BIOMARKER RESEARCH IN THROMBOEMBOLIC STROKE

BRUITS

Thesis submitted to Imperial College London for the degree of Doctor of Philosophy

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Read in the name of thy Lord who created
Created man from a clot
Read! And thy Lord is most bountiful
Who teaches man by the pen
Teaches that, which he knew not

The first revelation of the Quran

Statement of originality

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Abstract

Introduction

Stroke is a leading cause of death and disability worldwide. Approximately one quarter of all strokes are secondary to carotid atherosclerosis. There is a clinical need to improve risk stratification of carotid atherosclerosis, to better target surgical or interventional therapy and prevent stroke. This study aimed to determine diagnostic biomarkers of high-risk carotid atherosclerosis, and ensure the validity of such markers in the presence of alternative phenotypes of atherosclerotic disease.

Methods

150 patients were recruited according to the following criteria:

Group 1: Symptomatic >50% carotid stenosis

Group 2: Non-carotid stroke/TIA

Group 3: Asymptomatic >50% carotid stenosis

Group 4: Asymptomatic controls with <50% carotid stenosis

Group 5: Abdominal aortic aneurysm

Group 6: Intermittent claudication

Disease groups were matched for age, gender, cardiovascular risk factors, haematological parameters, renal function and lipid status.

Blood and urine was collected from all patients and analysed through global metabolic profiling (1H-NMR Spectroscopy, HILIC-Mass Spectrometry and Lipid Profiling-Mass Spectrometry). Acquired spectra were compared across groups using computational multivariate data analysis to determine markers of high-risk carotid atherosclerosis.

Results

Statistical models derived from urinary spectra proved stronger than serum datasets, in particular with HILIC-Mass Spectrometry (positive ionisation mode). Application of computational OPLS DA resulted in discrimination of symptomatic carotid atherosclerosis from asymptomatic disease, aneurysmal disease, and intermittent claudication. Differentiating metabolites span a vast array of compounds including lipid derivatives, amino acid derivatives and nucleotide derivatives.

Conclusion

This is the first study to identify urinary metabolic biomarkers of high-risk carotid atherosclerosis, differentiating symptomatic carotid atherosclerosis from asymptomatic disease, and aneurysmal and peripheral arterial disease. Targeted temporal studies are now required for clinical validation and to determine the variation of acute biomarkers with time.

Table of Contents

List of Figures

List of Tables

Abbreviations

1H-NMR	Proton Nuclear Magnetic Resonance Spectroscopy
AAA	Abdominal aortic aneurysm
ABPI	Ankle Brachial Pressure Index
A-FABP	Adipose Fatty Acid Binding Protein
AEA	Anandamide
AMA	Aminomalonic acid
B-FAB-P	Brain Fatty Acid Binding Protein
CB	Cannabinoid receptor
CE	Capillary electrophoresis
CEA	Carotid endarterectomy
cGMP	Cyclic guanosine monophosphate
Chol	Total cholesterol
Chol:HDL	Cholesterol:HDL ratio
CNS	Central nervous system
Cr	Creatinine
CRP	Capsular reactive protein
CV	Coefficient of Variance
CVA	Cerebrovascular accident
Da	Dalton
DNA	Deoxyribonucleic acid
E	Energy
ECST	European Carotid Surgery Trial
E-FAB-P	Epidermal Fatty Acid Binding Protein

ESI	Electrospray ionisation
ESR	Erythrocyte sedimentation rate
EVAR	Endovascular Aneurysm Repair
FAAH	Fatty acid amide hydrolyase
GABA	Gamma-amino butyric acid
GC	Gas chromatography
GSA	Guanidinosuccinic acid
GTP	Guanosine triphosphate
h	Planck's constant
HASU	Hyper Acute Stroke Unit
HDL	High density lipoprotein
HILIC	Hydrophilic Interaction Liquid Chromatography
HPLC	High Performance Liquid Chromatography
hs-CRP	High sensitivity Capsular Reactive Protein
HTA	Human Tissue Authority
ICAM	Intercellular Adhesion Molecule
IL	Interleukin
IMS	Imaging Mass Spectrometry
IMT	Intima-Medial Thickness
kDA	Kilodalton
LC	Liquid chromatography
LDL	Low density lipoprotein
LP	Lipid Profiling
Ly	Lymphocyte count

m/z	Mass/Charge Ratio
MALDI	Matrix Assisted Laser Desorption Ionisation
MCA	Middle cerebral artery
MDT	Multidisciplinary team meeting
MMP	Matrix Metalloprotease
Mo	Monocyte count
MRI	Magnetic Resonance Imaging
MS	Mass Spectrometry
MTBE	Methyl tert-butyl ether
NaN_3	Sodium Azide
NASCET	North Atlantic Symptomatic Carotid Endarterectomy Trial
Ne	Neutrophil count
NGAL	Neutrophil gelatinase-associated lipocalin
NHS	National Health Service
NMDA	N-methyl-D-aspartate
NMR	Nuclear Magnetic Resonance
NNT	Number Needed to Treat
NaOH	Sodium hydroxine
NaH_2PO_4	Sodium dihydrogen phosphate
NO	Nitric oxide
OPLS	Orthogonal Projection to Latent Structures
OPLS DA	Orthogonal Projection to Latent Structures Discriminant Analysis
PAD	Peripheral arterial disease
PCA	Principal Component Analysis

PET	Positron Emission Tomography
PLS	Projection to Latent Structures
PRISMA	Preferred Reporting Items for Systematic Reviews and Meta-Analyses
QC	Quality Control Sample
RNA	Ribonucleic acid
RP	Reverse Phase
rpm	Rounds per minute
SPE	Solid phase extraction
sTWEAK	Soluble Tumour Necrosis Factor-like Weak Inducer of Apoptosis
TIA	Transient ischaemic attack
Trig	Triglycerides
TSP	Trisodium phosphate
TWEAK	Tumour Necrosis Factor-like Weak Inducer of Apoptosis
UK	United Kingdom
UPLC	Ultra Performance Liquid Chromatography
Ur	Urea
UV	Unit Variance
υ	Frequency
υ_{res}	Resonant frequency
VCAM	Vascular Cell Adhesion Molecule
VLDL	Very low density lipoprotein
WCC	White cell count

I. Introduction

Chapter 1: Clinical Background – Carotid Atherosclerosis

1.1 Clinical Need

Stroke is the second leading cause of death worldwide(1) and the leading cause of serious disability in the United Kingdom(2). Stroke is estimated to cost the UK £9 billion annually in health and social care(3), representing 3% of the 2008-9 Department of Health budget of £82 billion(4). The national incidence of stroke is 130 000(5) and of these, approximately 20-30% are due to atherosclerosis of the carotid bifurcation and plaque rupture(6, 7). The European Carotid Surgery Trial found the mean age of patients with stroke secondary to carotid atherosclerosis in Europe to be 62 (SD±8) years(8).

Carotid atherosclerosis can be identified in an outpatient clinic simply and non-invasively with ultrasound(9). It is estimated that 100 000 people in the UK have this readily detectable and correctable risk factor for stroke(10). The annual risk of ipsilateral hemispheric symptoms from a newly diagnosed carotid plaque is 3% per year(11). Given the low event rate, current UK guidelines do not advocate routine carotid endarterectomy in all asymptomatic patients (12).

Of the 15,000 annual UK strokes caused by carotid thromboembolism, three-quarters will have been previously asymptomatic. There is a need to risk stratify patients with carotid stenosis, to identify high-risk patients with as yet asymptomatic carotid disease, in order to offer timely endarterectomy and prevent stroke.

1.2 Circulating biomarkers of carotid atherosclerosis

A biomarker is a "substance or characteristic that is objectively measured and evaluated and is used as an indicator of a biological state"(13). A good biomarker is sensitive, specific, and highly reproducible with stable and predictable release and clearance. With regard to carotid atherosclerosis, biomarkers could indicate presence of disease, risk of embolization or progression, and response to best medical therapy. The search for a biomarker of carotid disease has been the aim of extensive atherosclerosis research performed to date.

1.2.1 Biomarkers denoting presence of carotid atherosclerosis

Carotid atherosclerosis can be demonstrated simply, non-invasively and inexpensively with the use of carotid duplex ultrasonography. However, ultrasound is an operator-dependent modality, and less sensitive/specific for moderate stenosis. Therefore a large body of research has examined blood for the presence of biomarkers indicative of carotid atherosclerosis.

Atherosclerosis is an inflammatory disease, and markers of inflammation have been of interest to researchers studying carotid atherosclerosis. Circulating cells such as leukocytes, while found to be relatively increased in the presence of carotid atherosclerosis, still remain within normal levels, therefore lacking in specificity or clinical applicability(14).

TWEAK (Tumour Necrosis Factor-like Weak Inducer of Apoptosis) is a type II transmembrane protein that when proteolytically cleaved produces a soluble cytokine (sTWEAK), capable of inducing the expression of chemoattractant proteins including IL-8 and metalloproteinases such as MMP-9, as well as activating NF-kB signal transduction pathway(15). TWEAK is expressed in macrophages within carotid atherosclerotic plaques, co-localising with its receptor fibroblast growth-factor inducible 14 (Fn14)(15).

Proteomic analysis of supernatants from cultured human carotid atheroma and healthy mammary arterial controls identified TWEAK to be strongly expressed in healthy artery supernatant but not in atherosclerotic carotid supernatant(16). This trend was mirrored in the plasma of patients with severe carotid atherosclerosis compared with healthy controls: the presence of carotid atherosclerosis was associated with significantly reduced levels of plasma sTWEAK(16). These findings corroborate reports that TWEAK may guard against an excessive inflammatory response(17, 18).

CD163 is a member of the scanvenger recepter cysteine rich (SRCR) family with important anti-inflammatory properties, expressed exclusively on the surface of monocyte/macrophages. It has recently been recognised as a potential scavenger receptor of TWEAK. The soluble form of CD163 is a normal constituent of plasma. Morena et al. demonstrated the co-localisation of CD163 and TWEAK in carotid plaques, and further described the inverse correlation between the expression of these proteins(19). In vitro, CD163 expressing macrophages recognised and internalised sTWEAK. Plasma sCD163 levels are

negatively correlated with sTWEAK concentrations; and sCD163:sTWEAK ratio is positively correlated with carotid IMT(19).

The expression of cellular adhesion molecules is known to be perturbed in inflammatory states. VCAM-1 was significantly higher in patients with carotid atherosclerosis compared to patients without carotid plaque in a study comparing biomarker concentrations with duplex ultrasound parameters(14). A larger study analysing the relationship between carotid IMT and adhesion molecules found no correlation between VCAM-1 and carotid IMT, though the degree of carotid stenosis was not clearly defined in these patients(20). This 1997 study of 272 patients with carotid artery atherosclerosis, 204 patients with incident coronary heart disease and 316 non-atherosclerotic controls did find significantly higher levels of ICAM-1 and E-selectin in patients with carotid atherosclerosis as compared with controls. However, ICAM-1 and E-selectin were unable to distinguish carotid from coronary artery disease(20).

Lipid metabolism plays a central role in the development of atherosclerosis. Fatty acid binding proteins are low-molecular-weight intracellular lipid-binding proteins that are widely expressed and involved in the regulation of lipid metabolism as well as inflammation. Several subtypes are recognised, including adipocypte fatty acid binding protein (A-FABP), epidermal (E-FABP), and brain (B-FABP).

A-FABP has been shown to circulate in the blood stream, and an independent association has been found between A-FABP and carotid atherosclerosis in Chinese women(21). The same research group have, in a large study, discovered

significantly higher E-FABP concentrations in subjects with carotid plaques compared with those without carotid plaque(22).

1.2.2 Circulating biomarkers characterising carotid atherosclerosis

The unmet clinical need within carotid research is the detection of a biomarker that denotes vulnerability of the plaque. Discovery of such a marker would enable improved risk stratification of patients with carotid stenosis, allowing appropriate targeting of surgical intervention, and the realisation of potential therapeutic targets to develop drugs that prevent plaque rupture and stroke.

Markers such as leucocytes raised in the presence of carotid atherosclerosis do not necessarily correlate with clinical or ultrasonographic features of plaque vulnerability(23) (24). Furthermore, markers such as neutrophil gelatinase-associated lipocalin (NGAL) known to correlate with risk factors for atherosclerosis do not necessarily correlate with plaque vulnerability(25). Evidence of the importance of other inflammatory markers is contradictory: hs-CRP was found to be increased in the presence of carotid atherosclerosis, and higher still in patients with ultrasonographically characterised complex plaques(26); however, histological categorisation of plaques as stable or unstable has not been shown to correlate with hs-CRP(27), and hs-CRP was not found to be significantly altered between symptomatic and asymptomatic patients(24).

Prior to its association with TWEAK, the role of macrophage CD163 was recognised as the receptor responsible for haptoglobin-haemoglobin complex uptake(19). Plaque instability is frequently associated with intra-plaque haemorrhage. Despite evidence of the up-regulation of CD163 in unstable carotid plaques(28), serum sCD163 levels were not shown to be significantly different between asymptomatic and symptomatic patients with severe carotid stenosis(29).

Interferon-y released by T cells stimulates the guanosine-triphosphate pathway in activated macrophages, and neopterin is produced as a by-product. Original neopterin studies in coronary artery disease found elevated levels of this protein in the presence of coronary artery disease. Neopterin levels were further elevated in unstable vs. stable angina. However it was hypothesised that raised neopterin levels in stable angina may be secondary to the presence of carotid atherosclerosis, which formed the basis of an elegant Japanese study conducted by Sugioka et al(26). One hundred two patients with angiographically-proven coronary disease and stable angina pectoris (but no intercurrent inflammatory, infectious or malignant disease) underwent duplex ultrasonography of the carotid arteries. Serum levels of neopterin were determined and compared across well-matched groups of patients with no carotid stenosis, non-complex carotid atherosclerosis, and complex plaques (defined as ulcerated, irregular and/or with mobile components). The study demonstrated increased plasma levels of neopterin were independently associated with the presence of complex carotid plaques, but not non-complex plaques. This relationship persisted in both

diabetic and non-diabetic patients, and following adjustment for multi-vessel coronary disease and hs-CRP.

A subsequent Dutch study measured serum levels of neopterin in 100 patients undergoing CEA, grouped as asymptomatic, amaurosis fugax, TIA or CVA(29). While all included patients exhibited greater than 70% carotid stenosis, the characteristics of the plaque were not determined. Neopterin levels were lower in asymptomatic patients as compared with each of the three symptomatic subgroups, but this relationship failed to reach statistical significance. Of note, the time elapsed between symptoms and blood sampling in the symptomatic subgroups varied widely and averaged 85, 48 and 53 days for amaurosis fugax, TIA and CVA respectively, though the risk of recurrent ischaemic events is highest in the first two weeks following initial symptoms.

Matrix metalloproteinases (MMPs) degrade extracellular matrix components leading to plaque instability, intraplaque haemorrhage, increasing the likelihood of plaque rupture. Subtypes include collagenases, gelatinases, stromelysins, matrilysins, and membrane-type MMPs. They are inhibited by Tissue Inhibitor Metalloproteinases (TIMPs) but when latent may also form complexes with TIMPs leading to activation of MMPs such as MMP-2. It has therefore been hypothesised that both MMPs and TIMPs may serve as markers of plaque vulnerability, and their potential as biomarkers denoting stroke risk has been extensively investigated.

The gold standard measure of plaque vulnerability is histological analysis of parameters including lipid core and fibrous cap thickness. In a German study, Pelisek et al. investigated serum levels of MMP-1, -2, -3, -7, -8 and -9 and TIMP-1 and -2 in 101 patients undergoing carotid endarterectomy whose plaques were subsequently categorised as histologically stable (n=37) or unstable (n=64)(27). Although patients with unstable plaques were significantly more likely to be symptomatic, one-third of patients with histologically stable plaque also had neurological symptoms. Only MMP-1, -7 and TIMP-1 were significantly increased in patients with histologically unstable plaque.

These findings are contradictory to other studies of MMPs(30-32). As explained by Pelisek, one reason for this discrepancy may be the difference in plaque grading systems employed, or that MMP-2 and MMP-9 (gelatinases) better serve as biomarkers of presence of carotid atherosclerosis as opposed to biomarkers of plaque vulnerability.

A contemporaneous Dutch study analysed arterial serum MMP and TIMP subtype concentrations in 145 symptomatic and asymptomatic individuals with high-degree carotid stenosis. MMP-7 and TIMP-1 were again significantly enhanced in symptomatic patients as compared with a well-matched asymptomatic cohort(24). In addition, MMP-8 and MMP-9 were also significantly raised in symptomatic patients. However, the positive predictive value for each of these individual biomarkers to detect neurological symptoms varied between 57.0% (MMP-9) and 62.5% (TIMP-1). Combining prognostic biomarkers MMP-7, -8 and -9 improved positive predictive value to 73.8%, with 85.53% specificity.

Serum levels of TNF-alpha and IL-8 were found to be significantly higher in patients with histologically unstable carotid plaques compared with stable carotid plaques(27). The same study showed no significant differences in IL-1beta, IL-6, IL-10 and IL-12 between subgroups.

1.3 Urinary Biomarkers of Carotid Atherosclerosis

Despite extensive literature review utilising Medline and Embase databases, no putative urinary biomarkers denoting presence or vulnerability of carotid atherosclerosis were encountered.

1.4 The Need for a Systems Biology Approach

Traditional research methodology has to date failed to provide a blood or urine biomarker of high-risk carotid atherosclerosis with sufficient sensitivity and specificity for incorporation into clinical practice. Larger studies are required, incorporating a multitude of biofluids that can test a diverse range of potential biomarkers of varying nature simultaneously and in a cost-effective manner. These are the benefits that are intrinsic to systems biology techniques.

Chapter 2: Methodological Background – Metabonomics

2.1 Systems Biology and Metabonomics

Systems biology describes a holistic and integrative approach to cognising a physiological or pathological state by assessing the "net" biological effect imparted by the incumbent condition(13) – whether health, disease or intervention. It is a hierarchical science, the tiers of which represent organisational levels including DNA (genomics), RNA (transcriptomics), proteins (proteomics) through to metabolites representing endpoints of metabolism (metabonomics). As a generalisation, systems biology approaches combine high throughput analysis with computational modelling and multivariate statistical analysis, offering new prospects for determining the causes of disease and finding possible cures(33). However, statistical relationships between gene expression and protein levels can be weak and inconsistent, and indicative only of pathophysiological potential rather than phenotype. Mechanistic elucidation of one organisational level does not confer a systematic understanding of the next level, as it does not take account of feedback mechanisms or "external" influences (e.g. drugs, environment, context), as denoted by the principle of emergency complexity (13). In short, proteomics and genomics in the majority of diseases do not provide tangible endpoints for diagnostic biomarkers (33).

Metabonomics is a top-down systems biology approach involving the use of high resolution spectroscopic profiling of small (<1kDa) molecules in combination with computational data modelling permitting the study and modelling of metabolic responses to disease processes or interventions in complex living

systems(34-36). It is a hypothesis-testing or hypothesis-generating technique that attempts to elucidate physiological or pathological pathways by rapid and comprehensive analysis of metabolites, end products of metabolism, encountered in biological specimens.

Spectroscopy, or visualisation of the interaction between electromagnetic radiation and matter, is central to the acquisition of biological data in metabolic profiling. The subsequent application of computational mathematic techniques permits reduction of multiple dimensions, assisting visualisation of results. The extraction of features from the data can be subjected to further structural elucidation and mapping to their corresponding pathways.

Two techniques are relevant to this thesis: Ultra Performance Liquid Chromatography coupled to Mass Spectrometry (UPLC MS) and Proton Nuclear Magnetic Resonance spectroscopy (^{1}H-NMR).

2.2 Mass spectrometry

Mass spectrometry (MS) is a technique used to measure the mass/charge ratio (abbreviated m/z) and relative abundance of ions derived from a sample to be studied. The essential components of a mass spectrometer include an ionisation source, an accelerator, an electromagnet, a detector, and a data output mechanism.

The ionisation source converts molecules in the study sample to ions, permitting manipulation in an electromagnetic field, for detection by MS analysers. Electrospray ionisation (ESI) involves the application of a strong electric field (1-3kV) to a liquid sample in a capillary tube. The droplets created as the liquid passes through the capillary tube are highly charged due to the electric field. Inert gas such as Nitrogen is applied to the sample flow to aide the production of fine aerosol. Furthermore, heated gas is applied causing the solvent to evaporate. Like charges on droplet surfaces forces droplets to separate and become smaller. This continues until the analyte is in the gas-phase. ESL is utilised in both positive and negative phases.

If the ion produced is of an unstable nature, it may further split into fragment compounds. While this can aid the elucidation of molecular structure, if the unstable parent ion has too short a short lifespan (e.g. less than three milliseconds), it may not be detected.

The ions are then accelerated within a vacuum to identical velocities and manipulated into a beam (usually by like-charged plates). The beam of ions is passed through an electromagnetic field towards a detector. Based upon their charge and mass, ions are deflected by the electromagnetic field (the lower the mass and the higher the charge, the greater the deflection). The detector analyses the position at which each ion arrives at the detector, and this corresponds to the m/z ratio. Varying the strength of the magnetic field alters the degree of deflection.

The data output from MS is usually a bar chart depicting the relative abundance of each m/z detected. The most abundant m/z ratio is allocated a relative abundance of 100, and the remaining m/z abundances are distributed accordingly.

