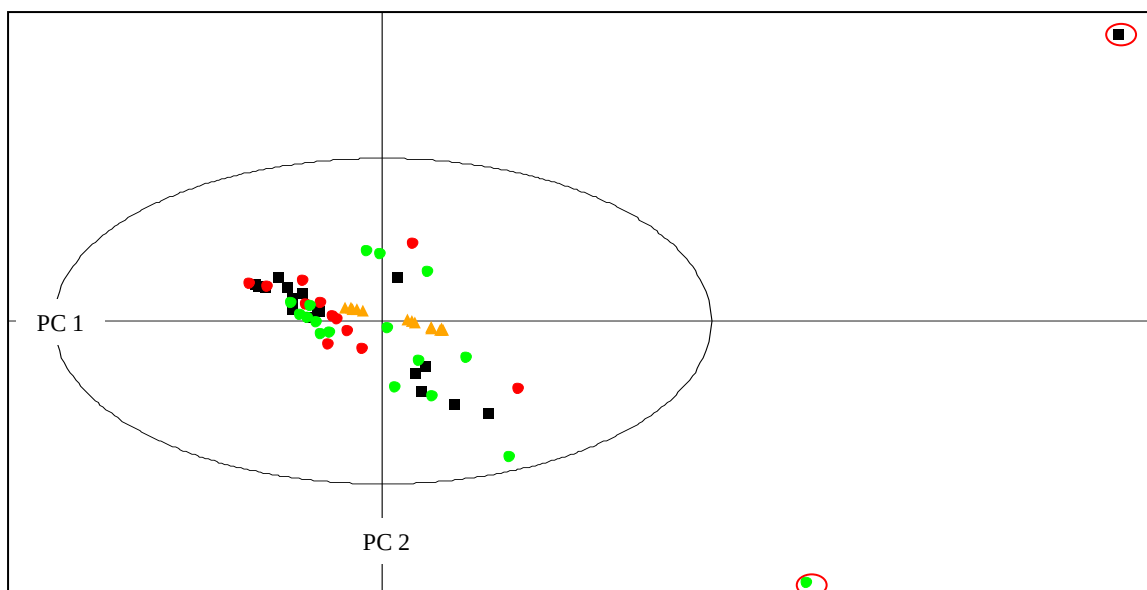


Figure 9-4. Negative ion mode: principal components analysis.

Key: Hepatocellular carcinoma (■), cirrhosis (●), healthy controls (●) and quality controls (▲). Outlying samples are circled in red. PC = principal component.

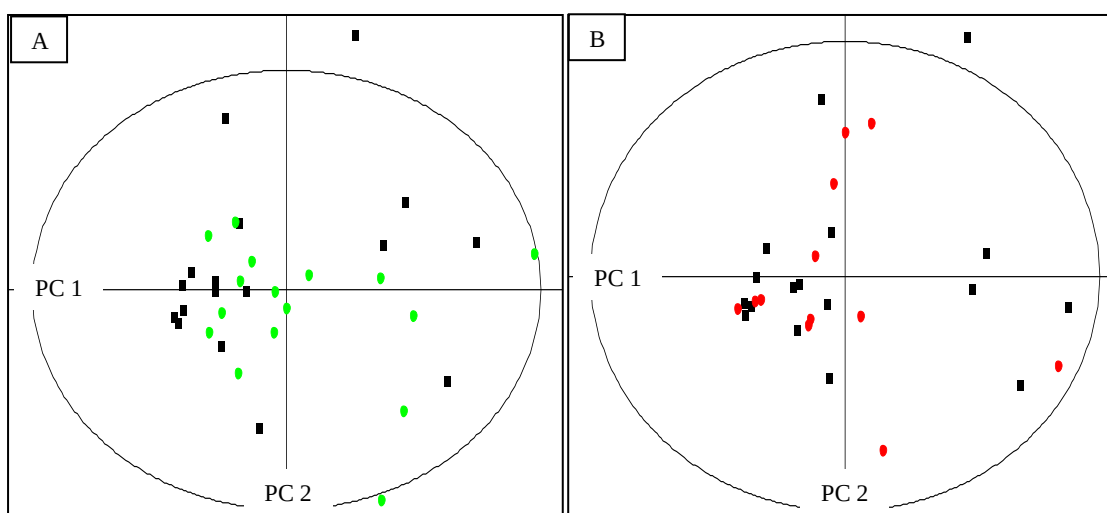


Figure 9-5. Negative ion mode: principal components analysis following exclusions.

Key: Hepatocellular carcinoma (■), healthy controls (●), cirrhosis (●). PC = principal component.

Table 9-8. Negative ion mode discriminatory variables.

HCC versus Healthy Controls	HCC versus Cirrhosis
M357-T235	M187-T258
M243-T61	M173-T203
M187-T258	M410-T280
M527-T236	M243-T61
M410-T280	M189-T185

9.4.3.2 XCMS generated univariate statistical analyses

The data matrix produced by XCMS is ordered in terms of statistical significance, by means of a student's *t*-test, of variables most altered between two designated groups. The five most significant variables for negative mode studies, are displayed in **Table 9-9**.

Table 9-9. Negative ion mode discriminatory variables comparisons generated by XCMS driven univariate analysis.

HCC versus Healthy Controls	<i>p</i>-value	HCC versus Cirrhosis	<i>p</i>-value
M481-T29	<0.001	M957-T194	<0.001
M583-T30	<0.001	M914-T41	<0.001
M335-T31	<0.001	M356-T35	<0.001
M449-T30	<0.001	M700-T39	<0.001
M409-T32	<0.001	M963-T194	<0.001

p-values calculated using student's *t*-test.

9.4.4 Variable identification and authentication

Based on PLS-DA and XCMS data, variables most significantly altered between groups were selected for MS-MS fragmentation studies. These are displayed in **Table 9-10**.

Table 9-10. Variables selected for tandem mass spectrometry studies.

Positive mode	Negative mode
M204-T67	M357-T235
M229-T94	M243-T61
M302-T208	M187-T258
M180-T77	M173-T203

Variables M180-T77, M357-T235 and M243-T61 produced poor chromatographic peaks and fragmentation patterns that could not be identified. Variables M302-T208, M187-T258 and M173-T203 produced distinct chromatographic peaks and fragmentation patterns; however, no match could be identified from databases or from a literature search. Variables M204-T67 and M229-T94 displayed clear chromatographic peaks and fragmentation patterns (**Figures 9-6 and 9-7**). **Figure 9-8** depicts the relative median abundance values for these variables in the three subject groups. Both variables are elevated in patients with HCC, reduced in healthy controls and negligible in patients with cirrhosis.

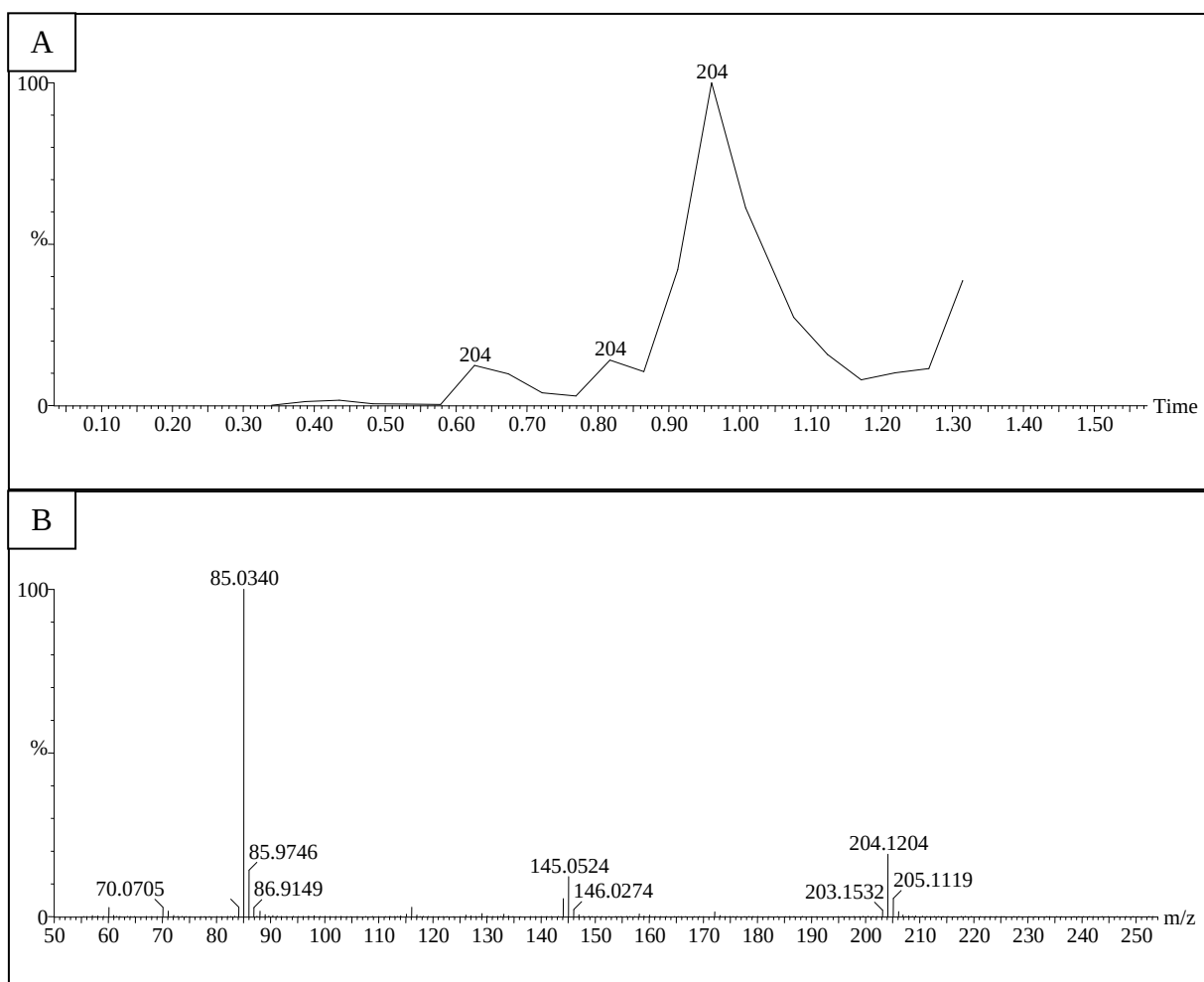


Figure 9-6. Total ion chromatogram and fragmentation spectrum of mass 204.

A. Total ion chromatogram for mass 204 m/z displaying a main elution peak at 0.96 minutes and B. Tandem mass spectrometry fragmentation spectrum of parent mass 204 m/z.

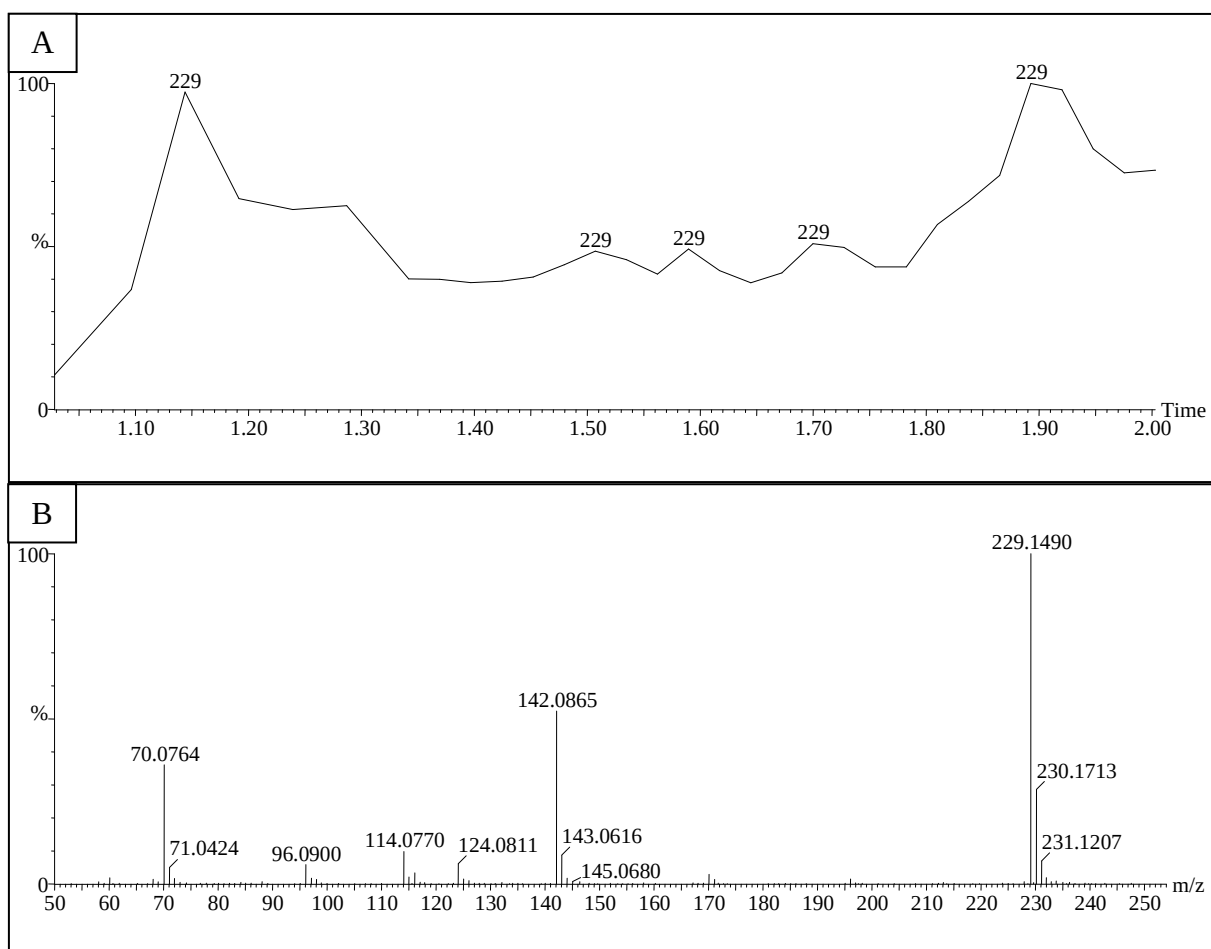


Figure 9-7. Total ion chromatogram and fragmentation spectrum of mass 229.

A. Total ion chromatogram for mass 229 m/z displaying a main elution peak at 1.14 minutes and B. Tandem mass spectrometry fragmentation spectrum of parent mass 229 m/z.

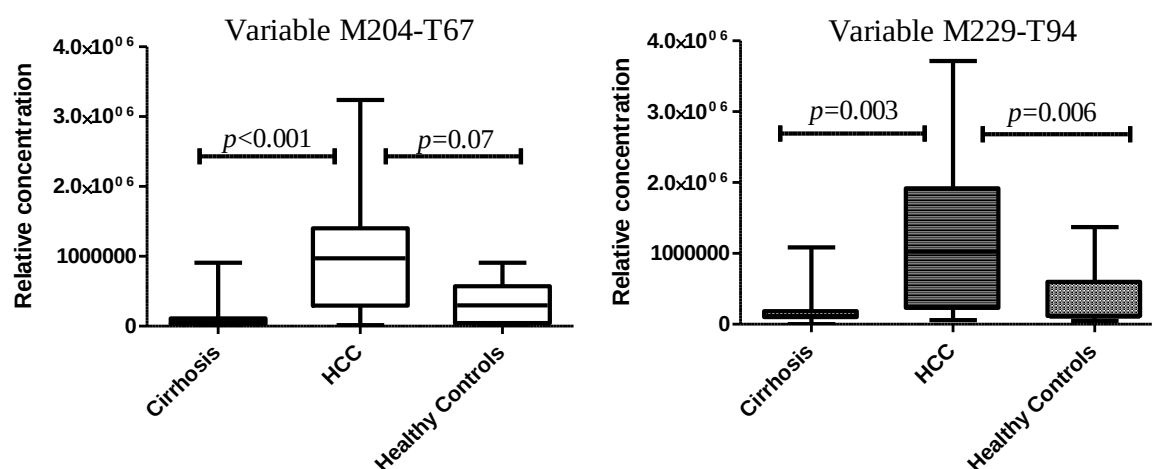


Figure 9-8. Relative concentration of variables M204-T67 and M229-T94.

p-values calculated using Mann-Whitney tests for non-parametric data.

Fragmentation experiments on mass 204 m/z displayed main fragments at 85.034 m/z and 145.05 m/z . Online databases confirmed this to be likely to represent acetylcarnitine, an acetic acid ester of carnitine.

Fragmentation spectra of mass 229 m/z revealed masses at 142.09 m/z , and 70.08 m/z . No match could be identified from online databases. Chen and colleagues, in an RPLC/HILIC MS urine study also identified a mass at 229.15 m/z elevated in patients with HCC which was attributed to leuacylproline, without standard authentication, a proline cycle metabolite (Chen *et al.*, 2009).

9.4.5 Standard authentication

The chromatographic trace and fragmentation pattern of mass 204 m/z is compared to that of an acetylcarnitine standard in **Figure 9-9**. The elution peak of the standard occurs at 1.10 min (66 sec) and is slightly delayed in comparison to the sample which elutes at 1.0 min (60 sec). This is likely to be due to the complex nature and multiple components of the sample, as opposed to the pure compound standard, affecting elution rates. The fragmented mass spectrum of mass 204 is highly correlative in both the standard and the sample, displaying peaks at 145.05 m/z and 85.04/5 m/z , confirming this molecule to be acetylcarnitine.

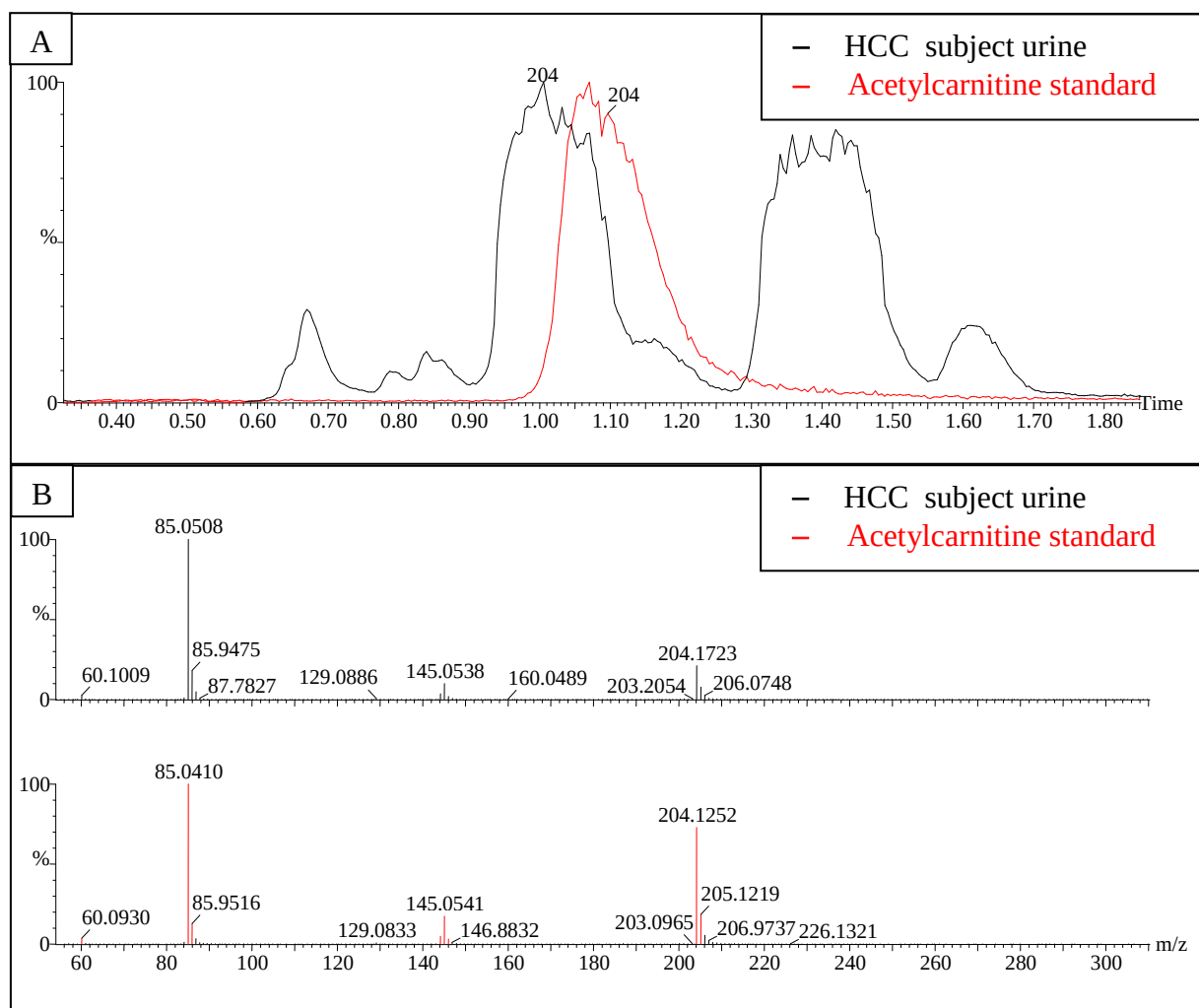


Figure 9-9. Total ion chromatogram and mass spectrum of acetylcarnitine.

A. Total ion chromatogram of mass 204 m/z in urine of subject with hepatocellular carcinoma (black) and authenticated standard (red). B. Mass spectrum of fragmented mass 204 m/z in urine of subject with hepatocellular carcinoma (black) and authenticated standard (red).

9.5 DISCUSSION

In contrast to ^1H NMR spectroscopy, UPLC-MS displays higher sensitivity and detects differing metabolites. Metabolite identification, however, remains more involved, in part owing to lack of comprehensive databases and the more complex nature of ionised metabolites which may be detected whole or fragmented. Given these differences, urine UPLC-MS metabolic profiles, in both positive and negative ion modes, could distinguish patients with HCC from those with cirrhosis and healthy controls with robust PCA and PLS-DA models. The loadings plots and regression vectors for these models and the XCMS generated data, identified influential analytes that were altered in HCC samples. As it is not possible to identify metabolites solely based on TOF-MS readings, and chromatographic retention times will deviate between experiments, fragmentation MS-MS was undertaken. Subsequently, the single metabolite that could be accurately identified and authenticated was acetylcarnitine.

Univariate statistical analysis confirmed that urinary acetylcarnitine (204 Da) was elevated in patients with HCC compared to those with cirrhosis ($p < 0.001$) and healthy controls ($p = 0.07$). These results bear similarity to the urinary levels of carnitine detected by ^1H NMR spectroscopy in both Nigerian (Shariff *et al.*, 2010) and Egyptian studies (Shariff *et al.*, 2011) (**Chapters 6 and 7**). Chen and colleagues also reported elevated carnitine and acetylcarnitine urinary levels in patients with HCC using HILIC and RPLC separations with MS (Chen *et al.*, 2009). The same group reported elevated carnitine and acetylcarnitine in the serum of patients with HCC (Yin *et al.*, 2009). Acetylcarnitine is an acetylated ester of carnitine that is intimately involved in the β -oxidation of fatty acids and transport of acetyl Co-A into the mitochondria for this purpose. Carnitine levels may rise with increased carnivorous consumption. Although dietary histories were not obtained in this Nigerian cohort, given that

the local diet very rarely contains meat, owing to its relatively high cost, it is unlikely that differences in meat consumption are responsible for the above observations. Urinary acetylcarnitine may therefore be a surrogate marker for alterations in oxidation of fatty acids by the body. **Figure 9-10** schematically illustrates fatty acid β -oxidation (Vaz and Wanders, 2002). Long chain fatty acids are present as Co-A esters in the cellular cytosol and are transesterified to carnitine, catalysed by carnitine palmitoyltransferase I (CPT I) allowing the passage of the resultant long chain fatty acid acylcarnitine esters through the outer mitochondrial membrane. The specific carrier protein, carnitine-acylcarnitine translocase (CACT) transports the esters through the inner mitochondrial membrane where they are trans-esterified back to long chain acyl-CoA, catalysed by CPT II. Carnitine that is released can be transported back into the cytoplasm via CACT or acetylated by carnitine acetyltransferase to produce acetylcarnitine which can also enter the cytosol via the CACT transporter.