2.2.1 Chromatography

Separation of study samples using techniques such as chromatography (gas or liquid) is usually necessary prior to MS analysis. Metabolites are of variable nature (i.e. chemically non-uniform) with considerable variation in their concentration dynamic range(37). Chromatography further increases sensitivity and specificity of analysis and allows detection of different isotopes that have the same m/z ratio. Direct infusion MS is possible, however for complex samples such as serum and urine, poor ionisation can result making large portions of the metabolome undetectable. Metabolites include a wide range of polarities, and ideally the separation technique needs to provide the most comprehensive metabolic profile(38). Three separation techniques are generally applied: gas chromatography (GC), capillary electrophoresis (CE) and liquid chromatography (LC).

Capillary electrophoresis is useful for the separation, identification and quantification of polar compounds and provides high-resolution results, but at present carries significant disadvantages relating to methodology as well as interfacing, thus further developments are necessary prior to this CE gaining widespread employment within metabonomics.

GC serves to separate volatile and semi-volatile compounds. It provides high-resolution results and permits simultaneous analysis of different compound classes, but is not applicable to thermolabile metabolites and challenging for non-volatile compounds, particularly those with high molecular mass. It is a relatively lower-throughput technology(39).

LC permits easier sample preparation than GC prior to analysis and allows separation, identification and quantification of a diverse range of compounds. LC is reliant upon chemical interaction of the analyte with the solid components of the chromatographic column (the stationary phase) and the solvent mobile phase that flows through the column.

LC can be further stratified into High Performance Liquid Chromatography (HPLC) and its successor Ultra Performance Liquid Chromatography (UPLC). UPLC separation involves a stationary phase and mobile phase of differing polarity to separate and deliver hydrophobic and hydrophilic molecules for MS analysis. With its elevated pressures and reduced particle diameter, UPLC improves the efficiency of separation as well as resolution and sensitivity while reducing the analysis time (39).

2.3 Nuclear Magnetic Resonance Spectrometry

The numerous applications of NMR in the biological realm may broadly be organised into the following three territories(40):

- the study of structure (small molecules and proteins)
- the study of metabolism and analysis of complex mixtures (e.g. serum)
- as a non-invasive means to obtain anatomical and functional (e.g. functional MRI) information.

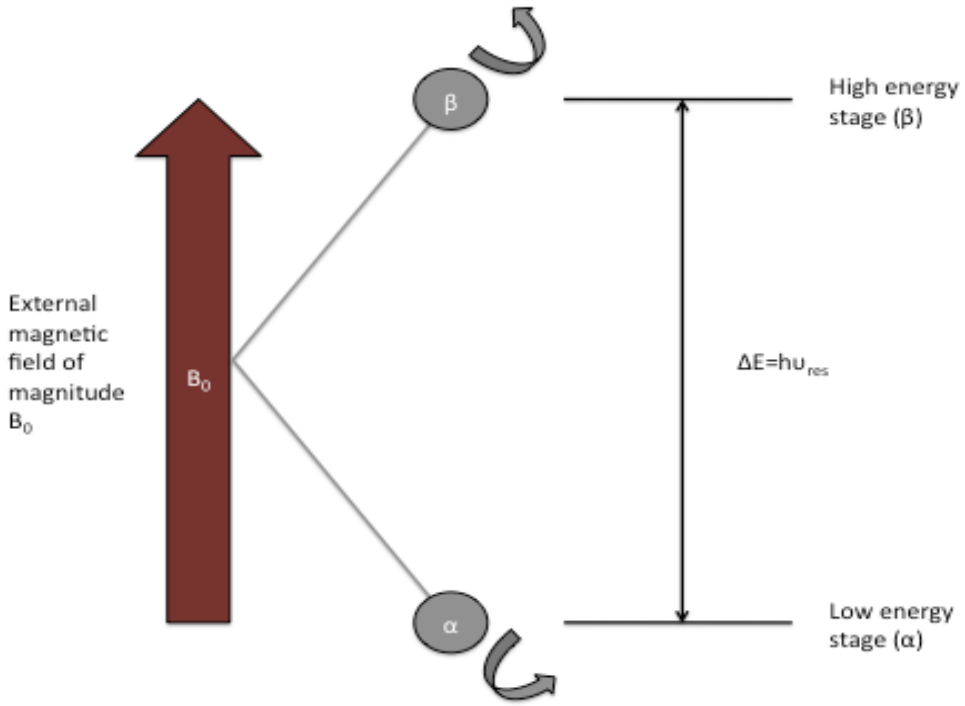


Figure 1: Two possible configurations of spinning nuclei on application of an external magnetic field (B_0). Low-energy configuration: α; high-energy configuration: β; energy required to convert from low-energy to high-energy configuration: ΔE; Planck's constant: h; resonant frequency: υ_{res}.

The fundamental basis of NMR is the property of protons and neutrons known as spin. Spin may be positive or negative and of magnitude in multiples of 1/2, thus nuclei with paired particles (protons or neutrons) where the spin properties of each particle "cancels" the other resulting in no net spin, are not detectable by NMR.

Nuclei that do possess spin behave as tiny bar magnets. Thus when an external magnetic field B_0 is applied, the nuclear spin vector positions itself at a particular angle relating to the direction of the magnetic field. Each spinning nucleus can adopt two possible configurations, one of low-energy (alpha, or +) and one of high-energy (beta, or -).

It is possible for nuclei to switch between configurations through the absorption or emission of quantised energy (Figure 1). The energy difference between the two configurations (ΔE) is related to its frequency υ as shown:

$$\Delta E = h\upsilon$$

where h is Planck's constant. The value for υ in NMR (termed resonance frequency, Larmour frequency, or υ_{res}) is typically between 10 to 80 MHz for hydrogen nuclei.

At equilibrium, there are equal numbers of nuclei in the alpha and beta energy states, and the net magnetism of spins is aligned with the external field. If ΔE then applied, in time T_1, at room temperature, the number of nuclei in the low energy state will outnumber those in the high-energy state. Time T_1 is termed spin-lattice relaxation time.

The ratio of alpha nuclei to beta nuclei (N^{α}/N^{β}) is related to the absolute temperature T (not to be confused with Time), the Boltzmann constant k, and ΔE.

$$N^{\alpha}/N^{\beta} = e^{-\Delta E/kT}$$

The magnetic field experienced by each particle is not homogenous, particularly when considering the motion of particles within a fluid sample. A collection of particles that each experiences the same magnetic field is termed a spin packet. Each packet spins at its own frequency, and thus the net magnetisation begins to dephase. The return to equilibrium is governed by time constant $T_{2,}$ which is termed the spin-spin relaxation time.

Each spinning nucleus is not only influenced by the applied magnetic field B_0, but also the fields produced by nuclei in the immediate vicinity (a concept known as spin-spin coupling), as well as electron clouds that may shield a nucleus from the magnetic field (shielding).

NMR spectroscopy is reliant upon capturing the net difference between ΔE absorbed and ΔE emitted. The data transduced shows net absorption at each resonant frequency. The frequency (Larmor frequency) is determined relative to a reference signal and is termed chemical shift. The Larmor frequency is influenced by the strength of the magnetic field, the type of nucleus, and the surrounding electrons. Thus in its simplest form, an NMR machine comprises a magnet, a radiofrequency generator, a detector, an amplifier, and a data output mechanism.

2.4 Combining UPLC MS and NMR

MS is a highly sensitive technique, which although destructive, can recognise compounds at very small (femtomole) concentrations. Quantification with MS alone can be tricky, since molecules can experience phenomena such as non-specific ion suppression or enhancement.

In comparison to UPLC MS, NMR has a lower sensitivity, but is extremely quantitative and reproducible. Sample preparation is not required prior to NMR, whereas this can be a time-consuming component of MS(41).

Combining the two techniques and employing statistical methods such as principal component analysis (PCA), it is possible to reliably differentiate sample groups based on metabolite composition(42). Targeted approaches are then required to quantify the metabolites.

2.5 Data Mining and Pre-Preprocessing

Data pre-processing is essential to derive accurate, interpretable statistical data from raw spectra. It is a sequential, computational process. Pre-processing serves as the stepping stone between spectral acquisition and multivariate statistical data analysis(43). There are subtle differences between the pre-treatment of MS- and NMR- derived spectral data, but the principals are largely similar.

Baseline correction of acquired spectra is necessary to improve the accuracy of subsequent data analysis.

Peak-picking is the process through which pre-processing software recognises the presence of a spectral peak and recognises it as a region of interest. In observations (samples) where an expected peak is absent, a small value may be designated to prevent statistical confounding introduced by a "zero" value. Grouping then identifies peaks that describe the same feature.

Alignment or retention time correction is the process by which peaks designated to the same metabolic feature across samples are "aligned" to correct for spectral shift during experimental analysis. Alignment stretches or compresses a signal to best match a reference standard(44).

There may be regions of spectra that are not of interest, and these may be removed. Historically, expulsion of data was performed in order to aid computational analysis and storage of data. This is now unnecessary, but regions are still removed to reduce unrequired influence in statistical analysis.

Normalisation, e.g. total area normalisation, is necessary to ameliorate the metabolic impact of variation in sample concentrations on spectral data – particularly relevant to, for example, urine spectra.

2.6 Multivariate Data analysis

Systems biology approaches involve generally high-throughput technology permitting a simultaneous analysis of a large number of samples (observations) producing a high volume of data with the number of variables (features or metabolites) numbering in the hundreds or even thousands. Metabolites are of widely varying classes and composition, although generally small molecules. Furthering this complexity is the absence of a complete catalogue of the human metabolome (or indeed those of less complex organisms) thus unknown compounds may be encountered during analysis. There may be considerable diversity between samples collected from the same organism at varying times, and between organisms with similar phenotypes. Computational data analysis is therefore required to analyse pre-processed data and permit meaningful statistical comparisons between groups to achieve three primary goals: visualisation of differences and relationships between sample and variables, determination of the statistical difference between groups, and identify the metabolites responsible for the observed effect

Two commonly utilised techniques are Principal Component Analysis (PCA) and Projection to Latent Structures (or Partial Least Squares – PLS) analysis. Both PCA and PLS attempt to differentiate between classes despite the presence of substantial intra-class variability (45).

2.6.1 Principal component analysis (PCA)

PCA aims to reduce the dimensionality of multivariate data while maintaining the variability of results that characterise class differences of samples. It is an unsupervised multivariate statistical method, meaning class identification is not applied to the analysis.

Spectroscopic data is considered as a matrix with observations (samples or sample derivatives) and variables (features or metabolites). Each observation represents one coordinate axis to plot the matrix in a Euclidean space. The data is then mean-centred by subtracting the variable averages from the data, such that the data average point becomes the coordinate origin. The first principal component is then the line passing through the origin in K-dimensional space that represents maximal variance of the data. Each data point is orthogonally projected to this component, generating a new coordinate termed a *score*. The second principal component is then plotted orthogonally to the first, and again data points are project to the second component. Two principal components define a plane wherein data-points are re-plotted according to their score values, thereby reducing the dimensionality of the data but maintaining variation. Increasing distance between observations denotes greater dissimilarity.

The contribution of particular variables to the generation of scores is deduced through a loadings plot. Loadings are calculated for each variable from the cosine of the angle between each principle component and the variable coordinate. The greater the distance of each variable from the origin of the loadings plot, the

greater the influence of the variable on separation within the score plot. Correlated variables are close together, and inversely correlated variables are in diagonally opposite quadrants of the loadings plot.

2.6.2 Projection to Latent Structures

PCA is useful to identify data outliers, but its ability to separate classes is dependent upon lower intra-class variability in comparison with inter-class variability. Separation in PCA and the direction of the model generated is based upon the direction of greatest variance, which may not be the direction defining separation of classes (46). PLS utilises a linear multivariate model to relate two data matrices; typically in metabonomic studies, the first matrix (X) is spectral data and the second (Y) may comprise response, toxicity, or class etc. The basic assumption underlying PLS is that X and Y are related.

In PLS analysis, observations are plotted in the Euclidean space as with PCA, but the first principal component is situated such that it correlates well with the nebula of observations in the X matrix as well as the direction of data in the Y matrix. The scores generated from the first principal component may be used to estimate response data in Y. The differences between estimated and measured response data are termed *residuals*(47) – the stronger the model, the smaller the residuals. Subsequent principal components are aligned such that they describe X with while correlating well with the residuals in Y.

When the second data matrix denotes qualitative information such as class identity of observations, then the purpose of PLS is to discriminate between

classes and is therefore termed Discriminant Analysis (DA). The accuracy of PLS-DA improves with an increasing number of variables, but the complexity of interpretation increases with greater number of classes (46).

Orthogonal PLS-DA (OPLS DA) is a recent descendant of PLS-DA that functions to remove variation not relevant to the biological class in order to better interpret the variables that define the class. This is accomplished by separating the systematic variation of X into two components – one that correlates with Y and one that is unrelated to Y(43).

2.6.3 Strength and interpretation of models

The explained variation of a model or "goodness of fit" is denoted by the R^2Y value, whereas the "goodness of prediction" is denoted by Q^2. R^2Y ranges between 0 and 1 and is usually higher than Q^2, but when the value of R^2Y disproportionately exceeds Q^2, the model is said to be "overfitted". Ideally, R^2Y should not exceed Q^2 by 0.2-0.3, but increases with the complexity of the model, the number of variables and number of components and will rapidly approach 1. On the other hand, Q^2 will rise prior to an eventual fall. The ideal number of components provides the peak value of Q^2 without overfitting the model (Figure 2).

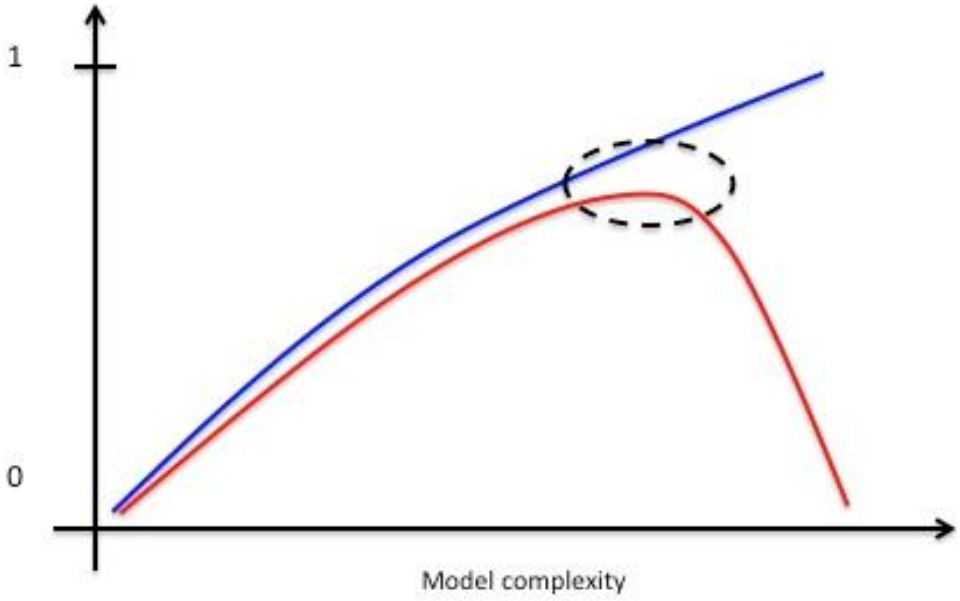


Figure 2: The theoretical relationship between R^2Y and Q^2 and model complexity. The blue line represents R^2Y and the red line represents Q^2. The optimum number of components provides peak values of Q^2 without overfitting the model (dashed oval).

Chapter 3: Application of Metabonomics to Vascular Disease

Atherosclerosis offers several niche roles for metabonomic biomarkers. These include screening for detection of disease, risk assessment in the presence of disease, prognostic in established or symptomatic disease(48), perhaps even failure to respond to treatment(49). It has been shown that pre-pathological conditions such as hypertension carry a distinct diagnostic metabolic profile(50, 51). Although a definitive and independent biomarker of risk remains elusive, metabonomic research in cardiovascular sciences is advancing.

The pioneering studies of metabolic profiling in atherosclerosis were applied to ischaemic heart disease. The potential of metabonomic biomarker research in myocardial ischaemia was explored by Sabatine et al in an LC MS study including 18 patients with suspected ischaemic heart disease (based on positive exercise-ECG testing) and 18 controls with no evidence of inducible ischaemia. Blood was collected from patients before, during and after exercise-ECG testing, and concentrations of metabolites were compared before and after exercise between cases and controls. Six metabolites were identified that differentiated cases from controls with high accuracy(52). More recently a gas chromatography (GC) MS study has identified palmitate as a biomarker of stable atherosclerosis in a Chinese population(53). Brindle et al showed that ^{1}H-NMR spectra of human serum could diagnose the presence and extent of coronary artery disease with sensitivity and specificity exceeding 90%. This technique can potentially be applied to rapidly and accurately diagnose the presence of coronary heart disease as a method of population screening, or to permit appropriate and effective targeting of risk-lowering medications (such as statins)(54).

3.1 Peripheral Arterial Disease

Relatively few metabonomic studies have been performed in peripheral arterial disease. However a recent plasma 1H-NMR study of patients with peripheral arterial disease has permitted the development of classification models predicting near-term all-cause death with overall accuracy of 83%, sensitivity 80% and specificity 86% (55).

3.2 Abdominal Aortic Aneurysm

Abdominal aortic aneurysm (AAA) is a common disorder thought to occur in 8% of the western population over the age of 65(56). Death secondary to aortic aneurysm rupture occurs in 1-2% of males(57), with other cardiovascular causes claiming responsibility for the majority of mortality in patients with AAA. Best medical therapy in aneurysmal disease is aimed at reducing cardiovascular mortality. Specific treatment to prevent aneurysm formation (primary prevention) or subsequent aortic dilation (secondary prevention) is not currently available.

Aneurysm development is a complex process, thought to involve inflammation, proteolytic extracellular matrix degradation, smooth muscle apoptosis, and oxidative stress(58, 59). The typical clinical risk factors for aneurysm formation (advancing age, male gender, smoking, hypertension, dyslipidaemia) are common to all atherosclerotic diseases, and it is uncertain why aneurysms may develop as opposed to, or in conjunction with, stenosing arterial disease (60).

There is evidence of a strong genetic component; family history is, after smoking, the most significant risk factor for AAA(61). Genetic studies indicate causality to be multifactorial, but aberrations relating to lipid and proteolytic pathways are frequently encountered(62).

In the UK, men are offered a screening ultrasound scan at the age of 65 to determine the presence of an abdominal aortic aneurysm. While screening reduces rupture-related mortality in men (not women, though AAA prognosis is worse in women(56)), it does not decrease all-cause mortality at 3-5 years(63). Ultrasound is an operator dependent modality, and abdominal views may be obscured by bowel gas, or challenging to obtain due to body habitus. Suprarenal aneurysms may be missed altogether. At present ultrasound is the only feasible option for screening, and there is no biomarker of adequate sensitivity or specificity to serve as a substitute.

Intervention is indicated for asymptomatic aneurysms when their diameter approaches 5.5cm, or if the aneurysm diameter increases by more than 1cm per year(64). The decision to intervene at 5.5cm is based upon data from now aged clinical trials that demonstrated the risk of rupture to exceed the risk of operative repair beyond this size(64). Although still adhered to, this data did not take into account current evidence-based medical prevention strategies for cardiovascular disease – statins(65). Furthermore, sub-threshold aneurysms can rupture and larger aneurysms may remain intact(66, 67). Pathological factors increasing the risk of rupture are poorly defined, and rates of growth are non-uniform within and between individuals(68). A meta-analysis published in 2013

has shown improved in-hospital or 30-day mortality following endovascular aneurysm repair (EVAR) and open surgery (1.3 % EVAR vs. 4.7% open repair; odds ratio (OR) 0·36, 95% confidence interval 0·21 – 0·61; P < 0· 001), but clinical thresholds for intervention remain unchanged(69).

Cardiovascular diseases such as aortic aneurysm and atherosclerosis obliterans are complex, multi-causal disorders that are the product of genetic predisposition, lifestyle and environmental influences – entities that are reflected in the variation of metabolites(34). Metabolic carries the advantage of delivering diagnostic biomarkers as well as unravelling physiological and pathological pathways that define disease phenotypes, generating possibilities for therapeutic modulation(70).

This section examines the scope of metabolic profiling within current aneurysm research, summarising existing evidence in domains of clinical need: diagnostic biomarkers, prognostic biomarkers, mechanisms of disease development, and potential therapeutic targets.

3.2.1 Methods

A systematic review has been performed adhering to PRISMA guidelines. Medline and Embase databases were searched using the Ovid portal for all aneurysm studies pertaining to metabonomics on 7th February 2016. The search string utilised was [(aort* AND aneurysm) AND (metabonomics OR metabolomics OR “metabolic profiling” OR “mass spectro*” OR “nuclear magnetic resonance spectro*” OR NMR)]. All original research articles applying metabolic

profiling to samples collected from patients with aneurysms were included. Non-human studies and reviews were excluded.

3.2.2 Results

Seven articles relevant for inclusion were identified (Figure 3). Table I summarises the scope of these studies. All studies utilised MS(71-77) and one additionally incorporated Proton NMR (1H-NMR)(74). Four studies investigated the metabolic profile of plasma or serum from patients with aortic aneurysms(71, 74-76), and three studies examined differential metabolic profiles of aortic tissue(72, 73, 77).