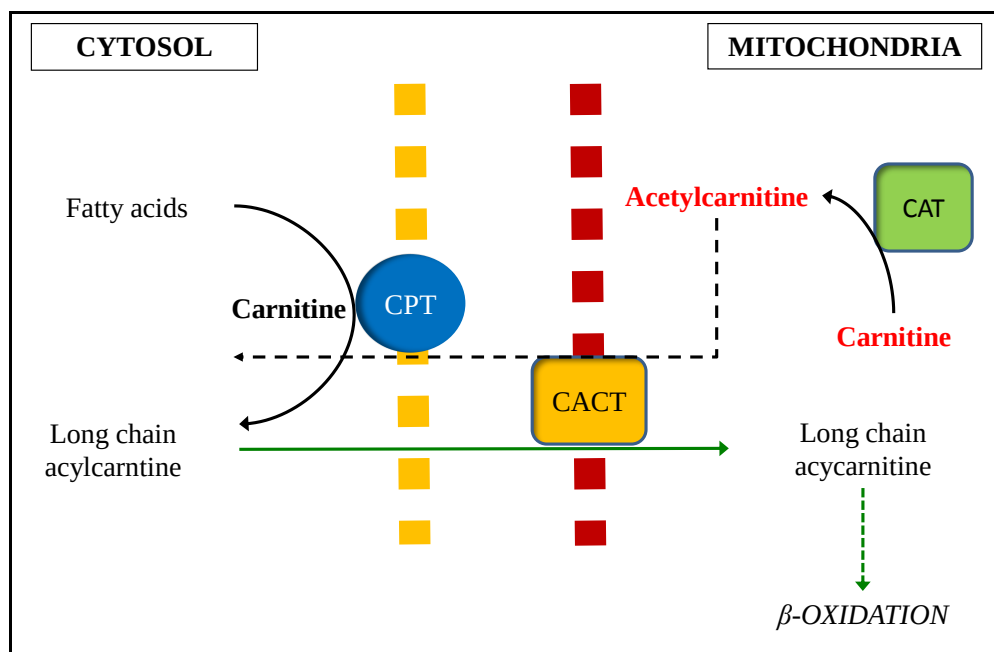


Figure 9-10. Fatty acid β -oxidation and carnitine and acetylcarnitine transport.

Key: CPT= carnitine palmitoyltransferase; CACT= carnitine-acylcarnitine translocase; CAT= carnitine acetyltransferase.

Peroxisome proliferator-activated receptor α (PPAR α) activation is an established mechanism of fatty acid oxidation stimulation through the induction of genes encoding proteins involved in the transport and synthesis of acylcarnitine. Patterson and colleagues administered fenofibrate, a PPAR α agonist, to healthy human volunteers for 14 days (Patterson *et al.*, 2009). Using UPLC-MS, urine was analysed at intervals and displayed depleted levels of acetylcarnitine by day 7, suggesting that increased fatty acid oxidation results in depleted urinary levels of acetylcarnitine. It is therefore theoretically possible that increased urinary acetylcarnitine is an indicator of decreased fatty acid oxidation. A mechanistic pathway of suppression of fatty acid acid oxidation in tumour cells was suggested by Debarardinis and colleagues in a study of interleukin-3-deprived hematopoietic cells. In this study, it was observed that phosphatidylinositol 3-kinase and Akt signalling, genes which have previously been found to be constitutively active in gastrointestinal and ovarian malignancies, suppressed expression of CPT I, thereby reducing the transport of fatty acids into the mitochondria for β -oxidation (Deberardinis *et al.*, 2006). They proposed that decreased fatty acid oxidation allows fatty acids to be available as building blocks for phospholipids in cell membranes of the rapidly proliferating tumour. This is certainly an attractive notion as it also gives basis to the theory proposed by Weinberg and Chandel that rather than oxidative phosphorylation being subjugated for wholly energy demand reasons, it occurs for the provision of glycolytic intermediates to the pentose phosphate pathway to provide nucleotide substrates for the growing tumour (Weinberg and Chandel, 2009). In the same manner, repression of β -oxidation provides fatty acids for the construction of cell membranes (**Figure 9-11**). Indeed, in the advanced stages of malignancy, energy reserves are low, so a reasonable explanation must exist as to why β -oxidation of fatty acids is suppressed.

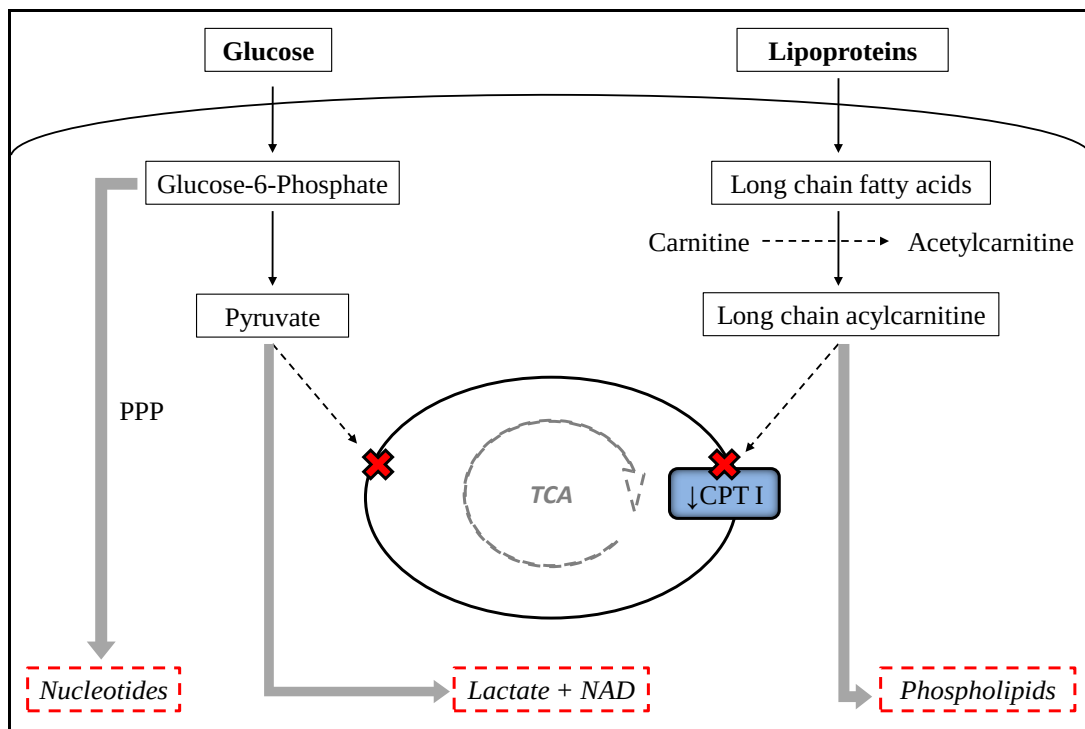


Figure 9-11. Altered glycolytic and fatty acid metabolism in tumour cells for the generation of cellular substrates.

Key: PPP= pentose phosphate pathway; TCA= tricarboxylic acid cycle; CPT I= carnitine palmitoyltransferase I.

9.6 CONCLUSIONS

This study has validated that UPLC-MS urinary metabolic profiles could generate metabolic profiles capable of distinguishing between patients with HCC from healthy controls and patients with cirrhosis. These data compliment the matched ^1H NMR spectroscopy study in confirming elevation in urinary carnitine molecules, in this case, acetylcarnitine. This result provides insight into the altered energy metabolism processes of HCC. In particular, it may suggest a subjugation of fatty acid metabolism to provide lipid building blocks for rapidly dividing tumour cells. Unfortunately, no further metabolites could be identified and this highlights the disadvantage of UPLC-MS. Despite its higher sensitivity, in comparison to ^1H NMR spectroscopy identification of metabolites is far more involved and of lower yield. Nevertheless, acetylcarnitine, which is distinguishable from carnitine, was not detected by ^1H NMR spectroscopy due to its lower abundance in urine. This strengthens the argument of the complementary nature of these two modalities in identifying as many discriminatory metabolites as possible.

10 THE URINARY METABOLIC PROFILING OF HEPATOCELLULAR CARCINOMA USING ULTRA PERFORMANCE LIQUID CHROMATOGRAPHY MASS SPECTROMETRY IN A UK POPULATION

10.1 ABSTRACT

Introduction: The study presented here describes UPLC-MS urinary metabolic profiling of UK patients with HCC and cirrhosis, in an attempt to validate previous findings. **Methods:** Urine sample chromatography was performed using a 1.7 μm high strength silica column. Mass spectrometry was performed in both positive and negative ionisation modes. Data were processed using XCMS, multivariate and univariate statistical analysis. Molecular species were identified using tandem mass spectrometry fragmentation experiments. **Results:** Following exclusions, 15 samples from aetiologically diverse patients with HCC, 24 (positive mode) and 26 (negative mode) samples from patients with cirrhosis were used for analyses. Multivariate urinary metabolic profile models displayed diagnostic sensitivity and specificity values of 80% and 95.8% of HCC diagnosis in positive mode and 93.3% and 96.2% in negative mode. Discriminatory urinary metabolites included elevated acetylcarnitine ($p=0.83$), reduced dimethylheptanoylcarnitine ($p=0.097$) and *p*-cresol sulphate ($p=0.05$). **Conclusions:** This study has provided evidence that UPLC-MS can identify a discriminatory urinary metabolic profile between patients with HCC and those with cirrhosis induced by a highly diverse range of aetiologies. This would suggest HCC-associated urinary metabolic changes are independent of the underlying disease. Further evidence was also provided of the role perturbed fatty acid oxidation and altered gut microbial activity, in the form of altered carnitine and *p*-cresol sulphate levels.

10.2 INTRODUCTION

In the previous chapters, UPLC-MS urinary metabolic profiling in a Nigerian population identified elevated acetylcarnitine as a discriminatory molecule. The population in this Nigerian study was aetiologically homogeneous, being mostly HBV infected. The findings reported in the study described in this chapter are of patients recruited in the UK, the same group that were enrolled into the parallel ^1H NMR study (**Chapter 8**). The aetiologies of these UK patients were more diverse, including alcohol and NASH induced liver disease. Furthermore, the control group selected is composed entirely of patients with cirrhosis matched for liver disease stage and aetiology.

The primary aim of this study was to identify HCC urinary biomarkers that are tumour specific and not as a result of liver disease aetiology and severity. The secondary aim was to identify any corroboratory findings to previous studies from other populations.

10.3 METHODS

10.3.1 Patient selection

Samples from patients recruited for the UK urinary ^1H NMR study (**Chapter 8**) were used for parallel UPLC-MS studies. All patients gave written informed consent in accordance with local Research Ethics Committee approval. Patients with HCC had been diagnosed with two confirmatory imaging modalities and patients with cirrhosis with biopsy or US, MRI or CT confirmation. Patients with HCC were staged according to the Barcelona Clinic Liver Cancer (BCLC) staging system (Bruix and Sherman, 2005). Patients with cirrhosis were staged according to the Child-Pugh staging system (Child and Turcotte, 1964; Pugh *et al.*, 1973).

10.3.2 Blood laboratory tests

Serum AFP, creatinine, ALT, total bilirubin, albumin and INR levels were measured at St Mary's Hospital, London as previously described (**Chapter 5**). Median and interquartile ranges (IQR) were calculated for each assay and median levels were compared using unpaired Mann-Whitney tests of significance.

10.3.3 Urine sample collection and preparation

15 mL of random urine was collected from each volunteer and prepared for storage at -80°C as described previously. 150 μL urine samples were prepared for UPLC-MS as described in **Chapter 9**. 5 μL of each urine sample were pooled to constitute three QC samples which were run at the beginning (the initial 10 runs), after every 10th sample and at the end of the experiment, in both positive and negative ion modes. Samples were run in random, non-grouped order.