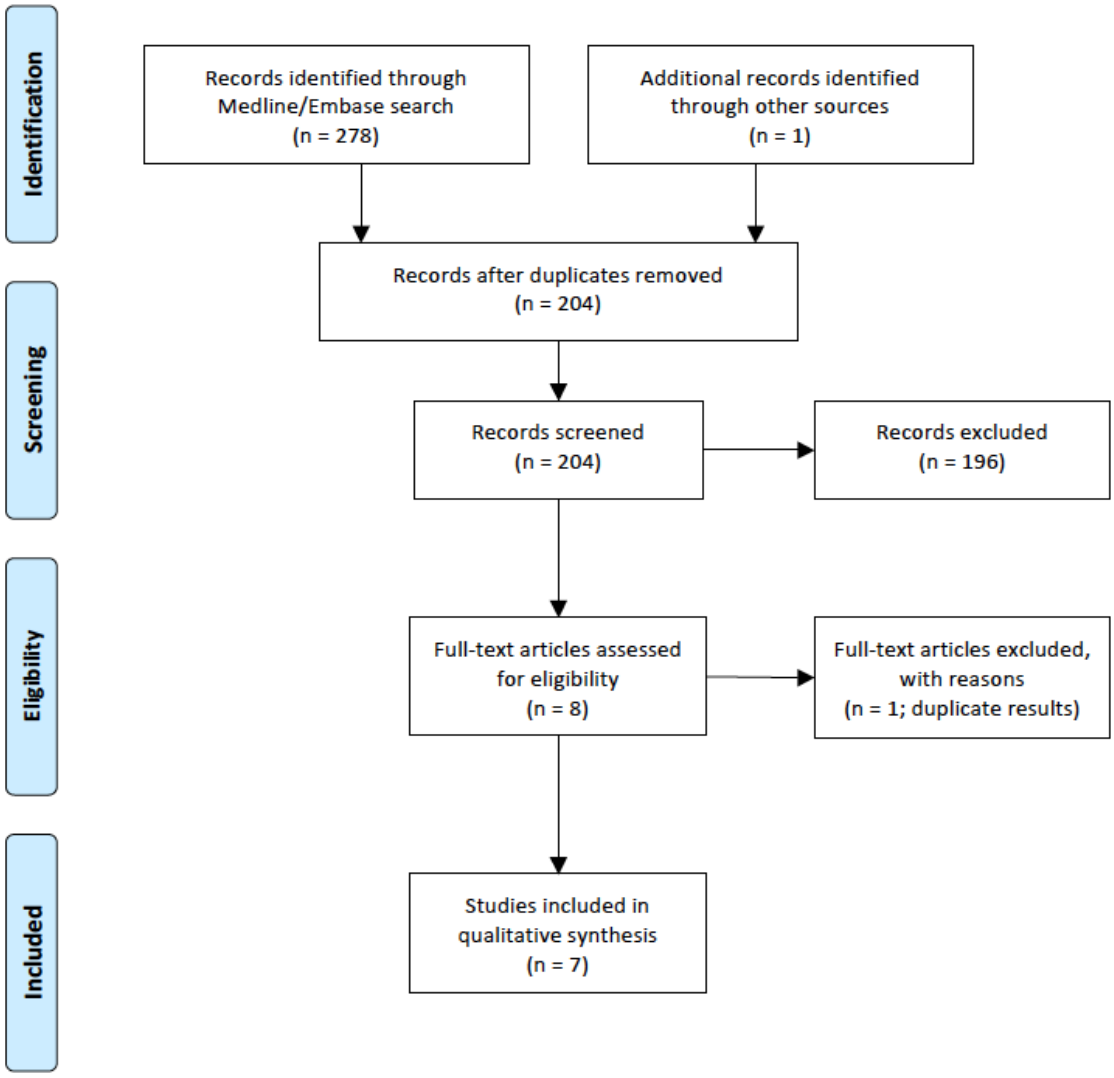


Figure 3: PRISMA diagram summarising search process resulting in 7 AAA metabonomic studies included for review.

Table I: Current metabolic profiling studies of aneurysmal disease

1st Author	Year	Origin	Description	Study size	Control group	Substrate	Technique	Targeted/ untargeted
Cibrowski (77)	2010	Spain	Comparison of metabolites secreted by aneurysmal aortic wall, healthy aortic wall and intraluminal thrombus	Aneurysm tissue: 8 Normal aortic tissue: 4 Intraluminal thrombus: 9	Cadaver derived macroscopically normal aorta	Tissue and thrombus	HPLC MS	Untargeted
Pillai (75)	2012	USA	Temporal comparison of metabolite variation following release of operative aortic cross-clamp	Patients: 15	Pre-clamp blood sample	Plasma	HPLC MS in conjunction with enzyme immunoassay	Targeted (Lipids)
Ruperez (74)	2012	Spain	Comparison of plasma metabolites between patients with small and large aneurysms and age- and sex-matched controls	Patients: 22 Controls: 11	Age and sex matched controls	Plasma	GC MS 1H-NMR	Untargeted
Cibrowski (71)	2012	Spain	Comparison of plasma metabolites between patients with small and large aneurysms and age- and sex-matched controls	Patients: 30 Controls: 11	Age and sex matched controls	Plasma	HPLC MS	Untargeted
Moxon (76)	2014	Australia	Comparison of lipid metabolites between patients with aneurysms and those with peripheral arterial disease	Patients: 161 Controls: 168	Peripheral arterial disease	Serum	HPLC MS	Targeted (Lipids)
Vorkas (72)	2015	UK	Demonstrates complementary expansion of metabolome coverage by incorporating complementary methods of chromatography when analysing tissue including AAA	Aneurysm: 26 Femoral plaque: 26 Carotid plaque: 52 Intimal thickening: 16	Intimal thickening tissue	Tissue	UPLC MS	Untargeted

Tanaka (73)	2015	Japan	Demonstrates spatial distribution of lipid metabolites in aneurysm sac as compared with aneurysm neck tissue	Patients: 30	Aortic tissue from normal diameter neck of aneurysm	Tissue	Imaging MS	Targeted (Lipids)

Table II: Circulating metabolites with >50% alteration in the presence of a large aneurysm

Author & Year	Metabolite	Technique	Change in large aneurysm	Coefficient of variance	Multiple testing correction	Goodness of fit	
Ruperez 2012 (74)	Pyruvate*	GC MS	Increased	Not given	None	0.577	0.118
	2-Hydroxybutyrate*	GC MS	Increased				
	Asparagine*	GC MS	Increased				
	Caproate*	GC MS	Increased				
	Caprylate	GC MS	Increased				
	Phosphate	GC MS	Increased				
	Aminomalonate*	GC MS	Increased				
	Citrate*	1H NMR	Increased	Not given	Not given	0.846	0.313
	Creatine	1H NMR	Increased				
	Acetoacetate*	1H NMR	Increased				
	Formate	1H NMR	Increased				
	Glycerol*	1H NMR	Increased				
	Methanol	1H NMR	Decreased				
Cibrowski 2012 (71)	Decanoylcarnitine	HPLC MS	Decreased	6	None	0.852	0.369
	Dodecanolycarnitine	HPLC MS	Decreased	15			
	Tetradecanoylcarnitine	HPLC MS	Decreased	6			
	Palmitoylcarnitine	HPLC MS	Decreased	11			
	Linoleylcarnitine	HPLC MS	Decreased	7			
	Vaccenylcarnitine OR oleoylcarnitine	HPLC MS	Decreased	13			
	Stearoylcarnitine	HPLC MS	Decreased	46			
	Guanidinosuccinic acid*	HPLC MS	Increased	14			
	Cholanoic acid derivative*	HPLC MS	Decreased	21			
	C27 bile acid	HPLC MS	Decreased	11			
	Hydroxydecadienediynoic acid	HPLC MS	Decreased	10			
	LysoPCs	HPLC MS	Decreased	8 - 42			
	LysoPEs	HPLC MS	Decreased	8 - 20			
Moxon 2014 (76)	Triglyceride 18:2/18:2/18:2	HPLC MS	Increased	<30	Benjamini-Hochberg correction	Not given	

*Metabolites with opposite direction of change in small aneurysms as compared with controls

Circulating biomarkers of aneurysmal disease

Three studies performed metabolic profiling of plasma or serum to identify metabolites associated with aneurysm presence, incorporating GC MS, HPLC MS and ^{1}H-NMR(71, 74, 76). Table II summarises statistically significant metabolites associated with large aneurysm presence and altered by at least 50% from controls. These metabolites pertain to pathways predominantly relating to glycolysis, cholesterol and lipid metabolism, and amino acid metabolism.

A biomarker is a "substance or characteristic that is objectively measured and evaluated and is used as an indicator of a biological state"(13). A good biomarker is sensitive, specific, and highly reproducible with stable and predictable release and clearance. Therefore endogenous molecules with very small variations from control levels are unlikely to serve as credible clinical markers. Perplexingly, a considerable number of metabolites found to be significantly and markedly altered in the presence of a large aneurysm, showed the opposite trend in patients with small aneurysms: pyruvate, citrate, 2-hydroxybutyrate, asparagine, acetoacetate, caproate, glycerol, aminomalonate and guanidinosuccinic acid were increased in patients with large aneurysms but decreased in patients with small aneurysms when compared to controls(74). Cholanoic acid derivative was decreased in patients with large aneurysms, but increased in patients with small aneurysms. Such paradoxical alterations serve as a considerable hindrance to biomarker utility – as normal levels may occur in the absence of an aneurysm, or perhaps with an enlarging aneurysm.

Plasma metabolites associated with the largest difference between control and disease levels for each metabonomic separation technique are aminomalonic acid (AMA; GC MS)(74), guanidinosuccinic acid (GSA; HPLC MS) (71) and glycerol (1H-NMR)(74).

GSA was increased in the plasma of patients with large AAA as compared to controls (309% increase; p=9x10-10)(71). Six of the 15 study patients with large aneurysms had intraluminal thrombus, whereas none of the patients with small or no aneurysms demonstrated intraluminal thrombus. The same research group had shown preferential release of GSA from intraluminal thrombus as compared with aortic aneurysm wall and healthy wall in an earlier study(77). GSA is produced by the transamidination of arginine in the urea cycle and is increased in the plasma of uraemic patients. It is hypothesised that GSA exerts neurotoxic effects through the NMDA receptor and activation of nitric oxide and cGMP(78). Nitric oxide has long been associated with aneurysm progression (79-81). LC MS is a reliable and sensitive method of GSA detection and quantification(82), but despite demonstration of its presence, the role of GSA in thrombus and/or aneurysm formation is undetermined. Paradoxically, GSA plasma levels were decreased in patients with small aneurysms as compared with controls(71).

AMA plasma levels follow a similar pattern to GSA; AMA was decreased in patients with small aneurysms, but increased 830% in patients with large aneurysms(74). Despite association with atherosclerosis described as early as 1984(83), little is known about the role of AMA in cardiovascular disease. AMA is not produced by hydrolysis of any amino acid and is unlikely to be an

experimental artefact, though may be produced through oxidative damage to amino acids or errors in protein synthesis(84).

Glycerol is generated from the hydrolysis of triglycerides by lipase, also releasing fatty acids. 1H-NMR revealed a 127% increase in glycerol in large aneurysms as compared with controls, but again glycerol was decreased in patients with small aneurysms(74). The same study revealed all fatty acids except laurate to be significantly increased in patients with large AAA than controls, indicating a greater degree of lipolysis. These changes were mirrored by higher glucose, pyruvate and ketone levels, indicating a potential causative role for insulin resistance and metabolic syndrome. Although the study used fasting blood samples from groups that were age and sex matched, no information is provided regarding baseline patient characteristics, including pharmacotherapy, lipid levels or diabetic status(74).

Untargeted metabolic profiling studies of plasma have incorporated a small number of patients, with control groups unmatched for pertinent cardiovascular characteristics that can influence the metabolome, in particular pharmacotherapy. It is possible that patients with large aneurysms were more likely to be prescribed medication that may reverse metabolite alterations seen in smaller aneurysms. Although statistically significant metabolites were described, no correction method was applied to reduce the likelihood of false positive results (particularly prevalent in metabolic profiling as a large number of variables are analysed), and if the statistical significance is weak, it may not withstand multiple testing correction. Only Ruperez et al. provided Coefficient of

Variance (CV) values for significant metabolites in quality control samples, but those with higher CVs were not excluded from multivariate analysis (74). Goodness of fit of the multivariate statistical models is denoted by values of R^2Y (explained variance) and Q^2 (predicted variance). Thus plasma metabolites are generally weak to moderately predictive of AAA presence, with R^2Y values considerably larger than Q^2, implying the models are statistically over-fitted (71, 74).

Where individual metabolites lack sensitivity or specificity to serve as solitary biomarkers, combinations of metabolites (biomarker signatures) may be combined with clinical characteristics to develop risk scores for patients, as demonstrated by Moxon et al in a statistically robust targeted lipidomic study comparing serum metabolites derived from 161 patients with AAA (>4.0cm) and 168 controls that were highly representative of patients with non-aneurysmal peripheral arterial disease (PAD). AAA patients were less likely to be diabetic (thus less likely to be prescribed Metformin) (76). Very small variations in age, HDL, CRP and Creatinine between the groups nonetheless produced highly significant differences ($P<0.001$). As a result, the subsequent statistical analysis was adjusted for differing baseline characteristics, with P values generated from regression analyses (Benjamini-Hochberg correction for false discovery rate). Three diacylglycerols and three triacylglycerols were significantly different between AAA and PAD groups, and this difference was maintained in a subsequent age-matched analysis. Lipidomic features were combined with traditional risk factors to create classification models, which significantly improved prediction of AAA presence (mean area under receiver operator

characteristic curve 0.760 [0.756-0.763] compared with 0.719 [0.716-0.723] for traditional risk factors alone; P<0.05).

Mechanisms of disease development

The alteration of metabolites in aneurysmal artery wall as compared with normal arterial wall can generate hypotheses regarding mechanisms of disease development. Cibrowski et al. utilised HPLC MS to analyse secretomes from cultured aneurysmal and normal aortic wall tissue(77). They demonstrated increased secretion of leukotrienes, LysoPC, acylcarnitine and 5-oxoprolene from the aneurysmal wall, but decreased release of oleamide (with associated variations in fatty acid metabolites) and vitamin E from aneurysmal as compared to normal aortic wall(77). These metabolites highlight the importance of inflammation and lipid metabolism pathways in aneurysm formation. However, normal tissue was derived from cadaveric organ donors with macroscopically normal aorta with no further elaboration on the presence or absence of salient diseases that can either influence arterial structure (e.g. vasculitis or connective tissue disease) or alter the metabolome (e.g. malignancy). Baseline patient characteristics were also not available, and it is unknown whether differences in lipid profiles or other cardiovascular characteristics could account for these differences.

Metabonomic comparison of AAA tissue with carotid plaque and femoral plaque (dilating, embolising and stenosing disease, respectively) as well as intimal thickening (representing early atherosclerosis) has revealed interesting results.

Separation of the four tissue groups was apparent with supervised multivariate statistical analysis (72). The resultant models were well-fitted and demonstrated high predictive values. Marked similarities were observed between the femoral plaque and AAA groups. From a methodological viewpoint, this analysis demonstrates the importance of using a broad-based metabonomic approach when performing an untargeted study; here dual analysis with contrasting yet complementary methods of chromatography provided a wealth of additional metabolic information (72).

The spatial distribution of salient metabolites can be demonstrated with Imaging Mass Spectrometry (IMS) – a two-dimensional MS method that detects metabolites within tissue sections, with resultant data providing information regarding location of metabolites(85). In combination with MALDI (Matrix Assisted Laser Desorption Ionisation), IMS provides spatial resolution sufficient to provide cell-specific information(85). One such MALDI-IMS targeted lipidomic study compared tissue from aneurysmal sac with that of normal-diameter aneurysm neck(73). Although multiple testing correction was not applied, and the number of patients included were relatively small (30 aneurysm patients), this study benefited from the advantage that corresponding neck and sac tissue were excised as longitudinal strips from each patient undergoing elective (non-ruptured) open aneurysm repair – allowing patients to serve as their own controls. Results demonstrated preferential incorporation of triglycerides within adipocytes in the adventia – the arterial layer primarily contributing to vessel tensile strength, resulting in the disruption of adventitial collagen. Triglycerides were significantly increased in the adventia compared with the intima and

media, and also in the aneurysm sac as compared with the neck. Correspondingly, the number of Adipocyte-Derived Stem Cells was increased in the aneurysm sac compared with the neck, and much lower in normal aorta(73). In future, in vivo quantification of adventitial lipids or adipocytes could provide an individualised estimate of risk of rupture.

Patterns of variation in groups of circulating metabolites can also render hypotheses regarding aetiology of disease. The seesaw nature of metabolite alteration between small and large aneurysms may reflect the complexity of disease mechanisms. Ruperez et al demonstrated that plasma from patients with larger aneurysms had higher measured concentrations of glycerol, glucose, pyruvate, lactate, free phosphate and ketones, but lower concentrations of fructose(74). This suggests that glycolysis plays a more significant role in energy supply in patients with large aneurysms than in age and sex matched controls with normal aortic diameter. That such variations are also found in diabetic ketoacidosis serves as a paradoxical finding, as diabetes is thought to be protective against aneurysm formation(86), a consequence of glycolysation of the vascular matrix(87). In the absence of data pertaining to patient baseline characteristics, it is unknown whether a higher proportion of patients with large aneurysms were diabetic, and if diabetic control was suboptimal. Additionally, metabolites relating to anaerobic glucose metabolism were not discovered in the tissue analysis by Cibrowski et al(77).

Implications for surgical treatment

Pillai et al investigated the temporal relationships between chemical mediators of inflammation and resolution in patients undergoing open AAA repair by collecting sequential blood samples prior to aortic cross clamping and subsequently at 5-minute, 30-minute, 6-hour, 24-hour, 48-hour and 72-hour time intervals following unclamping of the aorta(75). A semi-targeted lipidomic analysis of plasma was performed in conjunction with enzyme immunoassays of cytokines, leukotrienes and prostaglandins associated with inflammation and resolution. Consistent trends in metabolite variations were obtained for patients, and the authors demonstrated that mediator profiles grouped all 15 study patients into two distinct phenotypes – a pro-inflammatory and a pro-resolver phenotype, distinguishable based on temporal trends in levels of Lipoxin-A4, 15-epi-Lipoxin-A4, Leukotriene-B4, -C4, -D4 -E4, inactive Thromboxane-B2 and Prostaglandin-E2(75). That the baseline levels of these mediators show distinct differences prior to aortic clamping implies a degree of predictability of subsequent time course. It remains to be seen whether the distinction between the two phenotypes translates into differential outcomes for patients, and larger studies would be required to confirm associations between metabolic signatures and, for example, peri-operative outcomes and length of stay.

No studies examined the metabolic consequences of endovascular aneurysm repair.

Therapeutic targets

The role of pharmacological modulation of AAA subjects has not been the subject of metabonomic interrogation. However inferences may be drawn from existing work generating hypotheses for future drug design.

The development of pharmacological treatment of AAA patients may stem from several strategic aims:

- Reduction of overall cardiovascular risk
- Primary prevention of AAA
- Prevention of AAA growth/rupture
- Adjunctive therapy to improve outcomes of patients undergoing intervention

Statins and antiplatelet agents are well established in reducing cardiovascular risk(88, 89), but both statins and antiplatelet agents each represent a broad class of drugs of differing properties(90). The inflammatory nature of aneurysms may benefit from statins(91-93) and antiplatelet agents with the most potent anti-inflammatory properties (94, 95).

AAA presence has been associated with perturbations in metabolites linked with the development of metabolic syndrome. Therefore primary prevention of atherosclerotic aneurysms is likely associated with treating and preventing the manifestations of metabolic syndrome(71, 74). Aneurysms resulting from

infection, local inflammation (e.g. discitis) or collagen disorders are uncommon entities and have not been included by metabonomics studies to date.

To determine metabolic variations associated with aneurysm growth, longitudinal metabonomic studies are required incorporating larger study populations with sequential biofluid sampling. Similarly, to determine metabolic associations with ruptured aneurysm, a comparison of metabolic fingerprinting of ruptured and non-ruptured aneurysmal tissue may provide further insights. Present studies do not permit further comment on growth or risk of rupture.

As an adjunct to surgical therapy, certain possibilities for therapeutic modulation have been generated, stemming from the demonstration that patients can exhibit either a pro-inflammatory or pro-resolving metabolic phenotype(75). Pillai et al. have outlined that some pro-resolving metabolites are inducible by aspirin. Of the 15 study subjects, 12 were prescribed aspirin, and it is not known whether there was a significant difference in this and other patient characteristics between the pro-inflammatory and pro-resolving groups. The influence of higher-dose aspirin on subjects undergoing AAA repair may then be considered (balancing the risk of peril-operative haemorrhage), or targeted to patients exhibiting a pro-inflammatory phenotype, correlating resulting metabolite variations with peri-operative outcomes.

3.2.4 Discussion

This review demonstrates the feasibility of metabonomics in AAA research. It also highlights the relative paucity of published studies within this domain – three of the seven identified studies stem from one research group(71, 74, 77). As such, it is not possible draw hard conclusions from identified publications.

As pilot studies, several limitations are identifiable in metabolic profiling aneurysm research to date: most studies incorporate small patient numbers (and smaller control groups) that are not matched for cardiovascular risk factors, which can cause in distortion of metabonomic studies and deliver results that do not withstand correction for relevant patient variables. Disease mechanisms in cardiovascular illness are complex, and this is reflected in the diverse, non-uniform trends observed in levels of significant metabolites.

Nonetheless, several further avenues of research are suggested from the initial results, but require verification and clinical correlation. In particular, the applicability of GSA, AMA and glycerol as potential diagnostic biomarkers of large AAA require further investigation(71, 74).

Future metabolic profiling studies of aneurysmal disease need to incorporate large cohorts with stratification of cardiovascular risk factors and consideration of therapy such as statins. Robust statistical analysis is required, incorporating multitesting correction strategies to minimise type 1 error. Sequential sampling will permit patients to serve as their own controls in longitudinal studies that correlate metabolites with aneurysmal growth rates. Tissue comparisons of

ruptured and non-ruptured aneurysmal wall will address as yet unanswered questions regarding tendency to rupture and broaden the possibilities for therapeutic modulation to prevent rupture. Studies examining the metabolic consequences of intervention should consider endovascular as well as open surgical treatment. Finally, incorporation of complementary omics techniques, such as genomics, transcriptomics, proteomics and metagenomics will allow elucidation of pathological pathways leading to disease development.

3.2.5 Conclusion

Current studies demonstrate the utility of metabonomic science in identifying potential biomarkers of aneurysm presence and elucidating underlying mechanisms underlying dilating arterial disease. Potential biomarkers of large aneurysm presence have been suggested and require verification. Further translational studies incorporating larger, matched cohorts are required for validation and to address the hiatus in understanding aneurysm growth, while developing effective treatment strategies.

3.3 The metabonomics of stroke

Optimising the management of stroke patients in the hyperacute setting carries the potential to significantly improve clinical outcomes. Despite the technological advancement of modern medicine, the diagnosis of acute stroke remains a challenging entity, subject to the expertise of the attending healthcare provider and the diagnostic resources available(96). Computerised Tomography imaging

of the brain is only 30% sensitive in the diagnosis of acute cerebral ischaemia. Diffusion-weighted imaging may confer greater sensitivity, however it is time consuming, costly, of limited availability and may not be feasible depending on the patient's circumstances. There remains a clinical need to develop an objective test to confer rapid and accurate diagnostic discrimination of stroke to enable the provision of rapid therapy and better direct further investigation.

3.3.1 Biomarkers of stroke

A good biomarker is sensitive, specific, and highly reproducible with stable and predictable release and clearance. In the context of acute stroke, the ideal biomarker would permit rapid objective diagnosis of stroke, differentiate between haemorrhagic and ischaemic stroke, and indicate short-term prognosis to dictate immediate management.

A vast number of potential biomarkers of stroke have been researched, but none have been incorporated into routine clinical practice(97). Although the design of many biomarker studies have merited improvement(97), it is important to remember that stroke is a heterogeneous condition afflicting a globally varied population and encompassing a wide array of causalities that include thrombosis, embolism and haemorrhage. Thus using a combination of biomarkers may be more feasible when the underlying pathology is so variable(98, 99).

Encasing this complexity is the impermeability of the blood brain barrier, the disruption of which in the context of stroke is non-uniform and dependent upon manifold factors that include the type and magnitude of stroke. This imposes a delay in the ability of neuronal and glial proteins to reach peripheral circulation. More generalised markers may also be released in a variety of stroke mimic conditions, thus failing in specificity(100).

3.3.2 Metabonomics and stroke

The extension of metabonomics to stroke research is a valid and necessary extension of current cardiovascular and neurological applications. The applicability of metabonomics in stroke research has been examined through exploration of existing metabonomics studies in this field.

3.3.3 Methods

A systematic review adhering to the principles of Preferred Reporting Items for Systematic Review and Meta-Analyses was performed. Medline and Embase databases were searched using the Ovid Portal on 29th August 2015 with no restrictions on date of study. The search string utilised was [(metabonomics OR metabolomics) AND ("cerebrovascular attack" or CVA or stroke)].

Inclusion and exclusion criteria

All full-length English language articles utilising MS or NMR for metabolic profiling in human stroke patients were included. The following types of studies were excluded:

1. Studies subdividing patient groups according to traditional Oriental medicine (Chinese and Korean traditional medicine)
2. Animal studies
3. Abstracts of non-published studies

Quality assessment

The quality of included studies was assessed using QUADOMICS – a quality assessment tool for systems biology diagnostic studies, based on an adaptation of QUADAS(101). Pre-existing standards for assessing the quality of diagnostic experimental design were ill-suited for omics-based disciplines. Systems biology research encounters hurdles both common and distinct from traditional diagnostic research. However QUADOMICS applies only to diagnostic, and not prognostic studies.

3.3.4 Results and discussion

Following application of the search strategy and deduplication, 104 articles were screened, and four studies were included for summation analysis (102-105) (Figure 4). Table IV depicts the characteristics of included studies and summarises perturbations in the metabolites of stroke patients. Three studies aimed to determine diagnostic biomarkers of stroke (102, 103, 105), of which one was a targeted mass spectrometry study (102), and two were untargeted MS and NMR studies (103, 105). Only one study explored biomarkers depicting recurrent stroke risk following TIA using MS (104). All included studies

examined blood (plasma or serum) biomarkers(102-105), and one diagnostic study also analysed urinary metabolites using NMR(104).

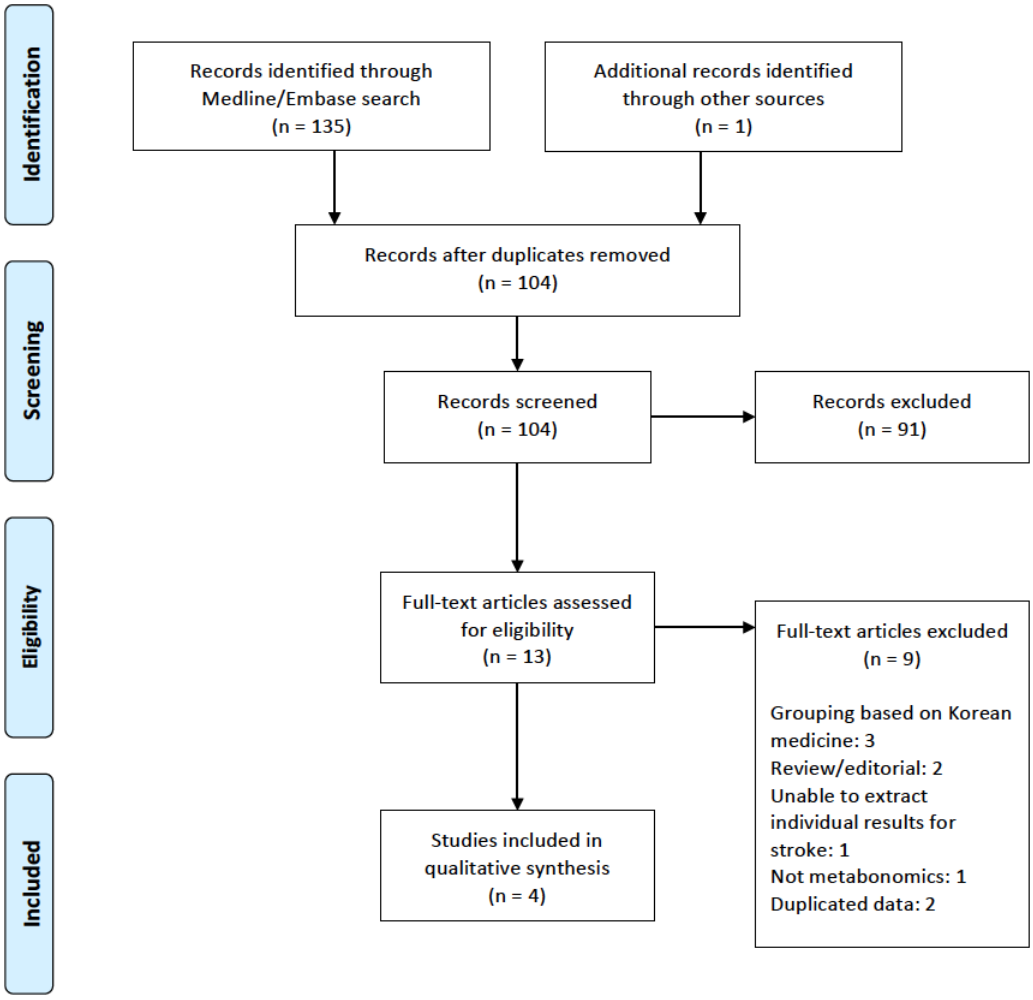


Figure 4: PRISMA diagram summarising search process resulting in 4 stroke metabonomic studies included for review

Quality of included studies

Table III itemises the QUADOMICS assessment of included diagnostic studies. No quality assessment criteria exist for prognostic omic studies, therefore the prognostic study by Jove et al. could not be included for quality assessment.

QUADOMICS was employed as the quality assessment tool as it was the only validated measure of quality of metabonomics study that was discovered following a literature review. A separate Medline search for "QUADOMICS" yields only four results(101, 106-108) – the original publication of the tool(101), its validation(107), and subsequent application in two systematic reviews(106, 108). The utilisation of QUADOMICS is therefore limited, despite employing a comprehensive assessment methodology.

In metabonomic stroke research (particularly hyper-acute stroke), aspects of QUADOMICS are inapplicable or inconsequential: a) stroke is a clinical diagnosis, there is no single reference standard that can be applied or objectively measured, particulary for hyper-acute patients; b) depending on the definition of stroke applied to a study, sample collection from hyper-acute patients will, by definition, be performed prior to diagnosis (if the criteria is simply neurological deficit resulting from focal cerebral ischaemia, persisting beyond 24 hours); c) acute stroke is seldom static in its symptomatology and it is probable that clinical fluctuations will occur between sample collection and final diagnosis/categorisation of subject; d) interpretation of index test is likely to require supervised multivariate statistical analyses (such as OPLS) where class specification is necessary, thus cannot proceed without incorporating the results of the reference standard; e) finally, high-throughput mechanisms inbuilt in omic science together with the sensitivity of technique (particularly of mass spectrometry) frequently necessitate analysis of all study samples together and detection of, in the order of, a thousand metabolites per sample – knowing the results of the reference standard is unlikely to bias results.

Table III Quality assessment of included diagnostic metabolic studies of ischaemic stroke

QUADOMIC QUALITY ASSESSMENT CRITERIA	Kimberly Stroke 2013	Jung Stroke 2011	Jiang Talanta 2011
1. Were selection criteria clearly described?	YES	YES	YES
2. Was the spectrum of patients representative of patients who will receive the test in practice?*	YES	N/A	N/A
3. Was the type of sample fully described?	YES	NO	NO
4. Were the procedures and timing of biological sample collection with respect to clinical factors described with enough detail?			
4.1. Clinical and physiological factors	NO	NO	NO
4.2. Diagnostic and treatment procedures.	NO	NO	NO
5. Were handling and pre-analytical procedures reported in sufficient detail and similar for the whole sample? And, if differences in procedures were reported, was their effect on the results assessed?	YES	NO	NO
6. Is the time period between the reference standard and the index test short enough to reasonably guarantee that the target condition did not change between the two tests?	YES	NO	NO
7. Is the reference standard likely to correctly classify the target condition?	YES	UNCLEAR	YES
8. Did the whole sample or a random selection of the sample receive verification using a reference standard of diagnosis?	YES	UNCLEAR	YES
9. Did patients receive the same reference standard regardless of the result of the index test?	YES	UNCLEAR	YES
10. Was the execution of the index test described in sufficient detail to permit replication of the test?	NO	YES	YES
11. Was the execution of the reference standard described in sufficient detail to permit its replication?	YES	NO	YES
12. Were the index test results interpreted without knowledge of the results of the reference standard?	NO	NO	NO
13. Were the reference standard results interpreted without knowledge of the results of the index test?	YES	YES	YES
14. Were the same clinical data available when test results were interpreted as would be available when the test is used in practice?*	YES	N/A	N/A
15. Were uninterpretable/intermediate test results reported?	N/A	N/A	N/A
16. Is it likely that the presence of overfitting was avoided?	UNCLEAR	YES	YES

*Criterion applies to Phase 4 diagnostic study only

N/A: Not applicable

Metabolic biomarkers of hyper-acute stroke

H1-NMR analysis of plasma and urine from 54 stroke patients compared with 47 healthy controls revealed significant differences in metabolites between the study groups(103). However, there were several disparities in the characteristics of the groups, as they were not matched for relevant risk factors such as previous TIA and hypertension. This study also excluded patients with concomitant vascular disease and diabetes – comorbidities present in a large proportion of stroke patients. Nonetheless, significantly elevated levels of lactate, pyruvate and formate and decreased levels of VLDL and LDL CH3, valine, lipid CH2CH2C=C and 4-hydroxymethyl acetate were found in plasma of stroke patients. Analysis of urine revealed higher levels of O-acetylcarnitine, trimethylamine-NO-oxide, betaine and carnitine in stroke patients, and decreased concentrations of citrate, dimethylamine, creatine, glycine and hippurate. Validation studies performed showed an average classification rate of 100% for stroke patients, and 96% for healthy subjects(103).

The reduction in circulating levels of valine in hyperacute stroke patients as well as other branched chain amino acids (leucine, isoleucine) has been demonstrated in a targeted mass spectrometry analysis of plasma(102). Fifty-two patients with mild or severe stroke were compared with 32 controls (patients presenting acutely with a final diagnosis of TIA or non-stroke). The magnitude of reduction correlated with size of infarct and clinical disability(102). Multivariate statistical analysis showed that branch chain amino acids were discriminatory in separating stroke from non-stroke or TIA patients. The researchers mention the reduction of branched chain amino acids in many critical illnesses as well as

heart disease (109). The patient groups are well representative of real-life acute attendances (making this a phase 4 study), but predictably patients were stroke and particularly severe stroke were more likely to suffer from diseases such as atrial fibrillation, and it remains to be seen whether the variation in branched chain amino acids are a consequence of stroke, or a reflection of patient comorbidities.

MS analysis of serum from 67 hyperacute stroke patients and 62 controls well matched for vascular risk factors revealed increased concentrations of cysteine, S-adenosyl homocysteine, oxidised glutathione, hydroxyeicosaetraenoic acid, and hydroxyoctadecadienoic acid, and decreased levels of folic acid, tetrahydrofolate, adenosine, aldosterone, deoxocathasterone, sucrose-6-phosphate, and betanin in stroke patients(105). Statistical validation again revealed a prediction accuracy of 100% in discriminating stroke patients from controls.

Homocysteine, a metabolite of S-adenosyl homocysteine, is a sulphur-containing amino acid synthesized from methionine(110). The regeneration of methionine from homocysteine is folate-dependent, thus homocysteine levels are governed by genetic predisposition as well as folate and B-vitamin intake. Elevated levels of homocysteine have been associated with numerous medical disorders, including various neurological disorders. As a proposed risk factor for stroke, homocysteine is hypothesised to induce oxidative injury to vascular endothelial cells, reduce the production of nitric oxide, enhance platelet adhesion to endothelial cells, and promote the growth of vascular smooth muscle cells(103).

Elevated homocysteine levels have been associated with poorer stroke outcomes in Chinese populations, where there is no folic acid nutritional fortification(111). However, retrospective studies have shown a stronger association between cardiovascular events and homocysteine levels than prospective studies (112). When considered with the finding that lowering homocysteine levels (e.g. with folic acid supplementation) does not appear to decrease the risk of stroke (113), it lends credence to the possibility of homocysteine as an acute-phase reactant. Prospective, large confirmatory studies are required in subjects that are disease-free at recruitment.

The role of lipid metabolism is of interest in stroke research, due to the close association with atherosclerosis, and the high lipid content of the CNS. Cerebral ischaemia results in anaerobic metabolism and excess glutamate release, leading to activation of phospholipases/ sphingomyelinases, phospholipid hydrolysis, ultimately hailing apoptopic or necrotic cell death(114). The association between stroke risk and fatty acid metabolism is highlighted by the prognostic stroke metabonomics study conducted by Jove et al(104). Plasma metabonomic profiles of TIA patients that suffered a stroke within one year were compared with TIA patients that did not develop subsequent stroke. The resulting discrepancies in metabolic profiles related primarily to fatty acid metabolism. Furthermore, risk of early stroke recurrence could also be differentiated from late stroke recurrence. The metabolite Lysophosphocholine 20:4 increased the sensitivity of the ABCD2 score from 64-67% to 71%, demonstrating clinical benefit(104).

Anandamide (AEA) is a long chain fatty acid, degraded by Fatty Acid Amide Hydrolase (FAAH), and linked with neuromodulatory effects in ischaemic brain injury(115). Naccarato et al. used mass spectrometry to compare plasma concentrations of AEA, as well as palmitoylethanolamide and 2-arachidonoyl glycerol in patients with hyper-acute stroke to well-matched healthy volunteers(116). Despite the utilisation of a spectroscopic technique to determine levels of metabolites, the fact that only three compounds were assessed and without the use of multivariate statistical analysis, it cannot be considered to be metabolic profiling or metabonomics. However, the results lend credence to the important association of lipid metabolism with CNS disease mechanism. AEA was significantly elevated in stroke patients as compared to healthy controls (3.42 +/- 2.71 pmol/lipid mg vs. 1.81 +/- 1.53 pmol/lipid mg; $p<0.05$). Furthermore, plasma AEA correlated positively with size of infarct, and degree of neurological impairment(116).

AEA is also an endocannabinoid, acting locally in the CNS on CB1 (modulating the actions of GABA and glutamate, postulated to reduce NMDA-mediated excitotoxicity in the penumbra, induce hypothermia, and reduce oedema(117)) and centrally and peripherally on CB2 receptors (immunomodulatory role triggered 24-48 hours after the onset of stroke). The beneficial effect of endocannabinoids in stroke is not certain; CB1 knock-out mice develop significantly larger stroke volumes with greater disability(118), but significant toxicity in relation to endocannabinoids has also been reported(119), therefore the balance of beneficial and detrimental effects requires further investigation.

Review limitations

Included untargeted metabonomics studies investigating biomarkers diagnostic of stroke compared stroke patients with healthy volunteers or patients attending for routine outpatient appointments – as such they are Phase 1 and 2 diagnostic studies(101), and patient groups are often not matched for vital characteristics, or stringent exclusion criteria imply that findings may not be translated into real-world practice. Risk factors such as diabetes, hypertension, dyslipidaemia and arterial disease confer distinct metabolic profiles; it is not known whether groups matched for such characteristics would retain the significant metabolic distinctions implied by Phase 1 studies, particularly given the small number of participants included. Furthermore, the use of pertinent medications, such as statins, was not considered by investigators. No included study elaborated on the timing of sample collection in relation to administration of hyper-acute stroke therapy (such as thrombolysis or aspirin, both of which might distort metabolic profiles).

The use of metabonomics as a research tool carries limitations common to diagnostic studies and systems biology studies, as well as those unique to metabolic profiling. Due to high throughput mechanisms, the cost per sample is low, but overhead maintenance and infrastructure demands are considerable(120). Variations in pre-analytic stages with regard to sample selection, collection, storage, and preparation can impact final results. Complex data interpretation tehniques are required (70), and error can result from statistical overfitting of data(101), particularly with small sample sizes. An

appropriately adapted scoring system is required to gauge the quality of stroke metabonomics studies – prognostic as well as diagnostic.

3.3.5 Conclusion

Although steeped in basic science, metabonomics offers a holistic overview of the physiological status of an individual, and provides a feasible platform to study a complex disorder with multi-causal pathology. Relatively few metabolic studies of stroke have been performed to date utilising different spectroscopic techniques in a variety of patient groups. The disparity in discovered biomarkers between studies may reflect the diversity of applied spectroscopic techniques, range of biofluid, differences in patient populations, disparate aetiologies of stroke, and variation in sampling time due to chronological variability of permeability of the blood brain barrier post-stroke. Certain metabolic pathways are however enunciated, particularly relating to branched chain amino acid, homocysteine, folate, anaerobic, and lipid metabolism. In future, prospective high-quality studies with groups well matched for cardiovascular risk factors, stratified for stroke aetiology, and with less stringent exclusion criteria are required for hyperacute biomarker delineation and improved understanding of the pathological processes that drive subtypes of stroke.