10.3.4 Chromatographic conditions

Chromatographic separations were performed on a Waters 2.1mm x 100mm ACQUITY™ 1.7 μ m HSS T3 column (Elstree, Herts, UK) maintained at 40 °C in an ACQUITY™ UPLC system (Waters MS Technologies, Manchester, UK) . The samples were maintained at 4 °C prior to injection of 5 μ L onto the column. A 12 mins gradient was employed with mobile phase A of 0.1a% formic acid (FA) in water and mobile phase B 0.1% FA in acetonitrile. The composition was increased linearly from 0 to 15% B over 1 min, then to 50% B over the next 2 mins, 95% B over the next 3 mins, remained 95% B until 9.9 mins then returned to 100% A over 0.2 min, and then re-equilibrated over the next 1.9 mins prior to injection of the next sample.

10.3.5 Mass spectrometry

Mass spectrometry experiments were performed on a Micromass LCT Premier™ (Waters MS Technologies, Manchester, UK) with ESI operated in both positive and negative modes. Data were collected in positive and negative ion mode centroid mode, the lockspray frequency was set at 200 s, and data were averaged over 10 scans. The mass spectrometer was operated with a capillary voltage of 2.4 kV for negative mode and 3.2 kV for positive ion mode, cone voltage of 35 V, nebulizer gas flow of 900 L/h, desolvation temperature of 350 °C, and source temperature of 120 °C. The instrument was set to acquire over the mass range m/z 50-1000 with acquisition time of 5 scans s^{-1} and an interscan delay of 100 ms.

10.3.6 Tandem mass spectrometry and feature identification

Tandem mass spectrometry (MS-MS) was performed on a Quadrupole Time of Flight (QTOF) Premiere™ instrument (Waters MS Technologies, Manchester, UK).

Chromatographic conditions were the same as preceding MS experiments and survey scans were performed for masses identified through multivariate analysis. A collision ramp was set for the argon filled collision cells from 5 – 30 eV to ensure passage and fragmentation of the parent ion selected by the quadrupole. Fragmentation patterns and retention times were then interrogated to identify candidate molecules using online databases for identification (<http://www.hmdb.ca/> and http://metlin.scripps.edu/metabolites_list.php).

10.3.7 Data pre-processing

Data underwent peak-picking, retention time correction and grouping using XCMS (Scripps Center for Mass Spectrometry, La Jolla, California, USA) (Smith *et al.*, 2006) as previously described (**Chapter 5**).

10.3.8 Multivariate analysis

The data matrix was exported to SIMCA (Umetrics, Umea, Sweden) for PCA and PLS-DA. All data were mean centred prior to multivariate analysis and outliers were excluded on the basis of exceeding the Hotelling's T^2 distance and causing heavy bias to the loadings components.

10.3.9 Univariate analysis

Data were exported to GraphPad Prism (La Jolla, California, USA) for univariate analysis in the form of Mann-Whitney t-tests comparison of medians between groups, assuming non-parametric distribution of data. Fisher's exact test was used to compare categorical data.

10.4 RESULTS

10.4.1 Subject recruitment, exclusion and demographics

A total of 50 patients were recruited, comprising two groups: 19 with HCC and 31 with cirrhosis. Prior to analysis, three patients were excluded from the HCC group: one who had undergone curative liver transplantation and thus no longer had HCC; a second who had undergone curative hepatic resection and a third who was co-infected with human immunodeficiency virus (HIV), the safety protocols at the department of biomolecular medicine prohibiting the analysis of HIV infected samples. From the positive and negative mode studies, a further sample was excluded following multivariate analysis from both ion mode data, the details of which are given below, leaving a total of 15 HCC samples. From the cirrhosis group, four patients were excluded prior to analysis, owing to insufficient radiological or biopsy evidence of cirrhosis; three samples were excluded following multivariate analysis of positive mode data, and one excluded after analysis of negative mode data, leaving a total of 24 and 26 in positive and negative mode groups, respectively. Subject samples used for the parallel ^1H NMR study (**Chapter 8**) were almost the same as those used for this study. As a means of comparison, **Table 10-1** details the subject demographics from each study.

Subject demographics for positive and negative mode studies are displayed in **Tables 10-2 and 10-3**. These data are directly comparable to multivariate analysis data as samples that were outlying according to PCA were excluded from both subsequent multivariate analysis and baseline demographics. Different samples were outlying in the two ion models; therefore two demographic data tables are displayed.

Table 10-1. Comparisons of subjects recruited for ^1H NMR and UPLC MS studies.

	HCC		Cirrhosis	
	^1H NMR	UPLC MS	^1H NMR	UPLC MS
<i>N</i>	13	15	25	26
Median age (yrs)	61	61	58	60
Male <i>n</i>	11/13	12/15	14/25	17/26
HCV	6	7	6	7
HBV	3	3	3	4
Alcohol	2	3	9	9
NASH	0	0	2	2
HCV + Alcohol	1	1	2	2
HBV + Alcohol	0	0	0	0
NASH + Alcohol	0	0	1	1
Haemochromatosis	1	1	1	1
Idiopathic	0	0	1	0
Median AFP	18.1	21	4.6	4.9

Table 10-2. Demographic profiles for samples used in positive ion mode.

Characteristic	HCC	Cirrhosis	p-value
<i>N</i>	15	26	-
Median age (range) (years)	61	60	0.9 ^a
Male n (%)	12 (80%)	17 (65%)	0.48 ^b
Median body mass index (kg/m ²)	26.3	27.2	0.24 ^a
Child-Pugh A	5 (33%)	17 (65%)	-
Child-Pugh B	4 (27%)	5 (19%)	-
Child-Pugh C	1 (7%)	2 (8%)	-
Non-cirrhotic	2 (13%)	0	-
Unknown due to missing data	3 (20%)	2 (8%)	-

Key: ^aMann-Whitney test for non-parametric data; ^bFisher's exact test for categorical data.

Table 10-3. Demographic profiles for samples used in negative ion mode.

Characteristic	HCC	Cirrhosis	p-value
<i>N</i>	15	24	-
Median age (range) (years)	61	60	0.9 ^a
Male n (%)	13/15 (87%)	15/24 (63%)	0.15 ^b
Median body mass index (kg/m ²)	26.3	26.5	0.42 ^a
Child-Pugh A	5 (33%)	16 (67%)	-
Child-Pugh B	4 (27%)	4 (17%)	-
Child-Pugh C	1 (7%)	2 (8%)	-
Non-cirrhotic	2 (13%)	0	-
Unknown due to missing data	3 (2%)	2 (8%)	-

Key: ^aMann-Whitney test for non-parametric data; ^bFisher's exact test for categorical data.

In both groups, median age and BMI were similar: 61 years and 26.5 kg/m² in the HCC groups; 60 years and 26.5 – 27.2 kg/m². There were proportionally more males in the HCC groups but not significantly ($p=0.48$ and 0.15). The majority of both cohorts consisted of Child-Pugh stage A and B patients (60% for HCCs and 84% for patients with cirrhosis).

Aetiological factors are displayed in **Table 10-4 and 10-5** and graphically, for positive mode samples, in **Figure 10-1**.

Table 10-4. Positive ion mode aetiological factors.

Aetiology	HCC	Cirrhosis
HCV	7 (46%)	7 (29%)
HBV	3 (20%)	4 (17%)
Alcohol	3 (20%)	8 (33%)
NASH	0	2 (8%)
Haemochromatosis	1 (7%)	1 (4%)
HCV + Alcohol	1 (7%)	1 (4%)
HBV + Alcohol	0	0
NASH + Alcohol	0	1 (4%)
Idiopathic	0	0
Total	15	24

Table 10-5. Negative ion mode aetiological factors.

Aetiology	HCC	Cirrhosis
HCV	7 (46%)	7 (27%)
HBV	3 (20%)	4 (15%)
Alcohol	3 (20%)	9 (35%)
NASH	0	2 (8%)
Haemochromatosis	1 (7%)	1 (4%)
HCV + Alcohol	1 (7%)	2 (8%)
HBV + Alcohol	0	0
NASH + Alcohol	0	1 (4%)
Idiopathic	0	0
Total	15	26

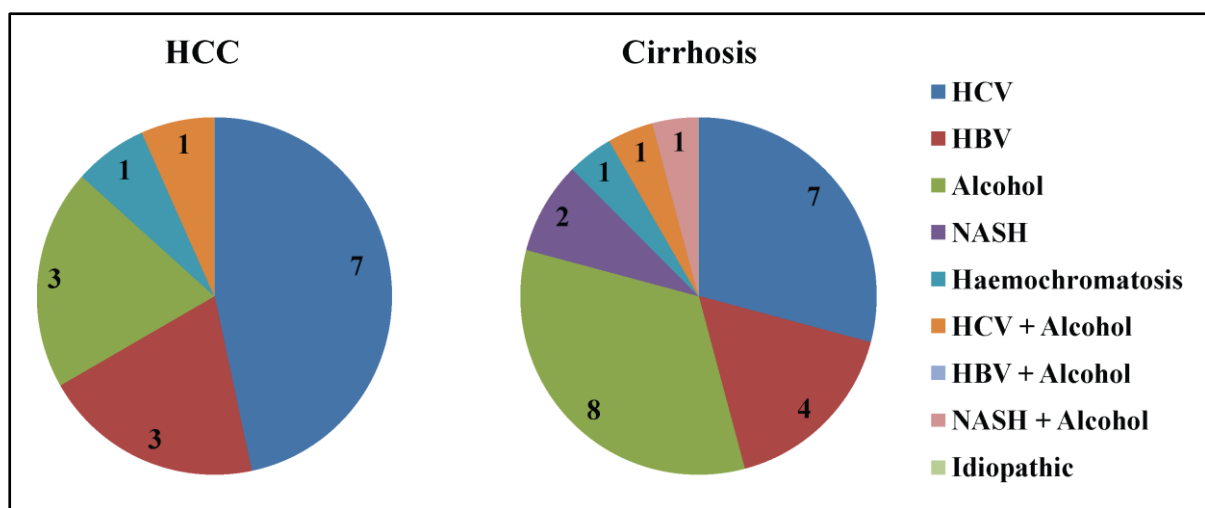


Figure 10-1. Pie chart of aetiological factors (positive mode).

HCV and alcohol induced liver disease were the major aetiological factors for both groups accounting for 66-73% of patients in all ion mode groups and patient cohorts. BCLC stages of HCC are depicted in **Table 10-6**.

Table10-6. Positive ion mode Barcelona Clinic Liver Cancer staging.

Stage	Positive mode (n)	Negative mode (n)
A	5	5
B	0	0
C	7	7
D	2	2
Unknown due to missing data	1	1
Total	15	15

10.4.2 Serum biochemical analysis

Patient cohort biochemical profiles are displayed in **Tables 10-7 and 10-8**, displaying similar biochemical profiles in HCC and cirrhosis groups, the only significant differences occurring in median serum albumin (35 gL^{-1} in cirrhosis and 30 gL^{-1} in HCC cohorts, $p=0.02$) and AFP (4.9 IU mL^{-1} in cirrhosis and 21 IU mL^{-1} in HCC cohorts, $p < 0.01$).

Table 10-7. Serum biochemistry profiles for samples used in positive ion mode.

	HCC	Cirrhosis	<i>p</i> -values
	Median (range)	Median (range)	
Serum Samples (<i>n</i>)	15	24	-
AFP (IU mL⁻¹)	21 (5.7-1230) ⁺⁺	4.9 (1.1-29.2) ⁺⁺⁺	<0.01
Creatinine (mmol L⁻¹)	77.5 (59-96) ⁺⁺	71 (48-132) ⁺	0.22
ALT (IU L⁻¹)	45 (7-147) ⁺⁺	35 (8-152) ⁺	0.37
Bilirubin (μmol L⁻¹)	11 (5-168) ⁺⁺	17 (6-87) ⁺	0.37
Albumin (g L⁻¹)	30 (22-40) ⁺⁺	35 (19-46) ⁺⁺	0.02
INR (IU)	1.1 (1-2) ⁺⁺⁺	1.2 (1-3.1) ⁺⁺	0.75

Key: Mann-Whitney test was used for non-parametric data, ⁺2 measurements missing, ⁺⁺3 measurements missing, ⁺⁺⁺4 measurements missing.