Table IV Studies included in thequalitative review of metabolic profiling of ischaemic stroke

Author	Year	Origin	Subjects						Bio-Fluid	Spectroscopic technique	Targeted/ Untargeted	Upregulated Biomarkers	Downregulated Biomarkers	Validation
			Stroke patients	Time from onset to sampling	n	Controls	n	Exclusion criteria						
Jung	2011	Korea	Cerebral infarction	</= 72 hours	54	Healthy volunteers	47	Diabetes Vascular disease	Plasma	H1-Nuclear magnetic resonance spectroscopy	Untargeted	Lactate Pyruvate Formate	VLDL CH3 LDL CH3 Valine Lipid CH2CH2C=C Glutamine Methanol	Training and prediction sets randomly selected and repeated three times
									Urine			O-acetylcarnitine Trimethylamine-NO-oxide Betaine Carnitine	Citrate Dimethylamine Creatine Glycine Hippurate	
Jiang	2011	China	Cerebral infarction	</= 6 hours	67	Routine outpatient attendees	62	Previous stroke Cancer Cardiac insufficiency Hepatosis Renal failure Respiratory failure GI haemorrhage	Serum	Ultra high pressure liquid chromatography and tine of flight mass spectrometry (negative ionisation mode)	Untargeted	Cysteine S-adenosyl homocysteine Oxidised glutathione Hydroxyeicosate-traenoic acid Hydroxyocta-decadienoic acid	Folic acid Tetrahydrofolate Adenosine Aldosterone Deoxocathasterone Sucrose-6-phosphate Betanin	Partial least square K-nearest neighbour (k=3); training and test division randomly carried out in 20 trials
Kimberley	2013	USA	Cardio-embolic stroke	< 9 hours	52	TIA or non-stroke (acutely presenting patients)	32	None stated	Plasma	High performance liquid chromatography HILIC mass spectrometry	Targeted	Glucose	Leucine Isoleucine Valine	
Jove	2015	Spain	Stroke following previous TIA	</= 24 hours of initial TIA	35	TIA patients that did not suffer subsequent stroke	258	None stated	Plasma	Liquid chromatography mass spectrometry	Untargeted	Myristoyl-ethanolamine	1-Monopalmitin Dodecanoic acid Mesoerythritol Threonate Lysophosphatidyl-choline	Second cohort of patients

3.4 Metabolic profiling of carotid atherosclerosis

The combined application of HILIC MS, lipid profiling MS and 1H-NMR to determine the metabolic profile of high-risk carotid atherosclerosis has not previously been undertaken. The combination of technological platforms and untargeted as well as semi-targeted approaches permit broad metabolic fingerprinting with the greatest potential to identify biomarkers of high-risk carotid stenosis. This would afford the following benefits:

- Personalised risk assessments for patients with carotid atherosclerosis
- Improved cost-utility of carotid endarterectomy with a reduction in NNT
- A means of monitoring plaque stabilisation therapy
- Reduced incidence of stroke

To understand the mechanisms leading to the development of symptomatic carotid stenosis, and how the tendency to embolise segregates from stenosing and dilating arterial disease, it is necessary also to compare the metabolic profile of symptomatic carotid atherosclerosis with those of peripheral arterial disease and aneurysmal disease. This is the fundamental purpose of this study.

Chapter 4: Hypotheses & Aims

4.1 Hypotheses

- Patients with high-risk carotid atherosclerosis demonstrate a distinct metabolic profile.
- The metabolic profile of high risk carotid atherosclerosis is distinguishable from patients with an alternate arteriopathic phentotype.

4.2 Aims

- To identify differential metabolic profiles for high-risk and low-risk moderate to severe carotid stenosis.
- To identify the metabolic features distinguishing embolising (symptomatic carotid) from stenosing (peripheral arterial) and dilating (aneurysmal) disease.

Accomplishment of these aims is dependent upon effective collaboration with clinical (vascular surgery) and scientific (metabonomics) experts. The overall structure, methodology and flow of this thesis is summarised in Figure 5.

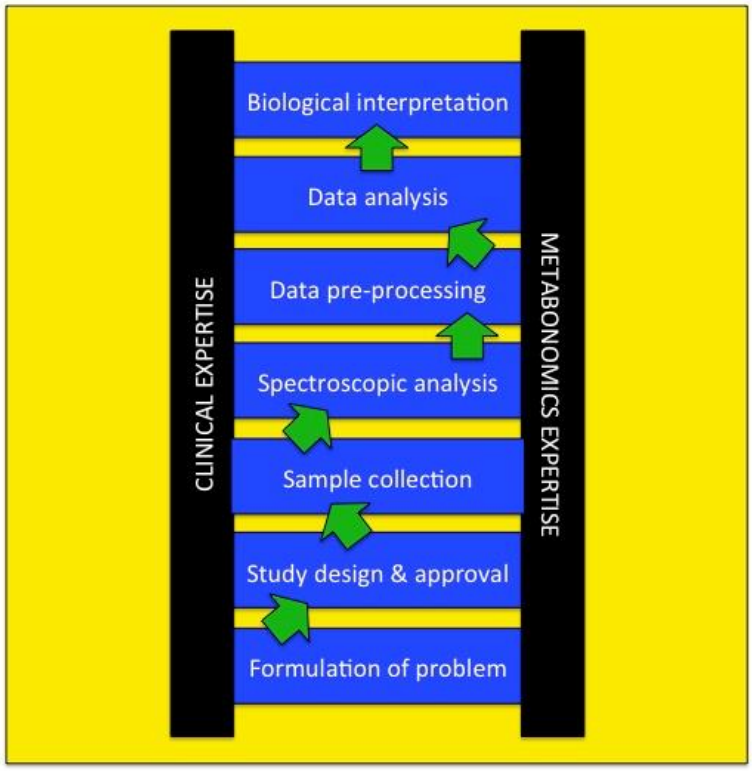


Figure 5: Summary of study methodology and flow, from formulation of clinical problem to the biological interpretation of data. The position of the arrows denotes relative contribution of clinical and scientific expertise.

II. BIOMARKER RESEARCH IN THROMBOEMBOLIC STROKE

Chapter 5: Patient recruitment

5.1 Clinical Setting

Imperial College NHS Healthcare Trust and specifically Charing Cross Hospital serves as a Hyper Acute Stroke Unit (HASU) that is the regional admitting centre for patients who have suffered an acute stroke, or experienced one or more transient ischaemic attacks (TIAs).

In addition, there are a number of TIA clinics that are filled through Emergency Department and General Practice referrals. Patients who are suspected by a stroke physician to have experienced a TIA are urgently investigated to determine the likely underlying cause (atrial fibrillation, cardiac thrombus, carotid stenosis, etc). The majority of HASU and TIA clinic patients undergo carotid duplex ultrasonography as part of their routine care in our vascular laboratory.

The Academic Section of Vascular Surgery, based at Charing Cross, is thus ideally suited for patient recruitment in stroke- and carotid atherosclerosis-related studies.

5.2 Recruitment and sample collection

5.2.1 Ethical approval

The Academic Section of Vascular Surgery at Imperial College London holds ethical approval for blood, urine and tissue collection from patients with

vascular diseases including carotid artery disease, aneurysmal disease and peripheral arterial disease (East Midlands Ethics Committee 13/EM/0011).

5.2.2 Recruitment

Subjects over 40 years of age were recruited into the following categories:

1. Symptomatic moderate to severe stenosis (50-99%)
2. Symptomatic patients with subthreshold (<50%) carotid plaque, not thought to be contributing to the patients' symptomatic status
3. Asymptomatic moderate to severe carotid stenosis (50-99%)
4. Asymptomatic individuals with subthreshold carotid stenosis
5. Patients with abdominal aortic aneurysm (>3cm)
6. Patients with symptomatic intermittent claudication

The decision as to whether a patient with carotid disease was classed as symptomatic or asymptomatic was based upon Multidisciplinary Team Meeting (MDT) outcome. The MDT is standard practice in the management of patients with carotid atherosclerosis.

Exclusion criteria included the following:

1. Atrial fibrillation
2. Active malignancy
3. Critical ischaemia
4. Tissue loss from non ischaemic causes (e.g. venous ulceration)

5.2.3 Patient selection

Carotid disease

Patients admitted to the Hyper Acute Stroke Unit at Imperial College Healthcare NHS Trust as well as patients presenting to the Vascular Outpatient Department and the Transient Ischaemic Attack clinic were invited to contribute to this study.

All patients underwent carotid duplex ultrasonography by an Accredited Vascular Scientist as part of their routine clinical assessment, enabling detailed characterisation of plaque and the resulting degree of stenosis (using NASCET criteria) of the extra-cranial carotid vasculature.

Peripheral arterial disease (claudication)

Patients with peripheral arterial disease manifesting as intermittent claudication and attending the outpatient clinics at Imperial College Healthcare NHS Trust were invited to contribute to this study. Patients with evidence of critical limb ischaemia (tissue loss, rest pain or gangrene) were not eligible.

Abdominal aortic aneurysmal disease

Patients admitted for elective repair of AAA, those undergoing surveillance of subthreshold aneurysms as part of outpatient follow up, and patients attending the National Abdominal Aortic Aneurysm Screening Programme were invited to contribute to this study.

5.2.4 Data recording

Following informed consent, baseline patient characteristics were recorded, using the proforma as depicted in Appendix 1. Patient characteristics were stored in paper form in a locked drawer within a locked room to which only the research team had access.

5.2.5 Baseline haematological and biochemical investigations

Baseline blood tests were routinely performed as part of the patient's clinical care. The following parameters were recorded at baseline:

- White cell count (WCC)
- Lymphocyte count (Ly)
- Monocyte count (Mo)
- Neutrophil count (Ne)
- Urea (Ur)
- Creatinine (Cr)
- Capsular Reactive Protein (CRP)
- Erythrocyte Sedimentation Rate (ESR)
- Total cholesterol (Chol)
- Triglycerides (Trig)
- High density lipoprotein (HDL) concentration
- Low densitiy lipoprotein (LDL) concentration
- Cholesterol:High density lipoprotein ratio (Chol:HDL)

5.2.6 Sample collection

Serum

Blood was collected from an upper limb vein with minimal tourniquet time using either a needle and syringe, or vacutainer technique. Two red-topped serum acquisition vacutainers were filled for each patient. The blood was allowed to rest for 30-40 minutes to permit the sample to fully clot. The vacutainers were then centrifuged at 5800rpm for 10 minutes to separate serum from cellular contents. Serum was pipetted in 1ml aliquots into 2ml eppendorfs and immediately frozen at -80*C until sample processing for metabonomic analysis.

Urine

Urine was collected from non-catheterised patients in universal sterile containers. This was pipetted in 1ml aliquots and stored in 2ml eppendorf containers, frozen immediately at -80*C until further processing for metabonomic analysis.

Despite best efforts, the maintenance of sterility for urine collection was challenging for many stroke patients due to impedance of coordination as a result of stroke. As a consequence of this, and a general reticence amongst many patients to provide urine, fewer urine samples were successfully collected than serum samples.

5.3 Human Tissue Authority Standards Compliance

Samples and patient data were subject to two separate internal audits as well as external audit by the Human Tissue Authority (HTA). Adjustments were made following the first internal audit to ensure all data and sample storing processes were compliant with HTA standards, and as a result, no deficiencies specific to this project were encountered by the HTA.

5.4 Patient metadata

In total, 150 patients were recruited for this study, but samples from 12 patients were not usable for analysis due to either haemolysis of blood or contamination of urine. One participant subsequently withdrew consent. Following MDT discussion, a further 4 patients were excluded as they did not meet the inclusion criteria.

Appendix 2 provides all baseline patient data, but group summaries are provided in Table V.

Table V Baseline data for recruited and included participants

Group	1	2	3	4	5	6	p values
Total	**46**	**18**	**26**	**11**	**18**	**14**	
Age	72.1	67.2	70.3	58.7	70.4	66.6	NS
Female (%)	28.3	44.4	30.8	45.5	16.7	21.4	NS
Statin (%)	73.9	72.2	92.3	18.2	77.8	71.4	NS
Antiplatelet (%)	91.3	88.9	88.5	45.5	50.0	92.9	8.56212E-06
Anticoagulation (%)	4.3	5.6	7.7	0.0	11.1	0.0	NS
HTN (%)	76.1	83.3	84.6	54.5	61.1	92.9	NS
IHD (%)	31.1	22.2	50.0	9.1	45.5	42.9	NS
DM (%)	19.6	38.9	46.2	0.0	45.5	21.4	0.017118759
Current smoker (%)	25.0	33.3	15.4	36.4	13.3	42.9	NS
Ever smoker (%)	82.2	55.6	76.9	36.4	87.5	100.0	NS
BMI	26.2	27.6	27.4	24.5	29.0	26.4	0.04337
White cell count	7.87	7.53	7.54	6.76	8.18	7.85	NS
Urea	6.45	7.81	6.82	5.17	7.32	6.06	NS
Creatinine	95.7	112.7	95.6	79.8	99.3	94.5	NS
CRP	8.27	8.25	3.70	1.50	19.16	3.21	NS
ESR	18.58	14.33	19.86	12.70	22.07	14.07	NS
Total cholesterol	4.64	4.69	3.89	5.59	4.09	5.06	NS
Triglycerides	1.60	1.30	1.49	1.37	1.49	1.79	NS
HDL	1.23	1.23	1.18	1.38	1.07	1.23	NS
LDL	2.74	2.90	2.03	3.56	2.35	3.23	NS
Cholesterol:HDL ratio	3.95	3.95	3.40	4.43	3.97	4.34	NS

Group 1: symptomatic >50% carotid stenosis
Group 2: patients with recent (<6 months) non-carotid stroke/TIA
Group 3: asymptomatic >50% carotid stenosis
Group 4: normal controls
Group 5: aortic aneurysmal disease
Group 6: intermittent claudication
NS: Non-significant

Group 4, normal controls, comprised patients presenting to the HASU with neurological symptoms that were deemed non-TIA/non-stroke. They had no clinical evidence of atherosclerotic disease. As such, these "normal controls" varied markedly in their cardiovascular risk factors compared with other groups. Secondly, this group included the least number of participants. The discrepancies in characteristics as well as low sample size made metabonomic comparison

between symptomatic carotid atherosclerosis and unmatched normal controls both clinically and scientifically less valuable.

Characteristics between diseased groups were relatively well matched. Chi squared was used to compare categoral variables, and ANOVA was used to compare continuous variables between symptomatic carotid atherosclerosis and other disease classes. The few statistically different parameters are highlighted in red font, with p values detailed.

Chapter 6: Metabolic profiling of urine

The non-invasive means of urine collection, together with abundant availability and relative ease of repeat sampling makes urine a clinically advantageous substrate for metabonomic analysis. Urine is a rich source of biomarkers, and its utilisation in metabonomic studies is increasing, with tremendous potential to improve the personalised prediction, diagnosis, prognosis, and understanding of disease (121).

Challenges pertaining to the analysis of urine include difficulty in standardising concentrations of metabolites as the concentration of urine itself is largely variable. In addition to concentration, the composition of urine is inconstant within individuals. Further challenges are encountered with regard to instrument sensitibity, chromatographic selectivity, optimisation of data extraction and metabolite identification and interpretation (122). However, less pre-analysis sample processing is required due to the lower concentration of protein in urine in comparison with serum (123).

Despite the application of urinary metabonomics in the investigation of a diverse array of diseases and states, there are relatively few urine studies of biomarkers of atherosclerotic disease. This chapter describes the global metabolic profiling of urine to determine biomarkers of high-risk carotid atherosclerosis, and to compare embolising carotid disease with stenosis and dilating arterial disease. To achieve this, HILIC MS, RP MS and ^{1}H-NMR analyses have been performed on random urine samples from patients consenting for study inclusion. Patients

were recruited and samples collected in the manner described fully in Chapter 5. Samples were stored at -80 degrees Celsium until analysis.

6.1 Urine reverse phase mass spectrometry

6.1.1 Sample preparation

Urine samples were retrieved from -80 degrees Celsius storage and allowed to defrost at room temperature (approximately 1 hour). A pooled urine sample was created by adding 200 microliters of each sample into a flask washed with demineralised water, LC MS water and acetonitrile (each three times).

Using 96 well MS plates, a random order was followed to aliquot 250 microliters of urine, each into a separate well. Six wells were left empty in each plate to incorporate pooled sample to serve as quality controls.

Dilutions were prepared from pooled sample through the addition of Chromasolve LC MS grade water, and added to empty wells in the second urine plate, in the following ratios:

- 1:2
- 1:3
- 1:4
- 1:6
- 1:8

Plates were centrifuged at 4500 rpm and 4 degrees Celsius for 30 minutes prior to MS analysis.

6.1.2 MS reverse phase urine experimental protocol

Individual components of the mass spectrometer source were cleaned using LC MS grade water and methanol to prevent contamination during analysis. The MS was then calibrated, and intensity optimised (injection volume and voltage) using pooled samples. The response to dilutions was tested to ensure accuracy. The MS was conditioned using quality control samples before started experimentation run. A quality control sample was analysed after every ten serum samples, with adherence to MS checklist to ensure appropriate progression of data acquisition (Appendix 3). Once complete, urine well plates were stored at -80 degrees Celsius until required for HILIC analysis.

6.1.3 Results: Urine Reverse Phase MS Negative Ionisation Mode

Data derived from RP MS were pre-processed and transferred to SIMCA for further statistical analysis.

Initial PCA of patient urine samples and QC samples showed co-localisation of QC samples (Figure 6), however, the clustering was improved by excluding features showing a coefficient of variance over 30% (Figure 7). Remaining analyses were performed with these features excluded.

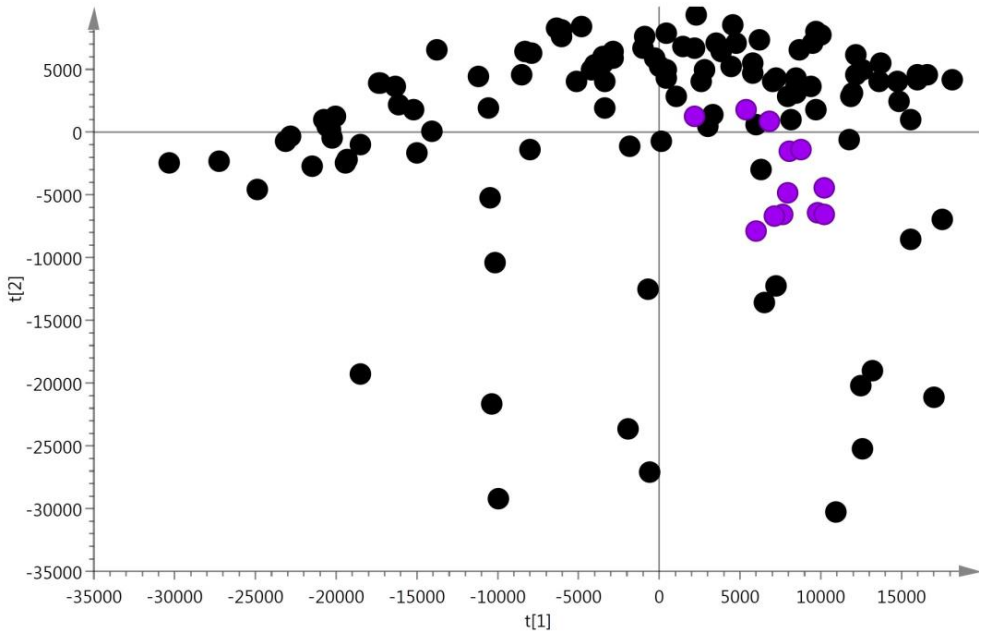


Figure 6: Pareto scaled PCA of all urine samples (black) vs. QCs (purple) analysed with RP MS negative ionisation mode.

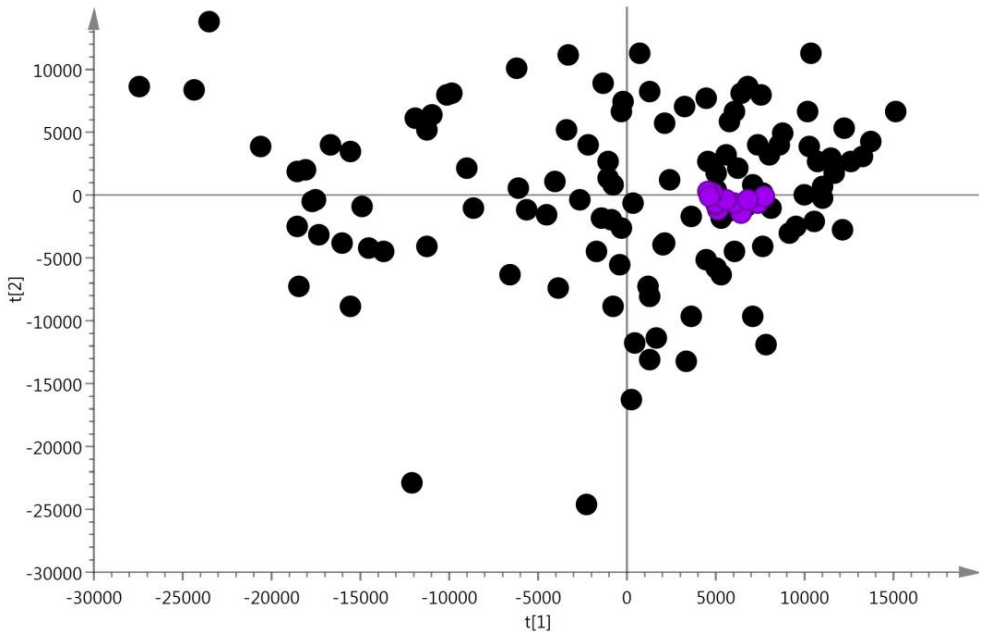


Figure 7: Repeat PCA of patient urine samples (black) vs. QCs (purple) analysed with RP MS negative ionisation mode with features exhibiting high coefficient of variance excluded.

PCA of samples, dilutions and blanks demonstrated tight clustering of Quality Control (QC) samples with sequential peripheralisation of dilutions towards blanks, also tightly clustered (Figure 8).