Table 10-8. Serum biochemistry profiles for samples used in negative ion mode.

	HCC	Cirrhosis Median	<i>p</i> -values
	Median (range)	(range)	
Serum Samples (<i>n</i>)	15	26	-
AFP (IU mL⁻¹)	21 (5.7-1230) ⁺⁺	4.9 (1.1-29.2) ⁺⁺⁺	<0.01
Creatinine (mmol L⁻¹)	77.5 (59-96) ⁺⁺	70 (48-132) ⁺⁺	0.21
ALT (IU L⁻¹)	44 (7-147) ⁺⁺⁺	35 (8-152) ⁺	0.43
Bilirubin (μmol L⁻¹)	11 (5-166) ⁺⁺	16.5 (6-87) ⁺	0.36
Albumin (g L⁻¹)	30 (22-40) ⁺⁺	35 (19-46) ⁺⁺	0.02
INR (IU)	1.1 (1-2) ⁺⁺⁺	1.2 (1-3.1) ⁺⁺⁺	0.7

Key: Mann-Whitney test was used for non-parametric data, ⁺2 measurements missing, ⁺⁺3 measurements missing, ⁺⁺⁺4 measurements missing.

The sensitivity of serum AFP, at all cut-off values, was poor, at best identifying only 46.2% of HCCs using a cut-off value of 20 IU mL⁻¹. This dropped to 23.1% using a cut-off value of 200 IU mL⁻¹. Specificity was high, at 95 - 100% for all cut-offs values (**Table 10-9**).

Table 10-9. Serum alpha-fetoprotein diagnostic sensitivity and specificity.

	Sensitivity	Specificity
AFP cut off 20 (IU mL⁻¹)	46.2	95
AFP cut off 200 (IU mL⁻¹)	23.1	100
AFP cut off 400 (IU mL⁻¹)	23.1	100

10.4.3 Chromatographic data

Representative total ion chromatograms from positive and negative ion chromatograms are displayed in **Figure 10-2**, displaying the variation in metabolite elution times between ESI modes and patient groups.

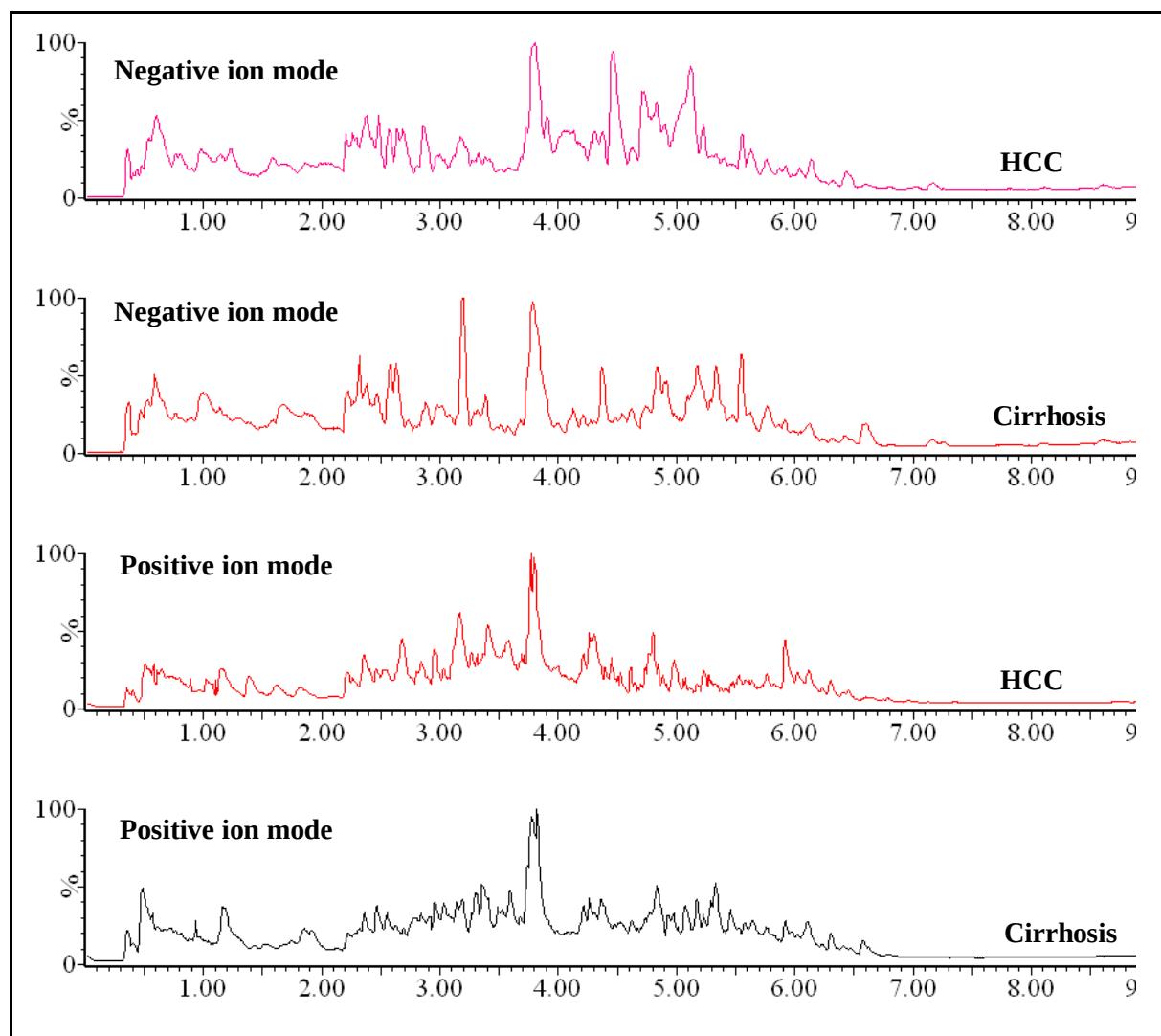


Figure 10-2. Representative positive and negative mode total ion chromatograms.

10.4.4 Multivariate statistical analysis

Principal components analysis of positive ion mode data identified three cirrhosis and one HCC outlying samples which heavily biased principal components. Examination of the loadings plots and the individual contributions plots of these samples identified features M 536 T293, M310 T316 and M172 T197 as major contributory features, elevated in these samples, causing them to be outliers. Given that the concentration of these features was unrepresentatively raised in these samples and not contributory to diagnostic biomarker profiles, their identity was not investigated further. Subsequent PCA is shown in **Figure 8-3**, displaying excellent clustering of QC samples indicating minimal retention time drift during the run.

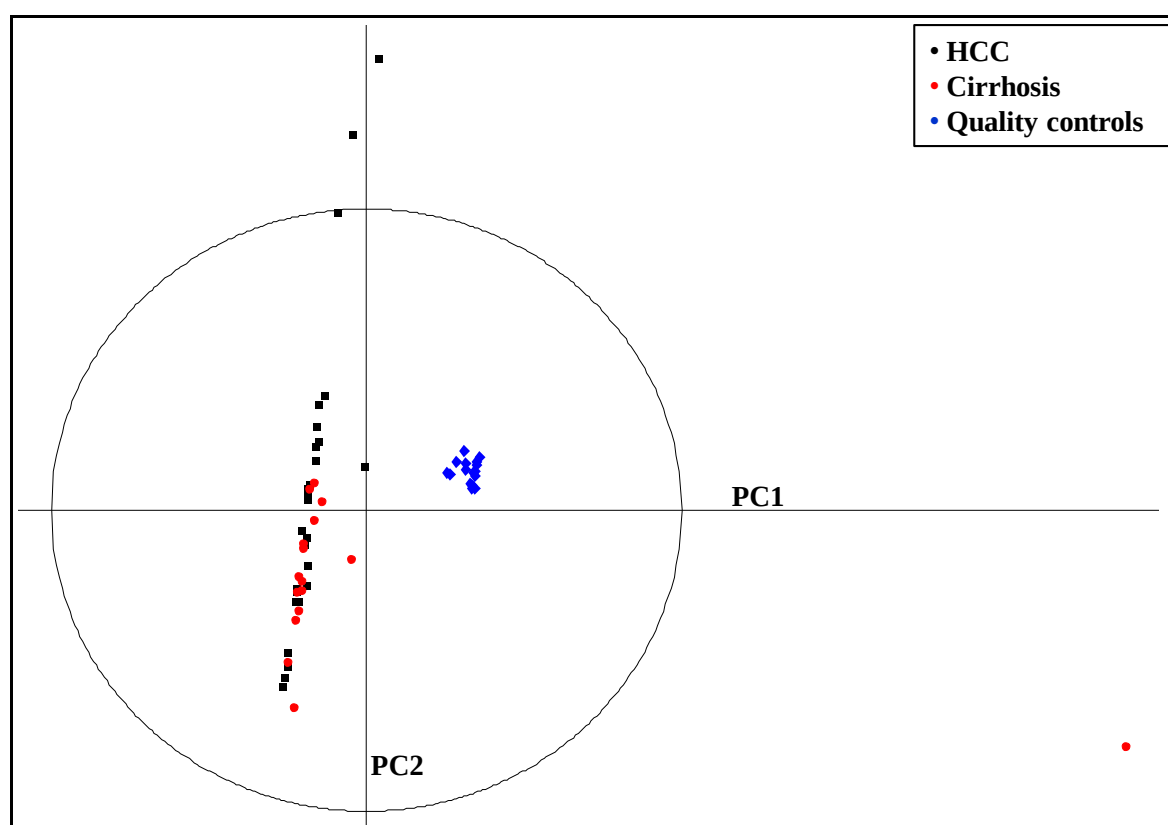


Figure 10-3. Principal components analysis of UK urine positive ion mode data.

Two group PCA models are displayed in **Figure 10-4**. In positive mode, little clustering is seen with poor R^2 and Q^2 values (0.3 and 0.04). In negative mode improved clustering is observed with only marginally improved R^2 and Q^2 values (0.4 and 0.04).

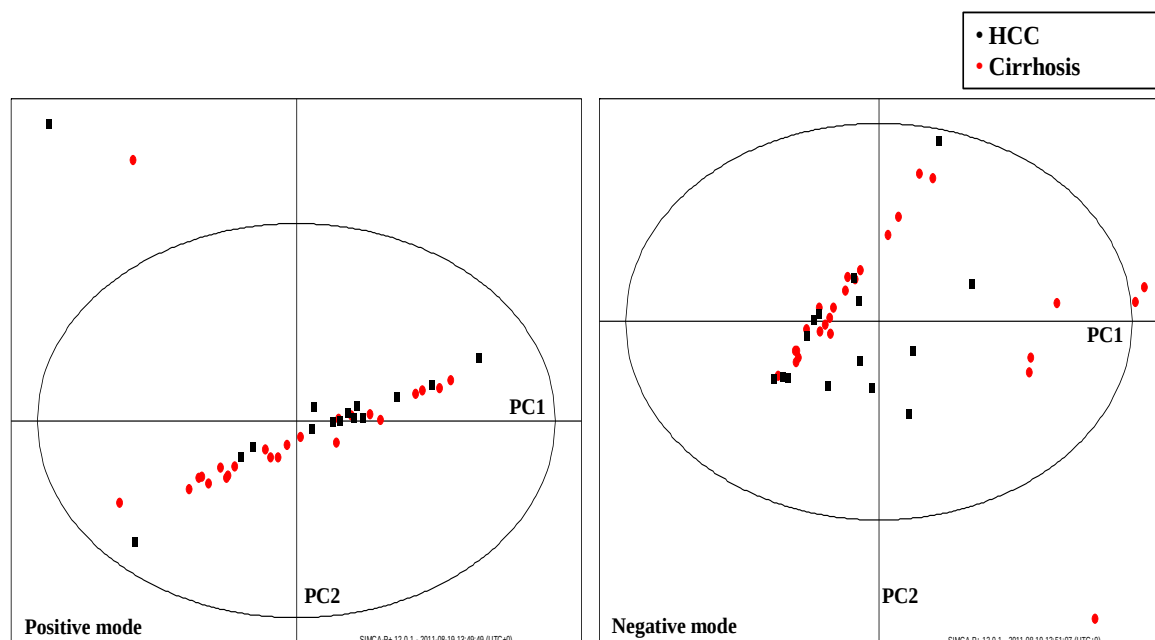


Figure 10-4. Principal components analysis of UK urine positive and negative mode data.

Partial least squared discriminant analyses, using 7-fold cross validation, revealed poor model parameters for positive mode data ($R^2 = 0.2$, Q^2 negative). The misclassification matrix of this model is displayed in **Table 10-10** displaying a sensitivity and specificity of 80% and 95.8% of HCC diagnosis. Negative mode data PLS-DA recorded improved model parameters ($R^2 = 0.6$, $Q^2 = 0.2$) with a sensitivity and specificity of 93.3% and 96.2% (**Table 10-11**).

Table 10-10. Positive ion mode misclassification matrix.

	Predicted as HCC	Predicted as Cirrhosis	
HCC	12	3	Sensitivity = 80%
Cirrhosis	1	23	Specificity = 95.8%

Table 10-11. Negative ion mode misclassification matrix.

	Predicted as HCC	Predicted as Cirrhosis	
HCC	14	1	Sensitivity = 93.3%
Cirrhosis	1	25	Specificity = 96.2%

Analysis of the loadings plots of these models identified a number of features that were highly contributory to the model. The extracted ion chromatogram for each of these features was inspected to ensure that the peak detection of XCMS had genuinely detected a peak. A signal to noise ratio of 10 was used as cut off for detection of true peaks. Features selected are displayed in **Table 10-12** alongside fragmentation data and probable identities based on fragmentation patterns.

Table 10-12. Discriminatory features identified from multivariate analysis.

m/z feature	Mode	Fragment Da (abundance)	ID
M204 T61	Positive	85 (100), 145.1 (10), 60.1 (5)	Acetylcarnitine
M265 T229	Positive	130.1 (100), 84.1 (70), 91.1 (50), 136.1 (20)	No match
M286 T297	Positive	85 ((100)	No match
M302 T334	Positive	85.1 (100), 243.2 (10)	Dimethylheptanoylcarnitine
M310 T316	Positive	85 (100), 121.1 (10), 79.1 (5)	No match
M187 T253	Negative	107 (100)	p-cresol sulfate

10.4.5 Tandem mass spectrometry and univariate analysis

Features selected from PLS-DA were subjected to tandem mass spectrometry and resultant fragmentation patterns were used for identification. Candidate features are displayed in **Table 10-12** alongside fragmentation data and probable identities. In positive mode, five features were identified and fragmented. Of note, four of these masses produced an 85 Da fragment, characteristic of carnitine compounds, as shown for acetylcarnitine in **Chapter 9**. Two features could be identified as acetylcarnitine and dimethylheptanoylcarnitine ($C_{16}H_{31}NO_4$), a long chain acylcarnitine. Fragmentation patterns of these molecules are displayed in **Figure 10-5**.

In negative ion mode, one identifiable molecule was detected, M187 T253. On tandem mass spectrometry studies, this fragmented to masses at 187 Da, 107 Da and 79 Da, a typical fragmentation pattern of *p*-cresol sulphate (Want *et al.*, 2010) (**Figure 10-6**).

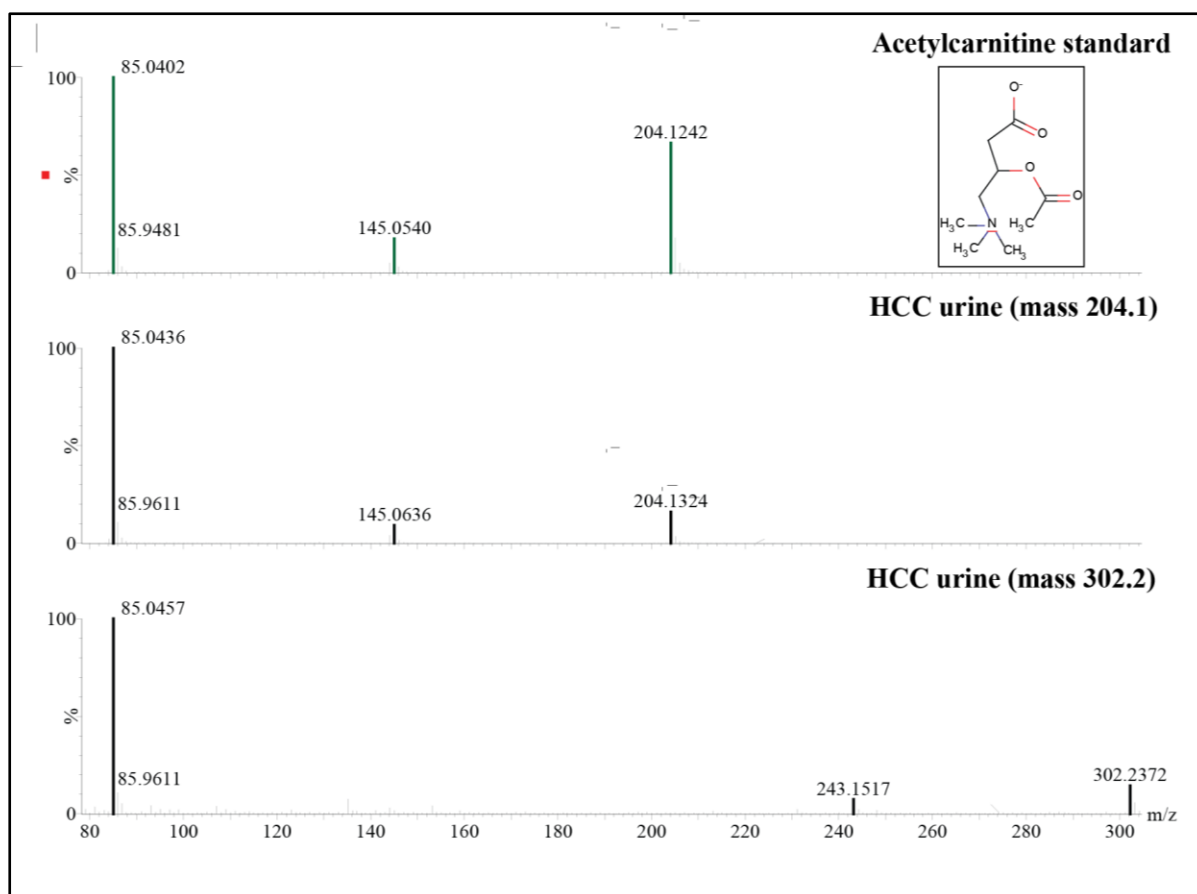


Figure 10-5. Mass spectra of acetylcarnitine standard compared to subject samples.

Key: Acetylcarnitine standard (top), feature M204 T61 from urine sample (middle) and feature M302 T334 (bottom).

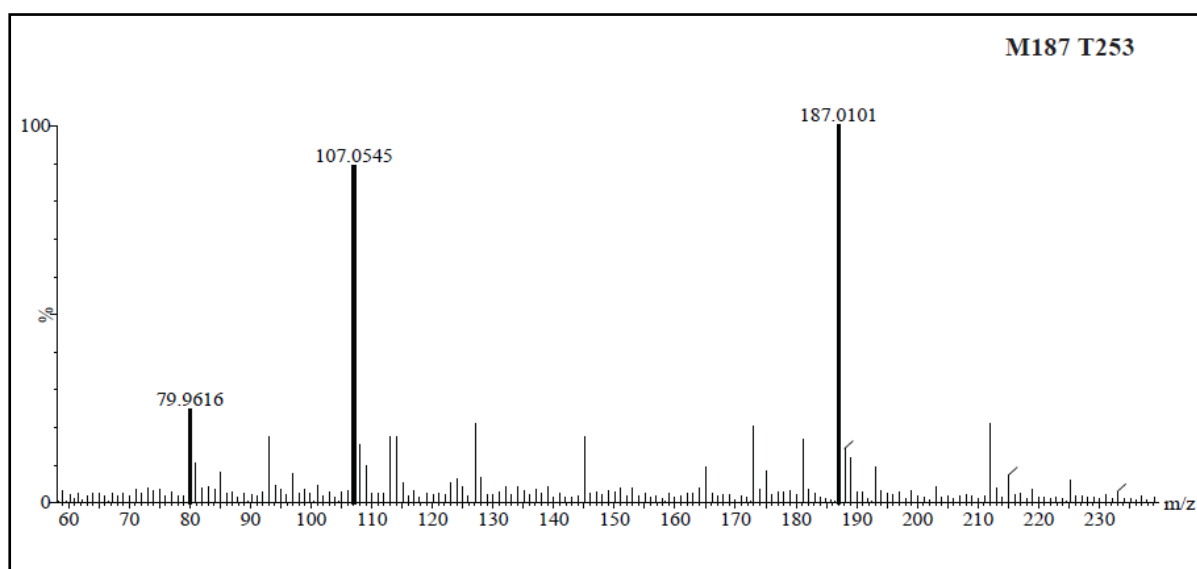


Figure 10-6. Mass spectra of feature M187 T253, likely to be *p*-cresol sulphate.

10.4.6 Univariate statistical analysis

The relative intensity levels for features M204 T61 and M302 T334 were compared for each group. Median levels were calculated and compared and these are represented as scatter bar charts in **Figure 10-7**. In positive mode, feature M204 T64 which represents acetylcarnitine, was elevated in the urine of patients with HCC, but not significantly ($p=0.83$). M302 T334, likely representing dimethylheptanoylcarnitine, was reduced, again not significantly ($p=0.097$). In negative mode, M187 T253, which is likely to represent *p*-cresol sulphate, was significantly reduced in the urine of patients with HCC ($p=0.05$). This finding is in agreement with the parallel ^1H NMR study which identified reduced urinary *p*-cresol sulphate in HCC patients, reported in **Chapter 8**.

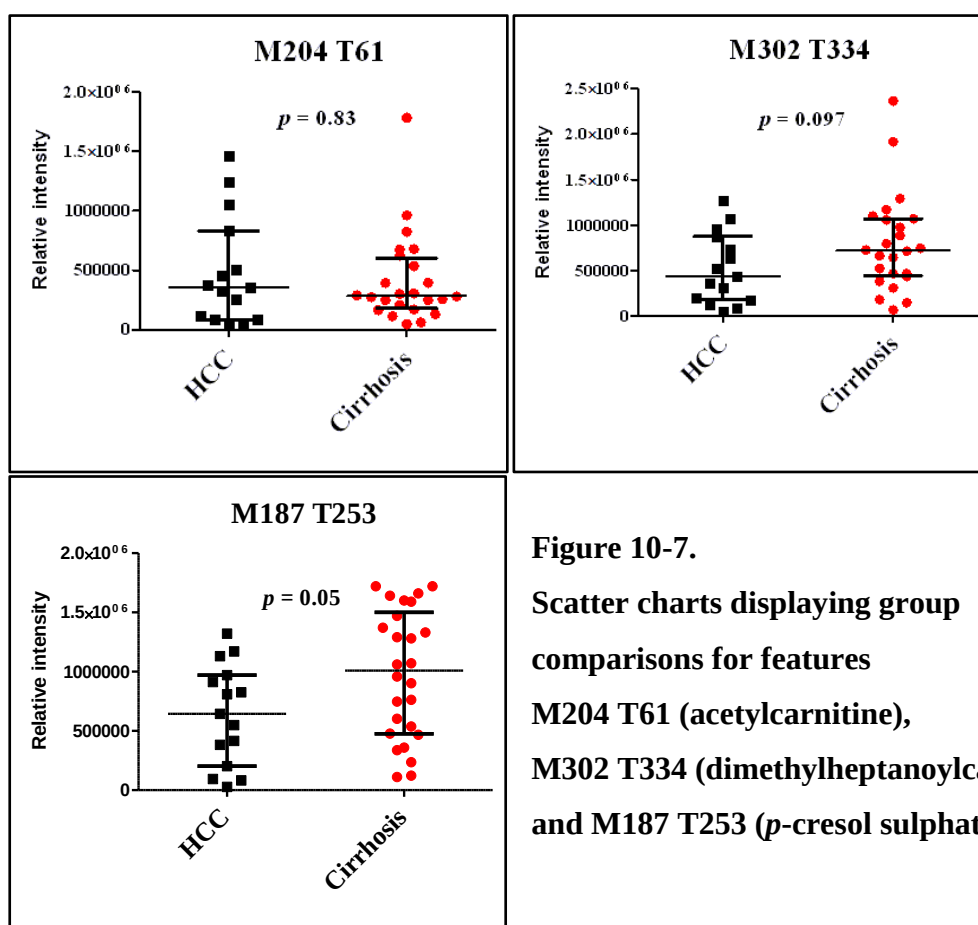


Figure 10-7.
Scatter charts displaying group comparisons for features M204 T61 (acetylcarnitine), M302 T334 (dimethylheptanoylcarnitine) and M187 T253 (*p*-cresol sulphate).