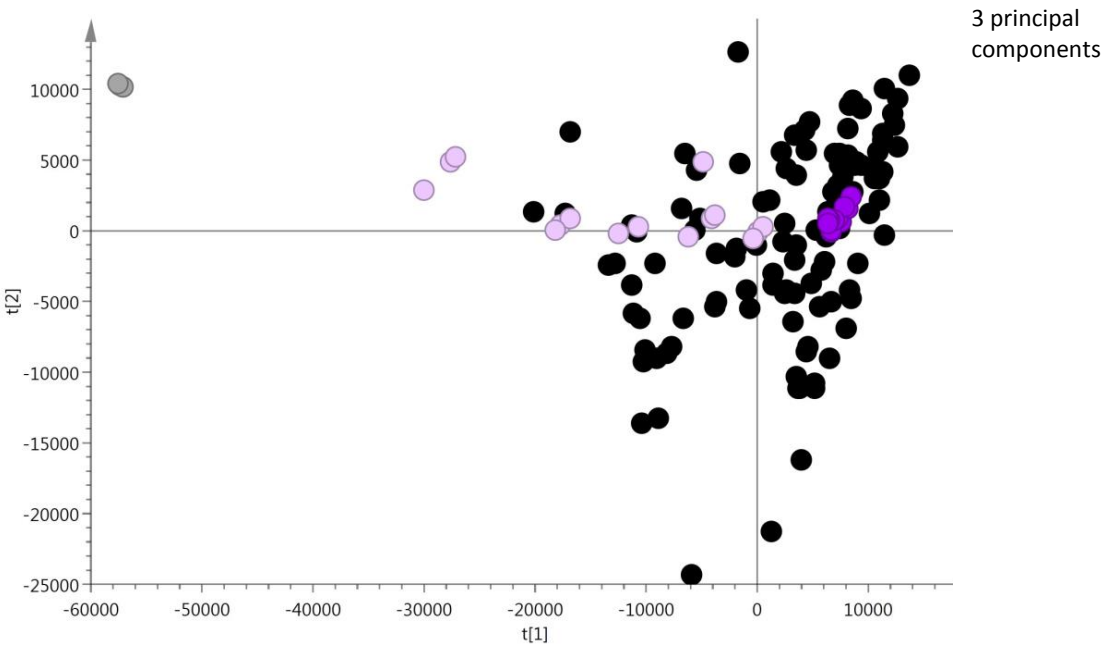


Figure 8: PCA of all urine samples (black), QCs (purple), dilutions (pink) and blanks (grey) analysed with RP MS in the negative ionisation mode.

Data was log transformed to reduce the impact of intense differences in magnitudes of concentration of features on statistical analysis. Further PCA of individual sample groups did not show visual separation, which is to be expected in complex patients where intra-class variability exceeds inter-class variability (Figures 9 and 10). Therefore OPLS DA was selected to enhance separation between classes.

6 principal components

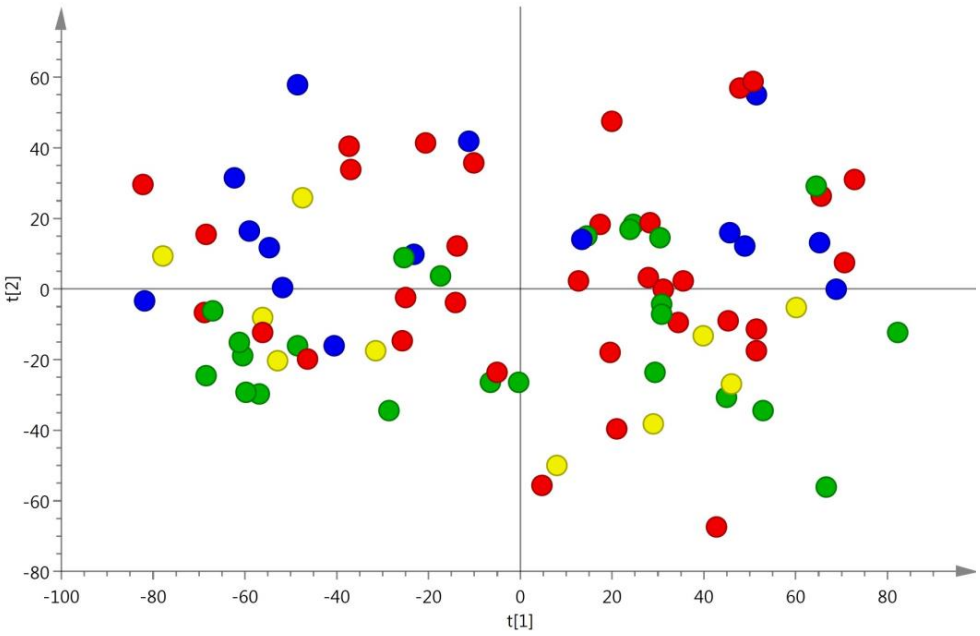


Figure 9: PCA of RP MS negative mode urine analysis from patients with symptomatic carotid stenosis (Group 1; red), non-carotid stroke or TIA (Group 2; blue); asymptomatic carotid stenosis (Group 3; green) and controls (Group 4; yellow).

6 principal components

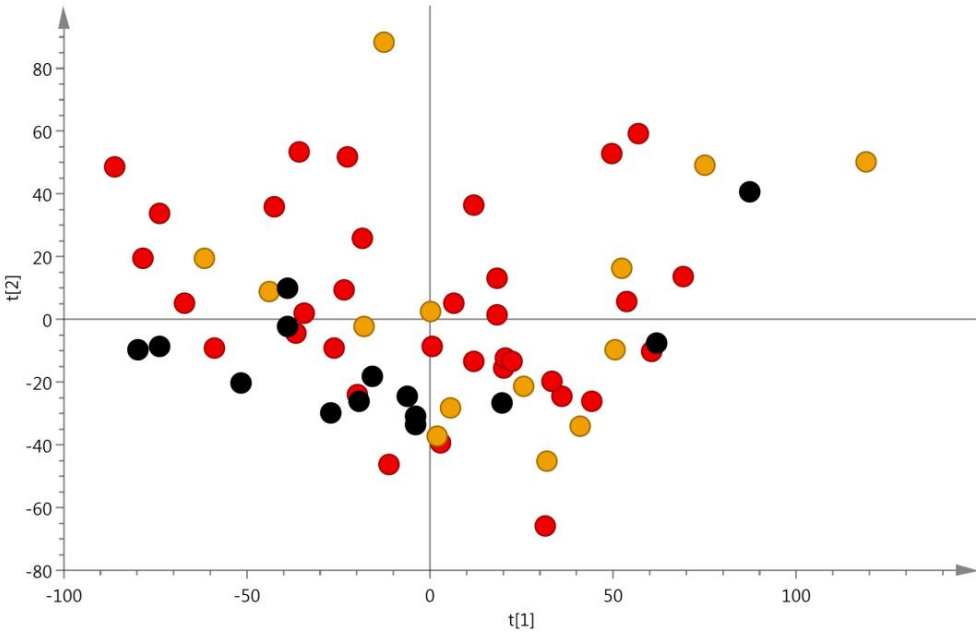
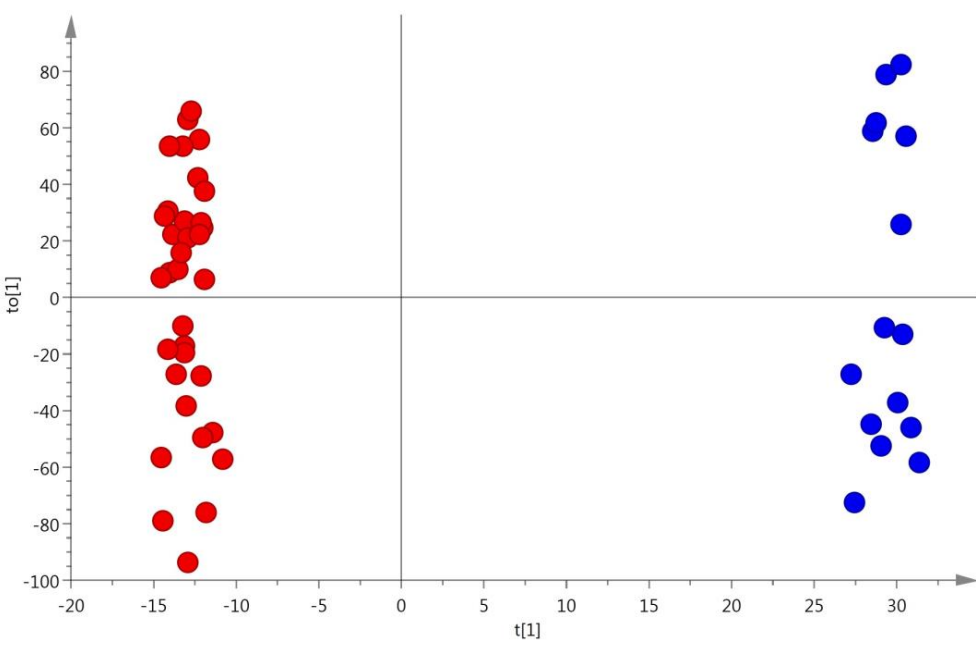


Figure 10: PCA of RP MS (negative mode) analysis of urine from patients with embolising carotid disease (red), aneurysmal disease (orange) and claudication (black).

OPLS DA of urine samples from Group 1 (symptomatic carotid stenosis) and Group 2 (stroke/TIA patients with insignificant carotid stenosis) was overfitted (R^2Y 0.997) and non-predictive (Q^2 0.0465) (Figure 11).



R^2Y	0.997
Q^2	0.0465
Principal components	5

Figure 11: OPLS DA of urine from patients with symptomatic carotid stenosis (red circles; Group 1) and non-carotid TIA/Stroke (blue circles; Group 2) analysed with RP MS negative ionisation mode.

Comparison of symptomatic carotid atherosclerosis with asymptomatic carotid atherosclerosis was weakly predictive and overfitted (Figure 12).

R^2Y	0.729
Q^2	0.107
Principal components	2

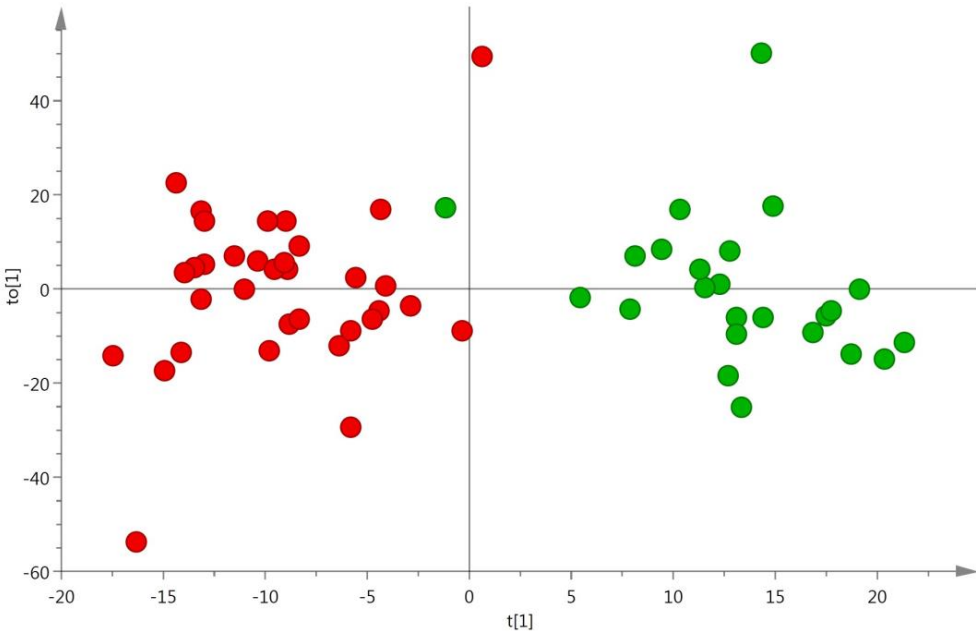


Figure 12: OPLS DA of log transformed data derived from urine RP MS analysis in the negative ionisation mode. Red circles: symptomatic carotid stenosis. Green circles: asymptomatic carotid stenosis.

However, comparisons of differing arterial phenotypes yielded stronger models. OPLS DA of embolising carotid stenosis (Group 1) compared with dilating arterial (aneurysmal) disease (Group 5) resulted in a Q^2Y value of 0.408, albeit the model was overfitted with an R^2Y value of 0.970 (Figure 13). Comparison of embolising with stenosing peripheral arterial disease (Group 6) was weaker, with a Q^2 value of 0.177 (Figure 14) .

R^2Y	0.970
Q^2	0.0408
Principal components	3

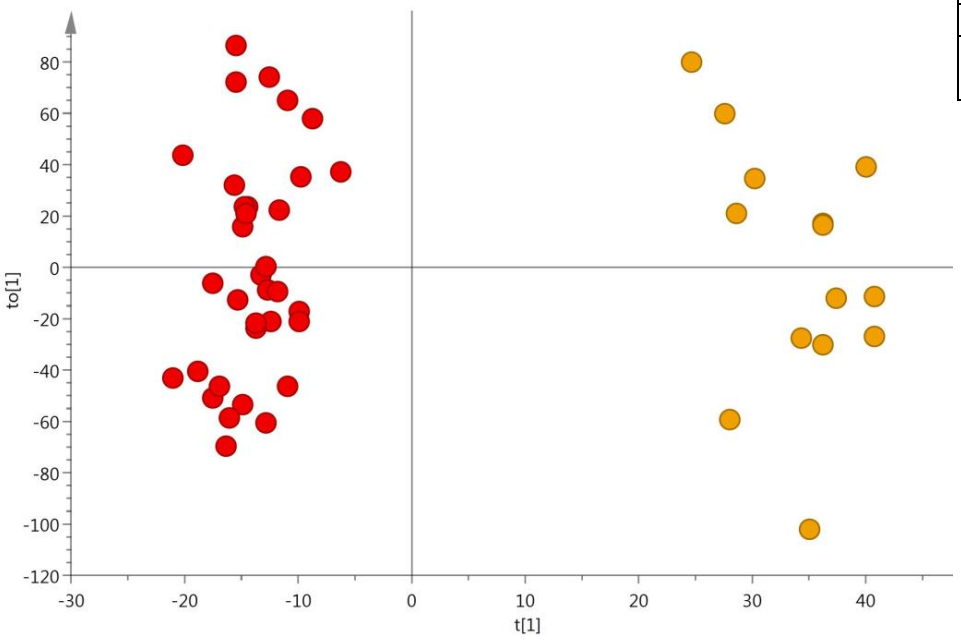


Figure 13: OPLS DA of log transformed data derived from urine RP MS analysis in the negative mode. Red circles: embolising carotid stenosis. Orange circles: aortic aneurysm.

R^2Y	0.787
Q^2	0.177
Principal components	2

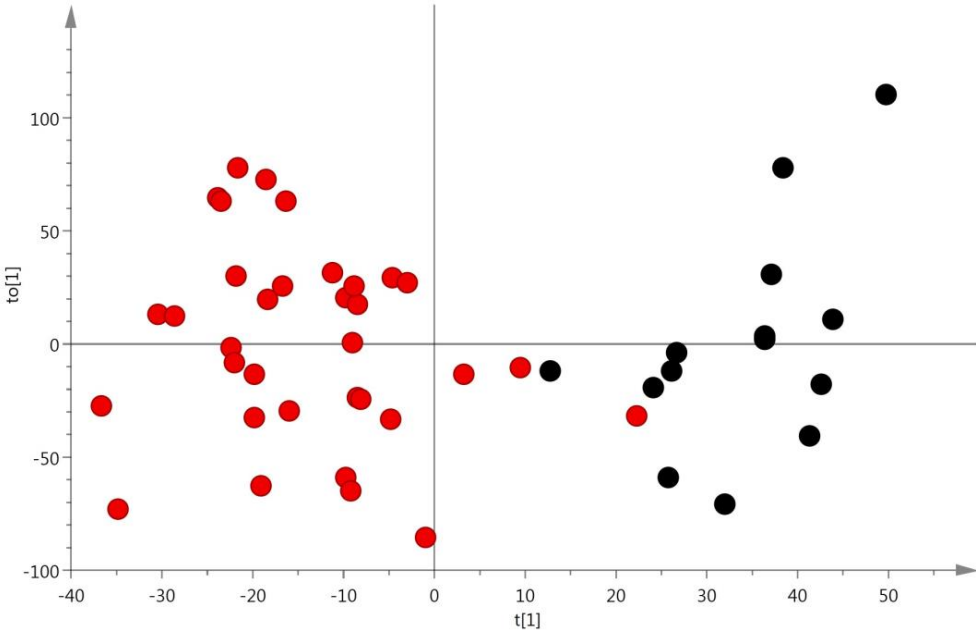


Figure 14: OPLS DA of log transformed data derived from urine RP MS analysis in the negative mode. Red circles: embolising carotid stenosis. Black circles: peripheral arterial disease.

6.1.4 Results: Urine Reverse Phase MS Positive Ionisation Mode

PCAs of all samples, dilutions and blanks in positive ionisation mode analysed with RP MS in the positive ionisation mode showed excellent visual clustering of QCs, dilutions and blanks indicating extremely technical quality of analysis (Figure 15). The clustering was tighter than in negative ionisation mode. Again as expected, PCAs of individual sample classes did not show visual separation and further statistical analyses were performed with OPLS DA. OPLS DA data were log transformed to aide statistical analysis.

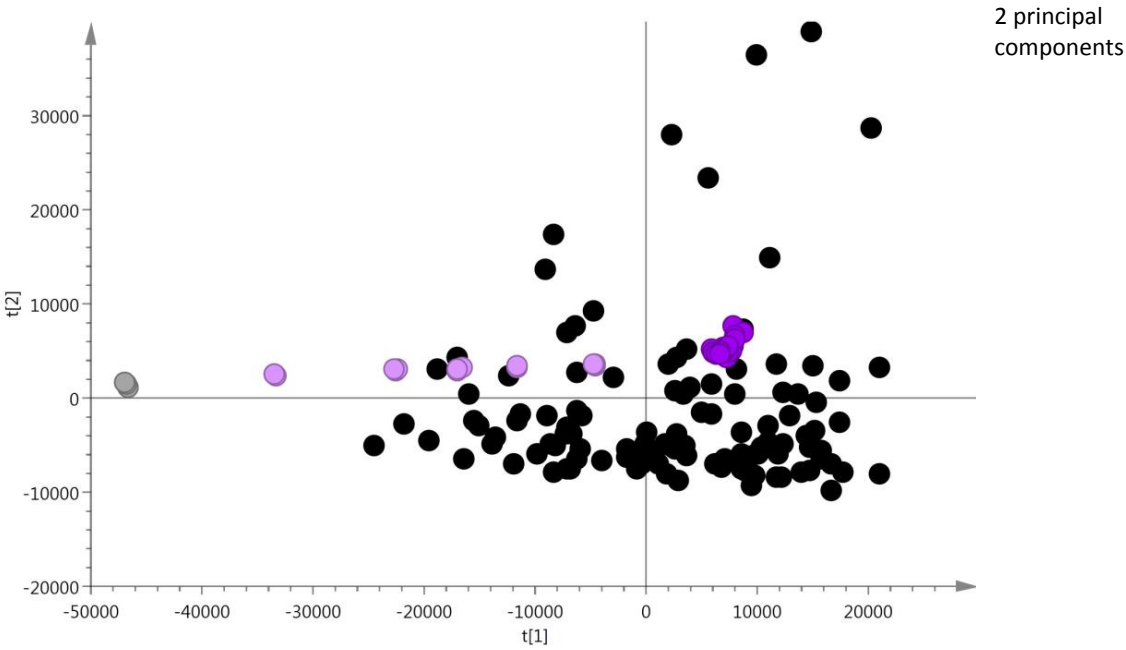


Figure 15: PCA of RP MS positive ionisation mode urine analysis showing appropriate tight clustering of QCs (purple) from samples (black), dilutions (pink) and blanks (grey).

10 principal components

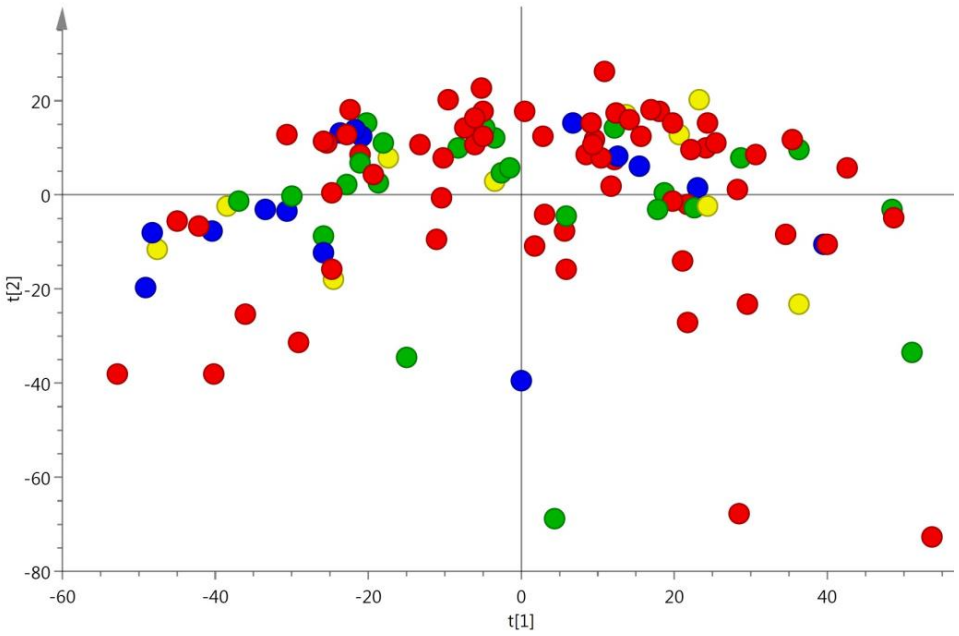


Figure 16: PCA of RP MS positive mode urine analysis from patients with symptomatic carotid stenosis (Group 1; red), non-carotid stroke or TIA (Group 2; blue); asymptomatic carotid stenosis (Group 3; green) and controls (Group 4; yellow).

3 principal components

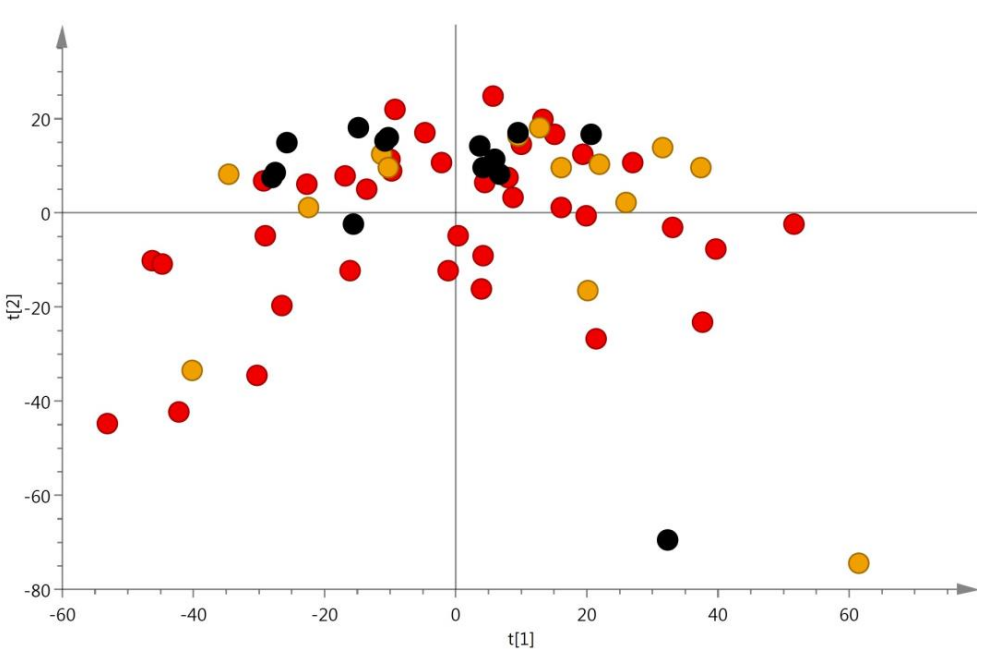


Figure 17: PCA of RP MS (positive mode) analysis of urine from patients with embolising carotid disease (red), aneurysmal disease (orange) and claudication (black).

Overall trends of results in positive ionisation mode were similar to negative ionisation mode. Comparison of symptomatic carotid disease with non-carotid stroke/TIA was non-predictive and R^2Y reached a value of 1 with negative values of Q^2 (Figure 18). Comparison of symptomatic and asymptomatic carotid stenosis was weakly predictive (Figure 19).

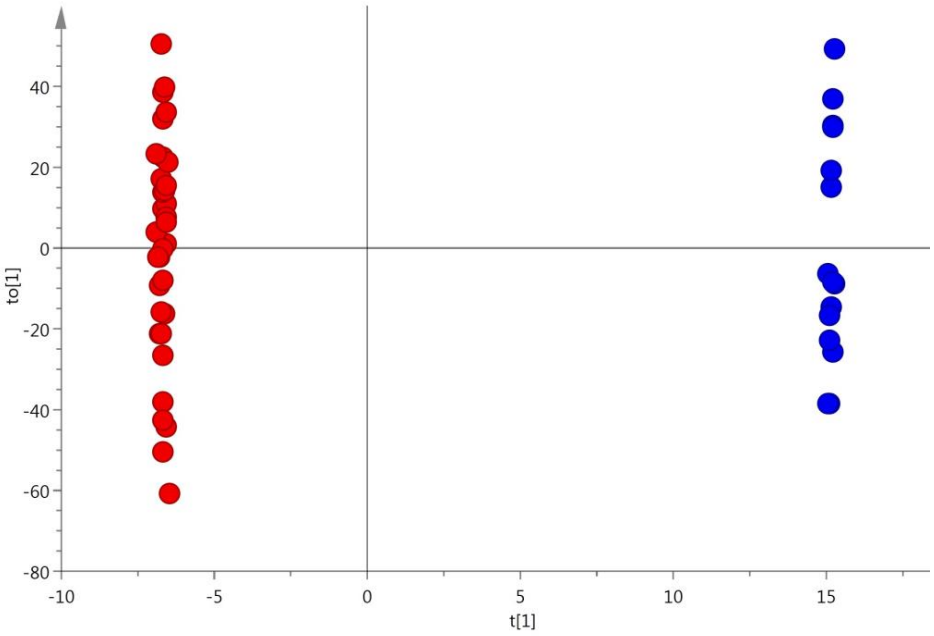


Figure 18: OPLS DA of log transformed data derived from urine RP MS analysis in the positive mode. Red circles: symptomatic carotid stenosis; blue circles: non-carotid stroke/TIA.

R^2Y	0.849
Q^2	0.129
Principal components	3

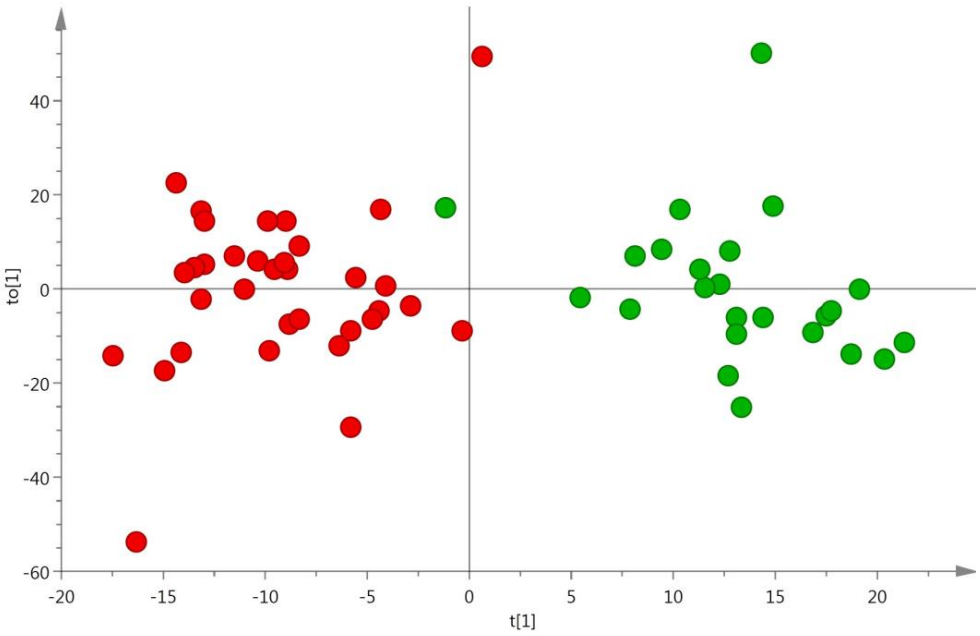
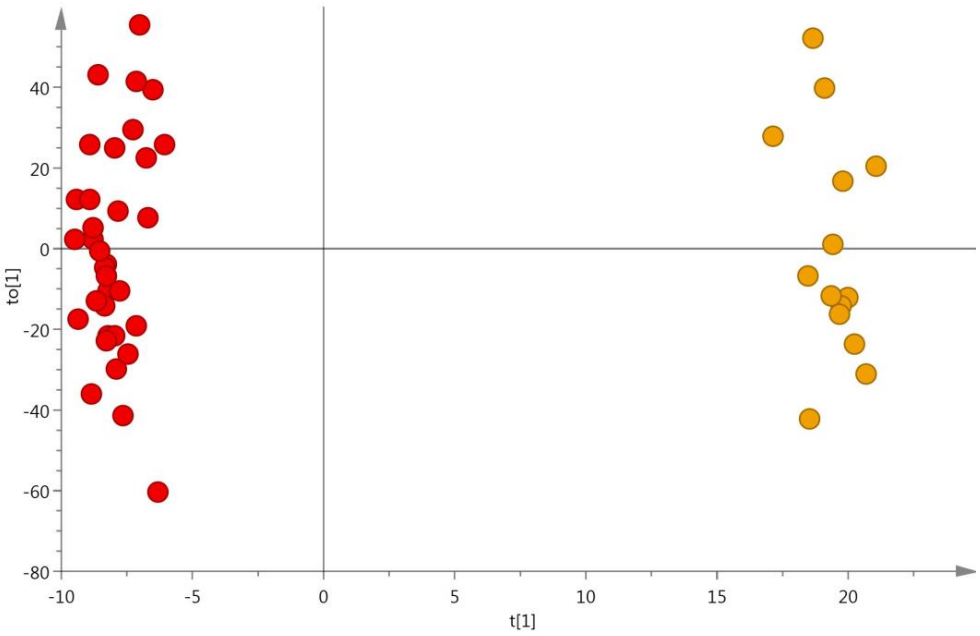


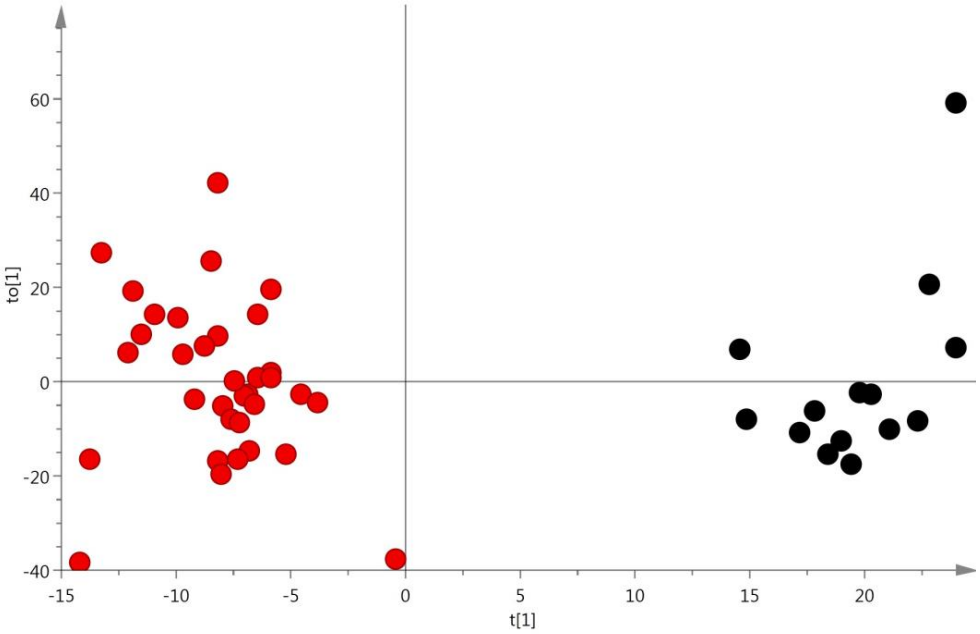
Figure 19: OPLS DA of log transformed data derived from urine RP MS analysis in the positive mode. Red circles: symptomatic carotid stenosis. Green circles: asymptomatic carotid stenosis.

OPLS DA models of embolising, dilating and stenosing disease were again stronger (Figure 20, Figure 21). Similar to negative mode, the strongest model acquired compared embolising (Group 1) urine with urine from aneurysmal patients (Group 5; Figure 20) but with an overfitted R^2Y value of 0.995.



R^2Y	0.995
Q^2	0.520
Principal components	3

Figure 20: OPLS DA of log transformed data derived from urine RP MS analysis in positive ionisation mode. Red circles: embolising carotid stenosis. Orange circles: aortic aneurysm.



R^2Y	0.951
Q^2	0.288
Principal components	3

Figure 21: OPLS DA of log transformed data derived from urine RP MS analysis in positive ionisation mode. Red circles: embolising carotid stenosis. Black circles: peripheral arterial disease.

6.2 Urine HILIC mass spectrometry

Complementary to RP MS, urine HILIC MS was performed to study polar urine metabolites.

6.2.1 Sample preparation

Urine well plates originally prepared for reverse phase MS were retrieved from -80 degree storage and allowed to defrost at room temperature (approximately 1 hour).

Quality control wells were topped up using pooled sample. Dilutations were reprepared using Chromasolve LC MS water and pooled sample, and volumes within well plates were replenished.

6.2.2 Mobile phase preparation for urine HILIC MS

Mobile phase A:

A volume of 4 L Mobile Phase A was prepared comprising 95% acetonitrile and 0.1% Formic Acid.

Containers and measuring cylinders were washed with demineralised water, Chromasolve LC MS grade water and acetonitrile.

A volume of 200ml Chromasolve was added to a prepared container to which 4ml of 0.1% formic acid was added, followed by 3800ml of acetonitrile. The mobile phase was mixed well, then sonicated for 20 minutes to aid degassing.

MS calibration failed to attain stability when using mobile phase A, therefore addition of ammonium acetate was deemed necessary. This resulted in stable acquisition of MS spectra.

Mobile phase B:

A volume of 2.5 L of mobile phase B was prepared comprising 50% acetonitrile. Containers and cylinders were prepared as stated for mobile phase A. To the prepared container, 1250ml of Chromasolve LC MS grade water was added. Ammonium acetate (MS grade, 1.927g) was weighed and added, followed by 2.5ml of formic acid and a further 1250ml of acenonitrile.

6.2.3 MS HILIC urine experimental protocol

Individual components of the mass spectrometer source were cleaned using LC MS grade water and methanol to prevent contamination during analysis. The MS was then calibrated, and intensity optimised (injection volume and voltage) using pooled samples. The response to dilutions was tested to ensure accuracy. The MS was conditioned using quality control samples before starting experimentation run. A quality control sample was analysed after every ten urine samples, with frequent checks using the quality control checklist.

6.2.4 Results: Urine HILIC MS negative ionisation mode

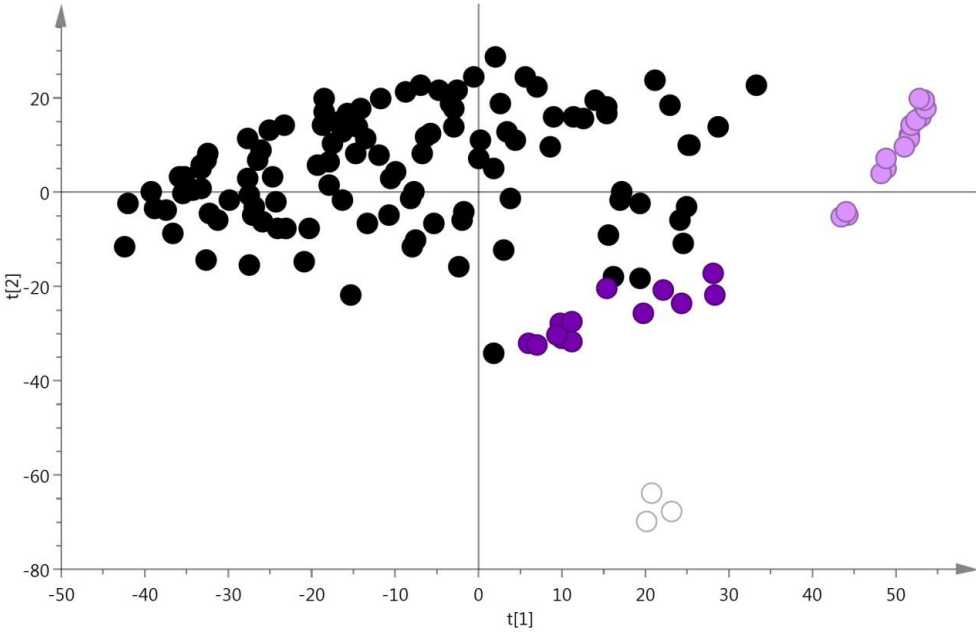


Figure 22: PCA of urine analysed with HILIC MS in the negative ionisation mode showing distribution of samples (black), QCs (purple), dilutions (pink) and blanks (grey).

The initial PCA of all urine samples comparing these to QCs, dilutions and blanks implies that the solvent contained within the blanks thus all samples is exerting a metabolic influence. The features correlating with blank samples (as determined by the loading plots and S-plots) were deleted from subsequent subgroup analyses (Figure 22).

Secondly, PCA of results from Group 1, Group 2, Group 3 and Group 4 identified outliers: sample 60 (Group 1) and sample 4 (Group 3). These were removed from subsequent OPLS DA analysis. There was no visual separation of study groups when compared with PCA (Figure 23, Figure 24).

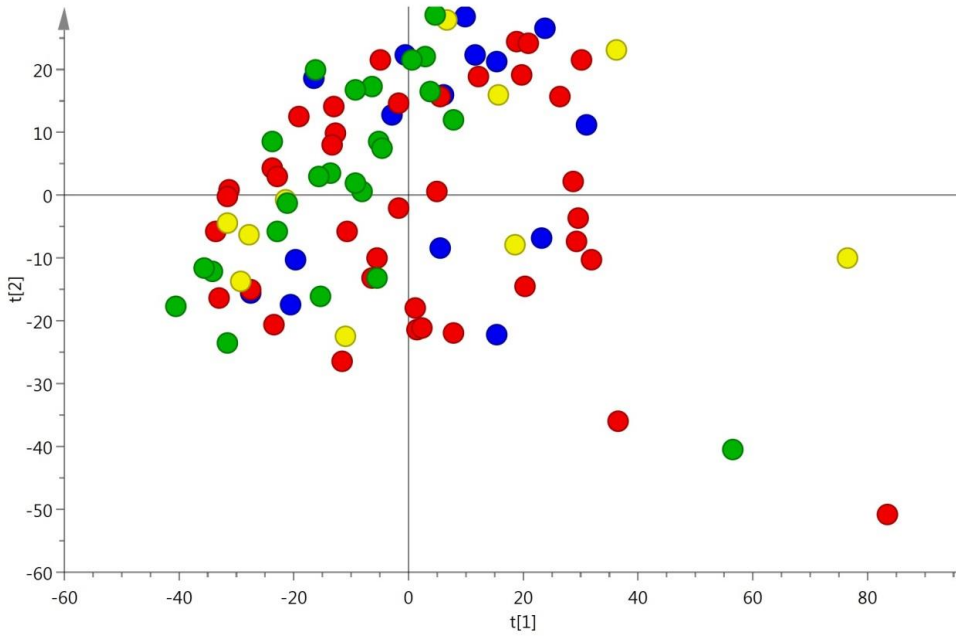


Figure 23: PCA showing distribution of urine samples analysed with HILIC MS negative ionisation mode from patients with symptomatic carotid atherosclerosis (Group 1; red), non-carotid stroke/TIA (Group 2; blue), asymptomatic carotid atherosclerosis (Group 3; green) and controls (Group 4; yellow).

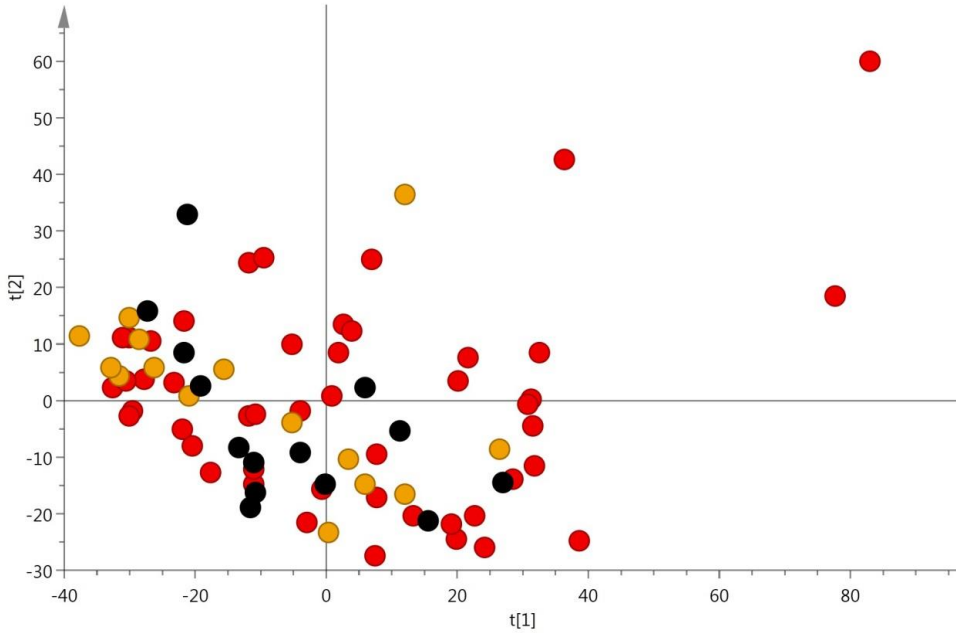
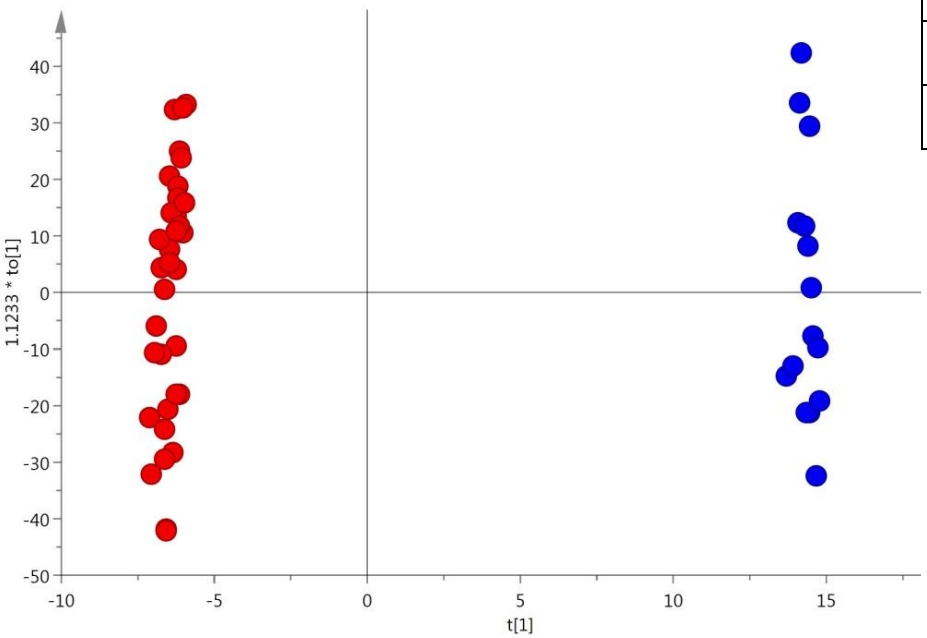


Figure 24: PCA (log transformed) showing distribution of urine samples analysed with HILIC MS in the negative ionisation mode from patients with embolising carotid atherosclerosis (red), dilating aneurysmal disease (orange) and intermittent claudicatiaon (black).