10.5 DISCUSSION

This pilot study has provided a number of informative findings relevant to the metabolic profiling of HCC. Firstly, there are no reported similar studies in a UK, European or US population and these results, therefore, provide novel insight into the urinary metabolic changes in a developed world population. It has also provided evidence that UPLC-MS can identify a discriminatory urinary metabolic profile between patients with HCC and those with cirrhosis induced by a highly diverse range of aetiologies, including alcohol and NASH induced liver disease. This would suggest that regardless of aetiology, alterations in metabolism are tumour-induced rather than aetiologically induced.

Secondly, multivariate analysis, in the form of cross validated PLS-DA provided predictive models for HCC diagnosis which out-performed serum AFP in terms of sensitivity (80% - 93.3% versus 23.1% - 46.2%). This result contrasts to that of the Nigerian population in which AFP was elevated, above 200 IU L⁻¹, in all HCC patients. This is not surprising given the knowledge that flares of chronic HBV, even without HCC, may cause a rise in serum AFP. Given the majority of tumours in this study occurred on a background of HCV or alcohol induced liver disease, it is expected that many of them will be non- secreting tumours. It is poorly understood why certain aetiologies encourage AFP secreting tumours and others do not. This may be as a result of the originator cell of the tumour, perhaps in the presence of HBV, being less differentiated and therefore more akin to the fetal state. It would appear, however, that urinary metabolic profiles are less affected by liver disease aetiology.

Identification of features remained challenging, but three discriminatory metabolites were characterised with reasonable accuracy. Acetylcarnitine was observed to be elevated in urine of patients with HCC, albeit not significantly ($p=0.83$). This finding is akin to those findings

in the parallel Nigerian UPLC-MS study (**Chapter 9**). In addition to this, dimethylheptanoylcarnitine, a long chain acylcarnitine was reduced in the urine of patients with HCC, again non-significantly ($p=0.097$). These results support the theory that *B*-oxidation of fatty acids is perturbed in HCC. A relative reduction in the level of long chain acylcarnitines could occur either through the decreased transesterification of long chain fatty acids by carnitine palmitoyl transferase 1 (CPT-1) in the outer mitochondrial membrane or an increased flux through the mitochondrial membrane with acetylcarnitine via carnitine-acylcarnitine translocase (CACT) (**Figure 9-10**). In either mechanism, it may be that alteration in oxidation of fatty acids may provide lipid substrates for cellular membranes in proliferating tumours.

The identification of a significant reduction in urinary *p*-cresol sulfate in negative ion mode provides validity confirmation to the parallel urinary ^1H NMR study in the same UK cohort. As discussed in **Chapter 8**, this finding was not evident in the Nigerian and Egyptian groups. *p*-cresol is a product of colonic bacterial metabolism (Clayton *et al.*, 2009) and it may be that because of dietary differences in Nigerian, Egyptian and UK populations, the gut microbiome is altered. This in turn may affect the production of urinary metabolites, such as *p*-cresol sulphate. It has been shown that urinary *p*-cresol sulphate levels were reduced in patients with Crohn's disease, a population where there are major alterations in the colonic gut microbiome (Williams *et al.*, 2009). Functionally, this finding may also be indicative of an underlying hepatic metabolic derangement, lower levels of *p*-cresol sulphate reflecting reduced sulfonation in those patients with HCC and worse liver function (**Chapter 8**).

Improvements in this study are possible. Sample numbers were small and this may explain why most metabolites identified displayed trends but not overt statistically significant

differences between groups. A larger study population number in follow up studies is essential. This would also allow subgroup analyses to be performed. In particular, it would be valuable to investigate whether the urinary metabolic profile of HCC patients with viral hepatitis differs from those with NASH, a condition intimately linked with the “metabolic syndrome”.

Standard authentication of metabolites would be desirable. Unfortunately, standards for *p*-cresol sulphate and dimethylheptanoylcarnitine are not commercially available but can be constructed in the laboratory. Finally, given the identification of carnitine related molecules in this UK population and in the previously reported Nigerian population (Shariff *et al.*, 2010) it would be informative to perform a targeted carnitine and carnitine related molecule profile of urine from patients with HCC to identify any further alterations that may be present

10.6 CONCLUSIONS

This study has provided evidence that UPLC-MS can identify a discriminatory urinary metabolic profile between patients with HCC and those with cirrhosis induced by a highly diverse range of aetiologies, including alcohol and NASH induced liver disease. This would suggest that HCC induces urinary metabolic changes independent of the underlying disease. Urinary UPLC-MS multivariate profiles also provided predictive models for HCC diagnosis which out-performed serum AFP in terms of sensitivity (80 - 93.3% versus 23.1 - 46.2%).

Further evidence was also provided, in the form of altered carnitine moiety levels, of the role perturbed fatty acid oxidation may have in HCC. The identity of *p*-cresol sulphate, a gut microbial produced metabolite, also found in parallel ^1H NMR studies to be reduced in the urine of patients with HCC. This provides some validation for the use of the complementary techniques of ^1H NMR and UPLC-MS.

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The results of these studies reveal altered biofluid metabolic profiles of HCC indicative of heightened basal metabolic rate, cellular turnover, deranged lipid metabolism, chromosomal methylation and altered gut microbe metabolism. The hypotheses of this thesis were firstly, that the presence of HCC results in altered blood and urinary metabolic profiles which can be characterised using ^1H NMR and LC-MS and secondly, that these profiles can be modelled to allow accurate disease diagnosis. The latter hypothesis, for the individual populations studied, was upheld, with urinary metabolic profiles, in particular, displaying high diagnostic sensitivity and specificity. Whether the metabolites detected are directly from the tumour itself, or as a result of the tumour effects on its microenvironment or total body physiology remains unclear.

The use of both ^1H NMR spectroscopy and UPLC MS profiling highlighted the advantages and disadvantages of the techniques. Preparation for ^1H NMR spectroscopy, both for blood and urine, involved few stages and interpretation of results was straightforward given the availability of well validated ^1H NMR spectral databases. It also provided, in the urine of patients with HCC from Nigeria and Egypt (**Chapters 6 and 7**), the most accurate predictive results, albeit with relatively abundant metabolites (creatinine, creatine and carnitine) given the inherent poor sensitivity of ^1H NMR spectroscopy in detecting low concentration metabolites. Preparation for UPLC MS, however, was labour intensive, involving many steps. This was particularly the case for lipidomic profiling of blood (**Chapter 5**). Identification of candidate metabolites was difficult owing to the need of further fragmentation experiments and paucity of well characterised databases. Metabolites detected, however, were different to that of ^1H NMR and illuminating of the different metabolic pathways altered in the presence

of HCC. The multivariate results from UPLC MS studies, both in urine and blood, were less impressive than ^1H NMR. The following sections summarise the findings from each experiment group in further detail.

11.1 FISHER RAT MODEL OF HCC

The well-characterised Fisher rat model of HCC, induced by DEN and NMOR, was chosen because of the rapid onset of HCC, allowing studies to be undertaken in reasonable timeframes. Animals with tumours were over 100g lighter than healthy counterparts. Proton NMR spectra from these animals displayed marked and significant decreases in lipoproteins, unsaturated fatty acids, acetyl-glycoprotein, acetoacetate, and glucose ($p \leq 0.001$). Plasma citrate and formate levels were increased ($p = 0.02$). Ultra performance LC-MS lipid profiling studies of plasma confirmed that multivariate statistical modelling was robust in classifying animals with HCC, distinct from healthy control animals. Features M496 T104 and M759 T366 were identified as most contributory to multivariate models from animal studies, although the levels of these metabolites were not significantly different between HCC and control animals on univariate statistical comparison. M496 T104 is likely to represent LPC (24, 0, 0), a major cellular membrane component. M759 T366 could not be identified from available databases. The lipoprotein changes in the rat are likely to indicate a heightened basal metabolic rate, a marker of cancer cachexia, resulting in dramatic weight differences. These findings are not the same as for the human studies, where certain lipoproteins are increased and others diminished, in comparison to patients with cirrhosis and healthy volunteers.

While revealing in their own right, the results from an animal model of HCC have limitations for translation into human studies. The pathophysiology, genotype, phenotype,

environmental, dietary and co-morbid factors in humans are impossible to replicate in an animal model and it is probably the interplay between all of these factors that results in the carcinogenic process and influences metabolic profiles in man (Holmes *et al.*, 2008; Psihogios *et al.*, 2008; Wolk *et al.*, 2001). Furthermore, the onset of tumours in this particular animal model occurs as early as 10 weeks. This is due to the potency of the mutagens utilised. This is not akin to the human disease, which typically occurs decades after the introduction of an aetiological factor. A model, whereby the aetiological factor is more like the human disease may provide better translation. An example of such is a C57/Black 6 transgenic mouse model of HCC that contains the entire open reading frame of HCV under an albumin promoter, developed by the Pawlotsky group in France (Lerat *et al.*, 2002). This model displays liver-specific expression of the complete HCV RNA coding sequence. Since these animals are immune-tolerant to the viral proteins, there is no immune or inflammatory response to viral protein expression, similar to human disease. There is an increased rate of hepatocellular adenoma and carcinoma in these. Such models should be considered in future studies as the carcinogenic process is more translational when making species comparisons.

11.2 HUMAN BLOOD METABOLIC PROFILING STUDIES

The human ^1H NMR blood metabolic profiling studies provided evidence of predominantly altered lipoprotein levels. Differences between patients with HCC, patients with cirrhosis and healthy volunteers were detected in levels of LDL ($p=0.002$ and 0.12), VLDL ($p<0.001$ and 0.77), lactate ($p=0.03$ and 0.12), *N*-acetylglycoproteins ($p<0.001$ and 0.001) and acetoacetate ($p=0.52$ and 0.06). Efforts were made to match groups as closely as possible; nonetheless the HCC group were older than the healthy controls. Although this difference was not significant, the confounding effect of age on lipoprotein resonances cannot be ruled

out. Nevertheless, these findings are still likely to indicate an altered production or consumption (decreased peripheral breakdown of VLDL) of lipoproteins.

The serum lipidomic UPLC-MS studies identified LPC (24, 0, 0) as the lipid contributing most to multivariate discriminant models of HCC and cirrhosis. However, the results from Nigerian or UK groups did not produce significant results, which may have been because sample numbers in this study were small ($n \leq 10$ in each group). This result was most marked in the UK group, displaying a reduction in LPC (24, 0, 0) levels in patients with HCC ($p=0.4$). A very recently published study observed similar changes in LPC (24, 0, 0) levels in a group of 20 American patients with HCC using UPLC-MS and GC-MS (Patterson *et al.*, 2011). Lysophosphatidylcholine (24, 0, 0), is a major constituent building block of cellular membranes. Taken together with the blood and urine NMR data, this may indicate that lipids are being diverted from catabolism to anabolism in the form of tumour proliferation.

There were inevitable limitations in these studies. First, although efforts were made to match HCC, healthy and cirrhosis groups, given the demographic make-up of HCC it is challenging to precisely match groups. Secondly, as the UPLC-MS lipid profiling studies were performed as a pilot, partly to optimise the technique of lipid extraction, sample numbers were small. Larger studies are needed to tease out any significant differences between patients with HCC and controls. In retrospect, it would also have been illuminating to record markers of cancer cachexia (Tisdale, 2010) including percentage weight loss, adipose tissue loss, skeletal tissue loss, inflammatory and cytokine markers to definitively identify or refute any correlation with metabolite markers of HCC. The hypothesis that HCC cells secrete metabolite markers could also be explored further. A study comparing peripheral blood metabolic profiles to that of blood collected in proximity to the tumour (which could be

collected prior to trans-arterial chemo-embolisation therapy) in the same patient would be revealing. This would provide some proof, if metabolic characteristics were different, that the tumour itself is producing metabolite markers. Other cancers, which do not occur on the background of liver disease, were not included. It would not have been possible to distinguish which metabolic differences were due to HCC and which were due to cirrhosis when comparing HCC to other tumour types.

11.3 HUMAN URINE METABOLIC PROFILING STUDIES

The human urine ^1H NMR studies of Nigerian (Shariff *et al.*, 2010), Egyptian (Shariff *et al.*, 2011) and UK cohorts (*manuscript in progress*), were the first to identify metabolite profiles of HCC which shared similarities between the different populations. Indeed, the diagnostic accuracy of urine profiles in Nigerian and Egyptian groups was far better than that of blood. Diagnostic sensitivity and specificity of discriminating urine profiles of HCC from healthy subjects ranged from 90 to 100% and 90 to 95%. This was only slightly reduced in discriminating patients with HCC from those with cirrhosis: 80 to 90% sensitivity and 70 to 90% specificity. These results compare favourably to serum AFP, which carries a 39% to 65% sensitivity and a 76% to 94% specificity for tumour diagnosis (Daniele *et al.*, 2004). The UK urine study, in which patients with HCC were compared to those with cirrhosis, displayed poorer diagnostic power of metabolite profiles, with sensitivity dropping to 50%. This may have resulted from close matching of cirrhosis and HCC patients resulting in higher similarity of ^1H NMR spectra. Certainly, BMI was similar in both groups, so it is unlikely that cancer cachexia, which may have been a prominent contributor to altered spectral profiles in the Nigerian and Egyptian group, had a major influence in this study. Certain metabolites were similarly altered across the studies, such as a reduced urinary creatinine and raised creatine and carnitine levels. Reduced creatinine is likely to reflect reduced muscle mass in patients with cachexia, creatine to reflect increased energy consumption and carnitine, an alteration in fatty acid metabolism. Of note, in both the UK and Nigerian UPLC-MS studies, acetylcarnitine was found to be increased in the urine of patients with HCC, corroborating altered carnitine profiles from ^1H NMR spectroscopy studies. A previous study assessing the urine metabolic ^1H NMR profiles of healthy volunteers after stimulation of β -oxidation of fatty acids with oral administration of fenofibrate, recorded decreased acetylcarnitine urine levels in treated subjects (Patterson *et al.*, 2009). This suggests that β -

oxidation is suppressed in patients with HCC. This would be in keeping with reduced LPC (24, 0, 0) levels in the blood of patients with the tumour if lipids were being diverted away from catabolism and instead incorporated into cellular membranes of rapidly proliferating cells.

Certain urinary metabolites were discriminatory in the different populations. Urinary glycine, hippurate, citrate and TMAO were all decreased in the Egyptian patients with HCC. *p*-cresol sulphate was reduced in the urine of patients with HCC in the UK study, confirmed in both ¹H NMR and UPLC-MS urine studies. Individual explanations for the altered levels of these metabolites are given in relevant chapters. These sets of metabolite are not part of the same metabolic pathway so it is unclear why in one population and not the other, they may be altered. Dietary, co-morbid and aetiological factors are sure to play a role but it is currently unclear how they would contribute to these particular metabolite differences.

11.4 METABONOMICS IN OTHER CANCERS AND CACHEXIA

The studies presented within this thesis, as with the majority of metabolite profiling studies, are limited to comparative disease groups. Metabolites are often the common endpoint of common mammalian pathways and it is probably the case that the same metabolites will be deranged in very different diseases. Metabonomics, therefore, is reliant upon the diagnostic accuracy of panels of biomarkers, the ratios of which are thought to be more specific to the disease in question. Examples of metabolic studies in non-HCC cancer, cachexia and other diseases are described to allow comparison.

Serum metabolic profiling studies in patients with colorectal cancer (CRC), in age and gender matched individuals, have identified reduced glycine levels as discriminatory (Leichtle *et al.*,

2012) and elevated acetylglycoproteins, acetate, formate and reduced citrate, creatine, and lactate were found to be highly discriminatory in patients with metastatic CRC (Bertini *et al.*, 2012). Furthermore, urine metabolic profiling studies of patients with CRC have identified reduced histidine (Qiu *et al.*, 2010) and citrate, hippurate and *p*-cresol compared to matched healthy controls (Cheng *et al.*, 2012). A study of GC MS profiles in serum of patients with recurrence of breast cancer after treatment identified altered levels of glycine and formate in addition to a number of other metabolites, as discriminatory (Asiago *et al.*, 2010).

There have been few human metabolic studies of metabolic profiles in cancer cachexia but a study of GC and LC MS serum profiles in a rat model of model of malnutrition, identified perturbations in levels of creatine, citrate and trimethylglycine (Wu *et al.*, 2010).

Finally, Williams and colleagues examined the ^1H NMR urine profiles of patients with Crohn's disease and compared these to healthy age and sex matched controls. Levels of hippurate and *p*-cresol were reduced in Crohn's sufferers and in combination, were again highly discriminatory of disease in these groups (Williams *et al.*, 2009).

It is clear that certain metabolites, such as citrate, creatine, glycine, *p*-cresol sulphate and hippurate are altered in the cancer and non-cancer diseased state. This needs further investigation and comparative studies to allow control groups to include other diseases and cancers in age, sex and nutrition-matched analyses. Whether it would then be the case that the disease itself or cachexia/general illness are the discriminatory driving factors could be determined. Without these data, the diagnostic acumen of metabolic profiling will always be cast in doubt.

11.5 FUTURE DIRECTIONS

For both blood and urine studies larger cohorts of patients with well-characterised anthropometric and liver disease stages are required for validation of the studies presented herein. It will also be essential to characterise the metabolic profiles of HCC in patients with early stage disease, when the tumour is asymptomatic. It would also be valuable to include positive controls, those patients with non-HCC tumours, to see whether there is similarity despite the presence of liver disease. Two ongoing projects, which are a direct result of the work of this thesis, address these issues. Firstly, the UK Clinical Research Network (UKCRN) has recently approved specimen collection for metabolic profiling of blood and urine in patients with HCC, with collaborative centres collecting samples from patients with HCC, cirrhosis and healthy volunteers (<http://public.ukcrn.org.uk>, UKCRN ID 8357). Parallel proteomics and genomics studies are also planned which will aid in identifying the underlying molecular basis of metabolic derangement. Secondly, as part of a European Commission FP7-funded study into the effect of prevention of liver fibrosis in HBV-infected patient population in West Africa (Banjul in Gambia, Dakar in Senegal, and Jos in Nigeria) led by the Liver Unit at Imperial College London, a metabolic profiling arm of the study will oversee sample collection from 800 patients with HCC over 5 years following an initial baseline screening programme of 13,000 subjects (http://ec.europa.eu/research/health/medical-research/cancer/fp7-projects/prolifika_en.html). From the perspective of continuing the work from my thesis, in addition to Gambia and Senegal, the study is located in the same centre in Jos in Nigeria, where my samples were collected. These studies will include longitudinal sampling from those patients with established cirrhosis, a proportion of which will develop tumours during follow up. This will allow the assessment of very early stage tumours.

The work presented in this thesis has provided novel information on the ability of metabolic profiling of patient blood and urine to diagnose HCC and expose the effect HCC can have on human physiology. Certain questions remain on the nature and origin of these metabolic changes that follow-up studies aim to address.

Hepatocellular carcinoma remains the third highest cause of cancer-related death, worldwide, affecting the developed and developing world. Urgent attention to developing novel methods of diagnosis, particularly in early stage HCC, is required to improve the prognosis from this challenging disease. Ultimately, a urinary dipstick test would be the goal for patients in resource-poor settings as screening at a village level would be simplified.

12 PUBLICATIONS AND PATENTS

Listed below are publications, book chapters and filed patents that have arisen from work presented in this thesis and published or accepted before February 2012.

12.1 ORIGINAL ARTICLES

Shariff, M.I., Gomaa, A.I., Cox, I.J., Patel, M., Williams, H.R., Crossey, M.M., Thillainayagam, A.V., Thomas, H.C., Waked, I., Khan, S.A., & Taylor-Robinson, S.D. 2011. Urinary Metabolic Biomarkers of Hepatocellular Carcinoma in an Egyptian Population: A Validation Study. *J.Proteome.Res.*

Shariff, M.I., Ladep, N.G., Cox, I.J., Williams, H.R., Okeke, E., Malu, A., Thillainayagam, A.V., Crossey, M.M., Khan, S.A., Thomas, H.C., & 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.

12.2 PUBLISHED ABSTRACTS

Shariff M.I.F., Lewis M.R., Want E.J., Keun H.C., Mohamed F., Jalan R., Ladep N.G., Crossey M.M.E., 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. Accepted for abstract publication (*J.Hepatol.*), European Association of the Study of the Liver, April 2012.

Shariff M.I.F., Keun H.C., Beckonert O., Mohamed F., Jalan R., Khan S.A., Holmes E., Taylor-Robinson S.D. Plasma metabolite profiling in a rat model of hepatocellular carcinoma and the effects of selective gut decontamination. Accepted for abstract publication (*J.Hepatol.*), European Association of the Study of the Liver, April 2012.

Ladep N.G., Shariff M.I.F., Okeke E, Malu A, Cox I.J., Taylor-Robinson S.D. A urinary metabonomic study of hepatocellular carcinoma in a Nigerian population using proton magnetic resonance spectroscopy. *J.Hepatol.* 2009 50, Supplement No. 1, S299.

12.3 BOOK CHAPTERS

Shariff M.I.F and Taylor-Robinson S.D. Dipstick markers for diagnosis: Feasible or not? Clinical Dilemmas in Primary Liver Cancer. Wiley publishing, December 2011.

12.3 REVIEW ARTICLES

Shariff, M.I., Cox, I.J., Gomaa, A.I., Khan, S.A., Gedroyc, W., & Taylor-Robinson, S.D. 2009. Hepatocellular carcinoma: current trends in worldwide epidemiology, risk factors, diagnosis and therapeutics. *Expert.Rev.Gastroenterol.Hepatol.*, 3, (4) 353-367.

Patel, M., Shariff, M.I., Ladep, N.G., Thillainayagam, A.V., Thomas, H.C., Khan, S.A., & Taylor-Robinson, S.D. 2010. Hepatocellular carcinoma: diagnostics and screening. *J.Eval.Clin.Pract.*

12.4 OTHER NATIONAL AND INTERNATIONAL PRESENTATIONS

Ladep N.G., Shariff M.I.F., Crossey M.M.E., Okeke E.N., Malu A.O., Williams H.R.T, Khan S.A., Cox I.J., Taylor-Robinson S.D. 2009. Characterisation of urinary biomarkers of hepatocellular carcinoma using nuclear magnetic resonance spectroscopy in a Nigerian population. Association of Physicians of Great Britain and Ireland annual meeting, Birmingham, UK, April 2009.

Shariff M.I., Cox I.J. Patel M, Gomaa A.I., Ladep N.G., Williams H.R.T., Crossey M.M.E., Thillainayagam A.V. Waked I., Khan S.A., Thomas H.C., Taylor-Robinson S.D. Urine Metabolic profiling of hepatocellular carcinoma: Corroborative evidence from two populations. International Liver Cancer Association, Montreal, Quebec, Canada, September 2010.

Patel M., Shariff M.I.F., Ladep N.G., Cox I.J., Williams H.R.T., Thillainayagam A., Crossey M.M.E., Khan S., Thomas H., Rosenburg W., Taylor-Robinson S.D. Urine metabolite and enhanced liver fibrosis test profiling of hepatitis B-related liver disease in a Nigerian population. Medical Research Society, London, February 2011.

12.5 PATENTS FILED

Taylor-Robinson S.D., Shariff M.I.F, Khan S.A., Penfold H. Diagnostic Method: Diagnostic urine markers of hepatocellular carcinoma. P056352GB. Imperial Innovations Ltd. January 2011.

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