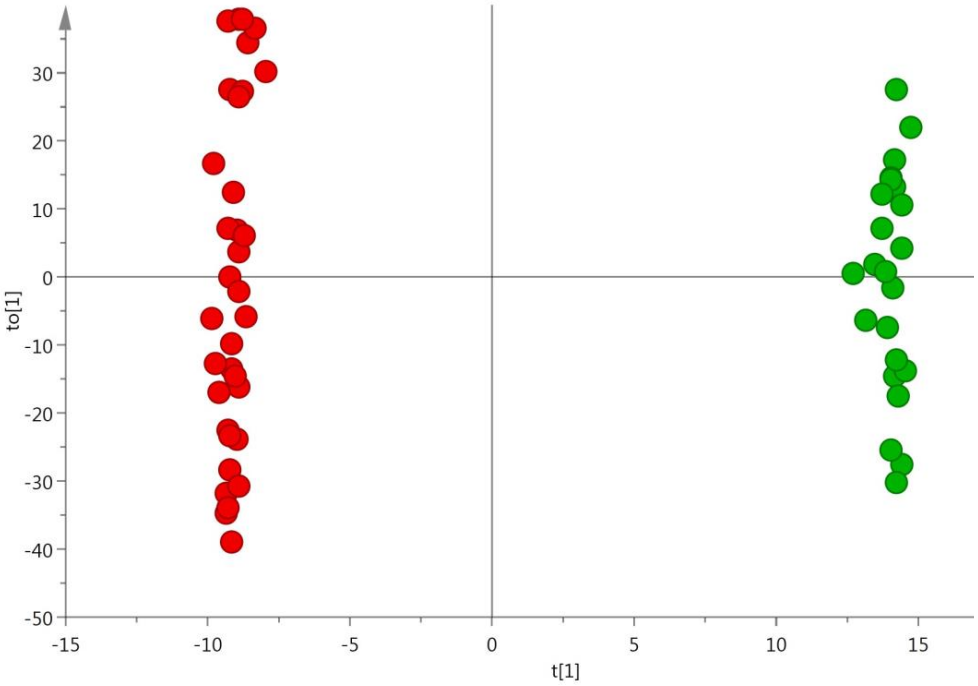
Comparison of urine from symptomatic carotid patients (Group 1) did not separate convincingly from non-carotid stroke and TIA patients (Group 2) with OPLS DA analysis (Figure 25). The model was statistically overfitted with a high R^2Y value.



R^2Y	0.996
Q^2	0.0565
Principal components	10

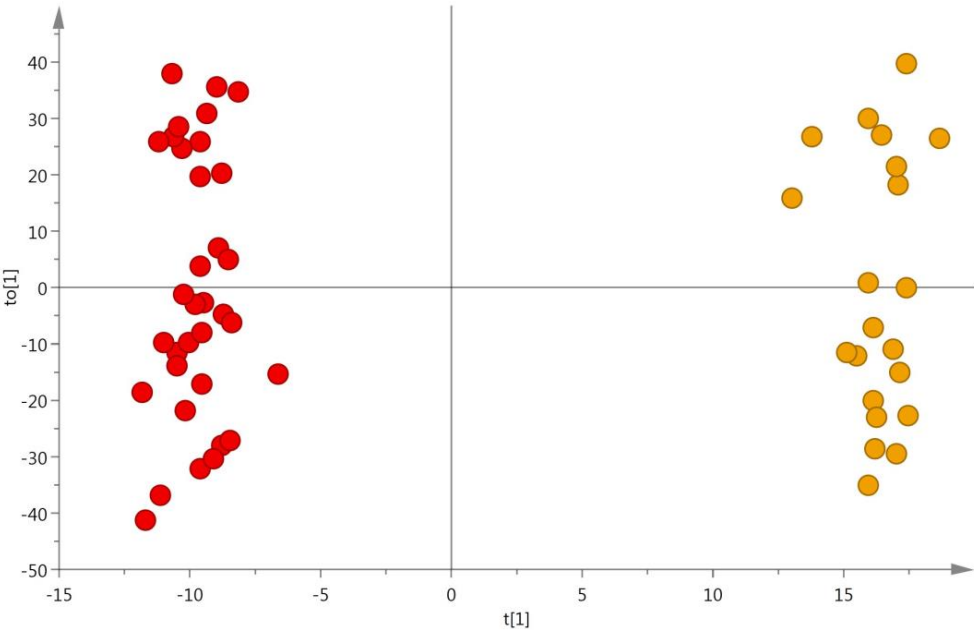
Figure 25: OPLS DA (log transformed) analysing data from urine HILIC MS negative ionisation mode from patients with symptomatic carotid atherosclerosis (red) and non-carotid stroke/TIA (blue).

However comparison of urine from patients with symptomatic and asymptomatic carotid stenosis yielded models with stronger predictive capabilities, but were statistically overfitted (Figure 26). Similar OPLS DA models were obtained for comparisons of embolising, stenosing and aneurysmal disease (Figure 27 and Figure 28), again with relatively high values of Q^2, but with R^2Y approaching 1. This is visually apparent with vertical stacking of observations in OPLS DA models.



R^2Y	0.998
Q^2	0.507
Principal components	6

Figure 26: OPLS DA (log transformed) showing separation of urine analysed with HILIC MS in the negative ionisation mode from patients with symptomatic carotid atherosclerosis (red) and asymptomatic carotid atherosclerosis (green).



R2Y	0.992
Q2	0.656
Principal components	4

Figure 27: OPLS DA (log transformed) showing separation of urine analysed with HILIC MS negative ionisation mode from patients with embolising carotid atherosclerosis (red) and dilating aneurysmal disease (orange).

R^2Y	0.997
Q^2	0.499
Principal components	5

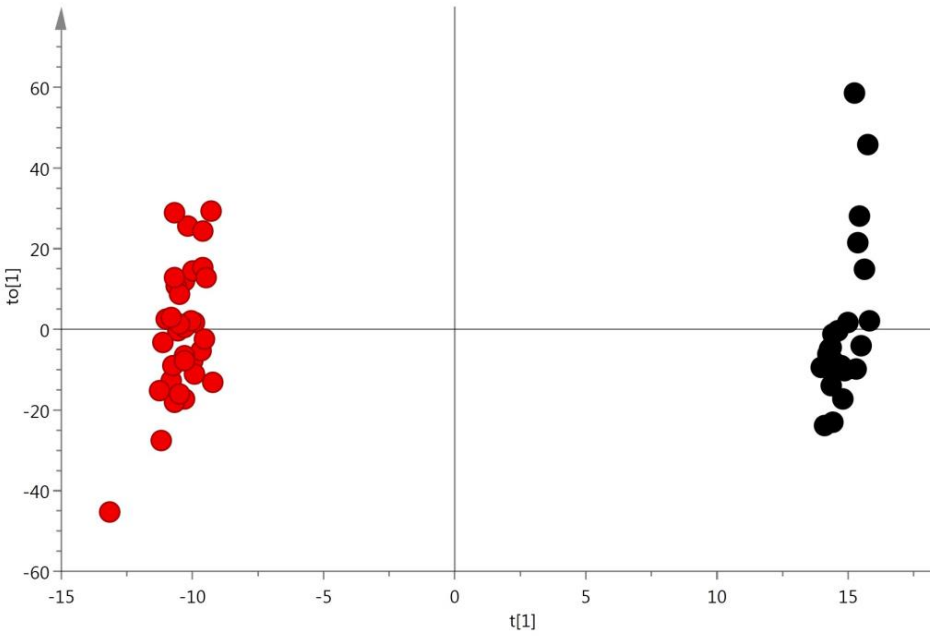


Figure 28: OPLS DA (log transformed) of urine analysed with HILIC MS negative ionisation mode showing separation of embolising carotid atherosclerosis (red) and peripheral stenosing arterial disease (black).

6.2.5 Results: Urine HILIC MS positive ionisation mode

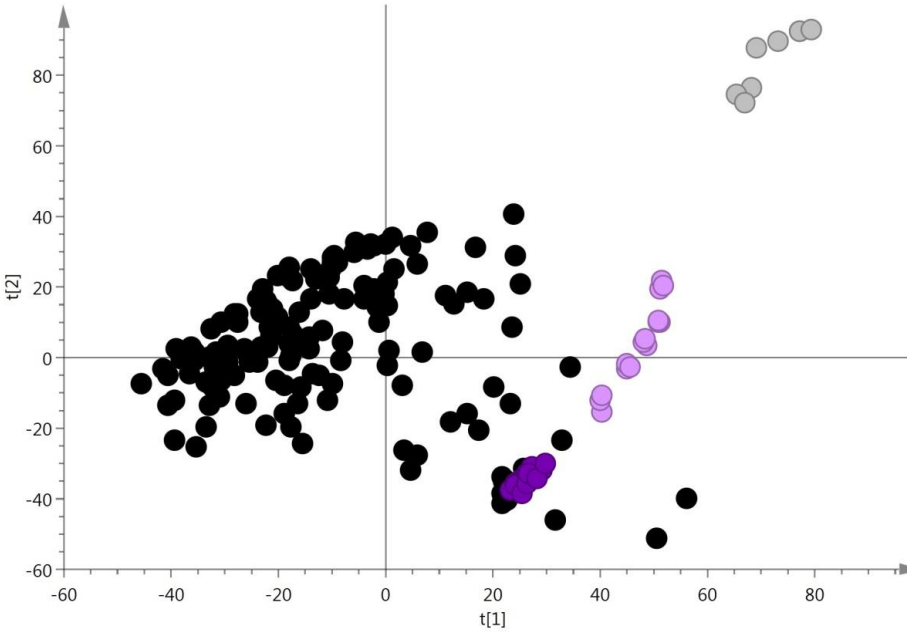


Figure 29: PCA showing distribution of urine samples analysed with HILIC MS positive ionisation mode (black), QCs (purple), dilutions (pink) and blanks (grey).

PCA of all samples compared with QCs, dilutions and blanks again determined outliers in Group 1 (sample 60), Group 3 (sample 4) and Group 4 (sample 63) (Figure 29). These samples were therefore excluded from further analysis. The PCA also demonstrated metabolic influence from solvent implying contamination, and these features were excluded from the subsequent analysis. As expected, PCAs comparing Groups 1 – 4, and Groups 1, 5 and 6 did not show visual separation of classes (Figure 30, Figure 31).

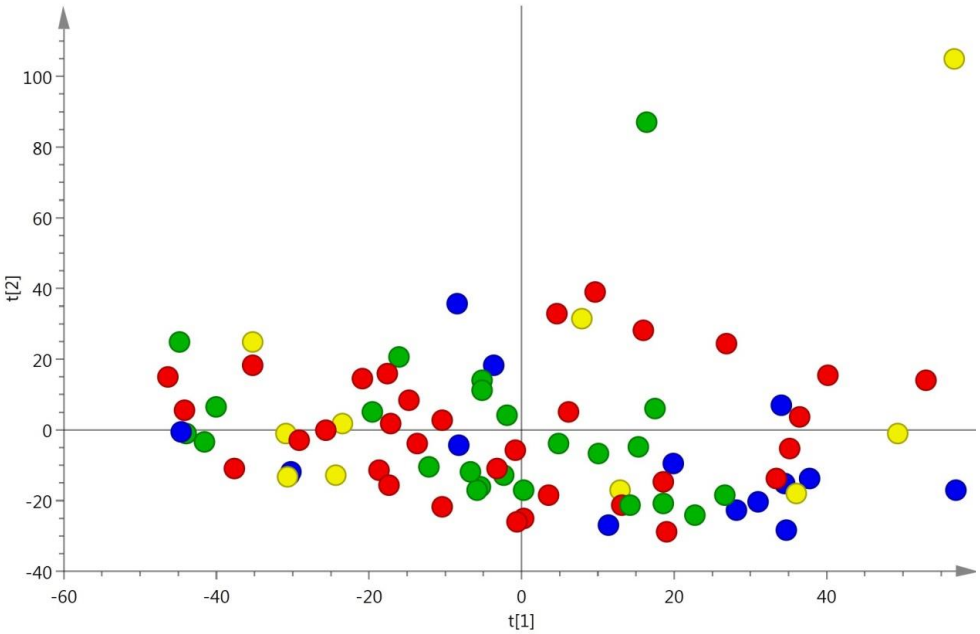


Figure 30: PCA showing distribution of urine analysed with HILIC MS positive ionisation mode from patients with symptomatic >50% carotid stenosis (red), non-carotid stroke/TIA (blue), asymptomatic >50% carotid stenosis (green) and controls (yellow).

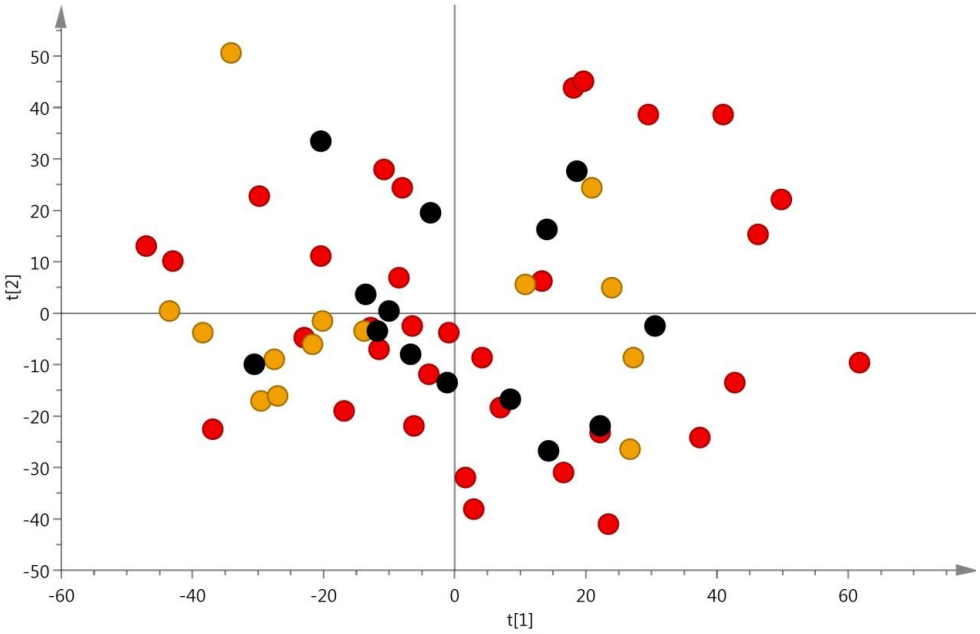
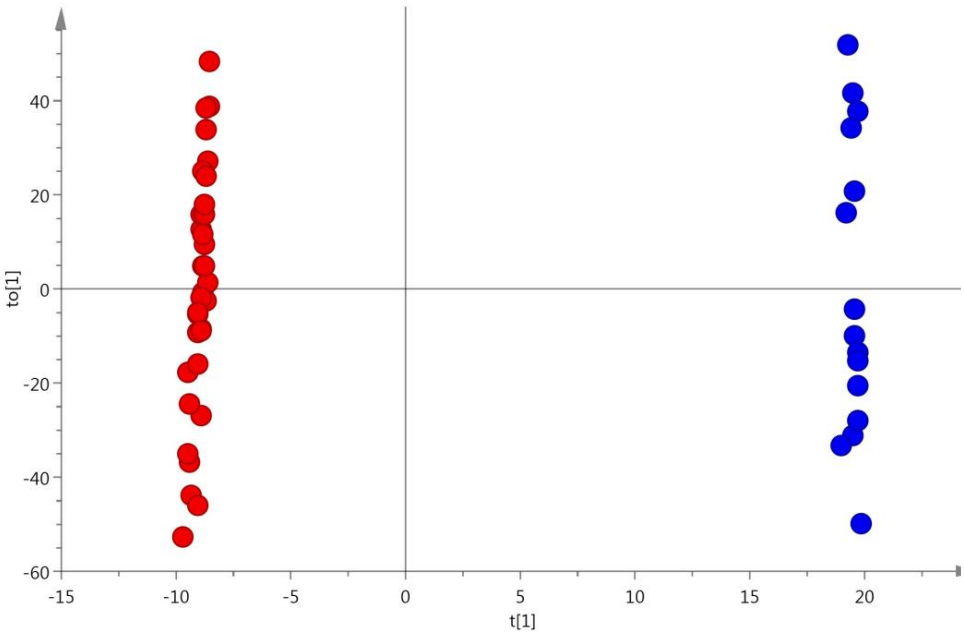


Figure 31: PCA showing distribution of urine samples analysed with HILIC MS positive ionisation mode derived from patients with embolising carotid disease (red), aneurysmal disease (orange) and stenosing peripheral arterial disease (black).

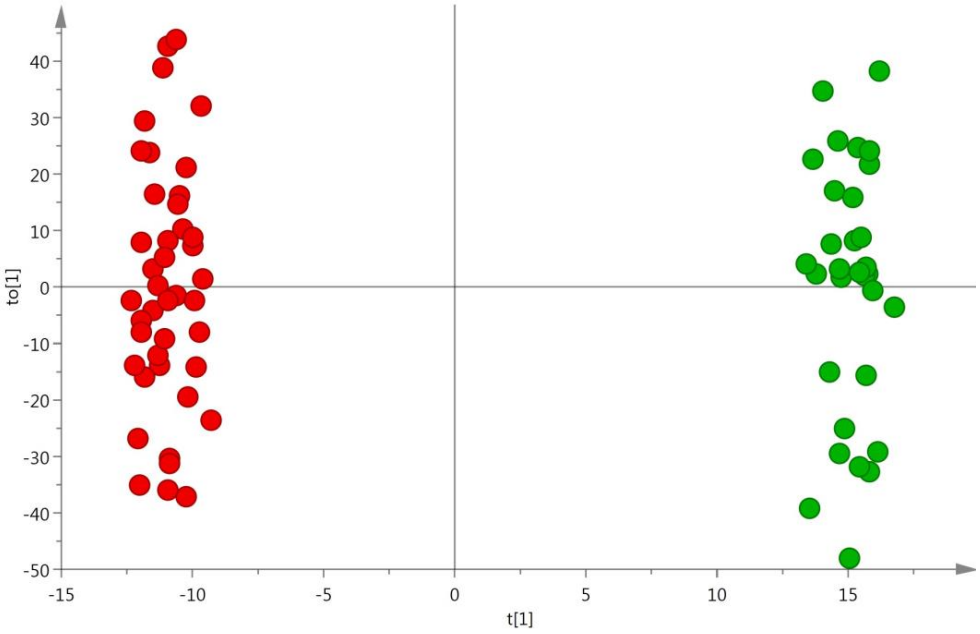
OPLS DA analysis comparing symptomatic carotid disease with all other classes followed similar trends to HILIC MS negative ionisation mode analysis. This result demonstrates consistency and reliability of technique.

Overall, acquired models were statistically stronger with HILIC MS of urine in the positive ionisation mode as compared with the negative ionisation mode. While separation was not seen between symptomatic carotid and non-carotid stroke/TIA participants (Figure 32), OPLS DA models comparing urine from symptomatic and asymptomatic carotid participants demonstrated excellent predictability, though again with relatively high R^2Y values (Figure 33).



R^2Y	0.999
Q^2	0.0199
Principal components	7

Figure 32: OPLS DA (log transformed) analying spectral features derived from analysis of urine analysed with HILIC MS positive ionisation mode from patients with symptomatic carotid atherosclerosis (red) and non-carotid stroke/TIA (blue).



R^2Y	0.996
Q^2	0.563
Principal components	5

Figure 33: OPLS DA (log transformed) separating urine samples analysed with HILIC MS positive ionisation mode from patients with symptomatic carotid atherosclerosis (red) and asymptomatic carotid atherosclerosis (green).

Permutation analysis was peformed with 20 permutations to cross-validate OPLS DA results for symptomatic and asymptomatic carotid stenosis (Figure 34). The flat gradient of R2 further corroborates the overfitted nature of the result, but nonetheless the analysis is statistically significant ($p = 4.492e^{-007}$).

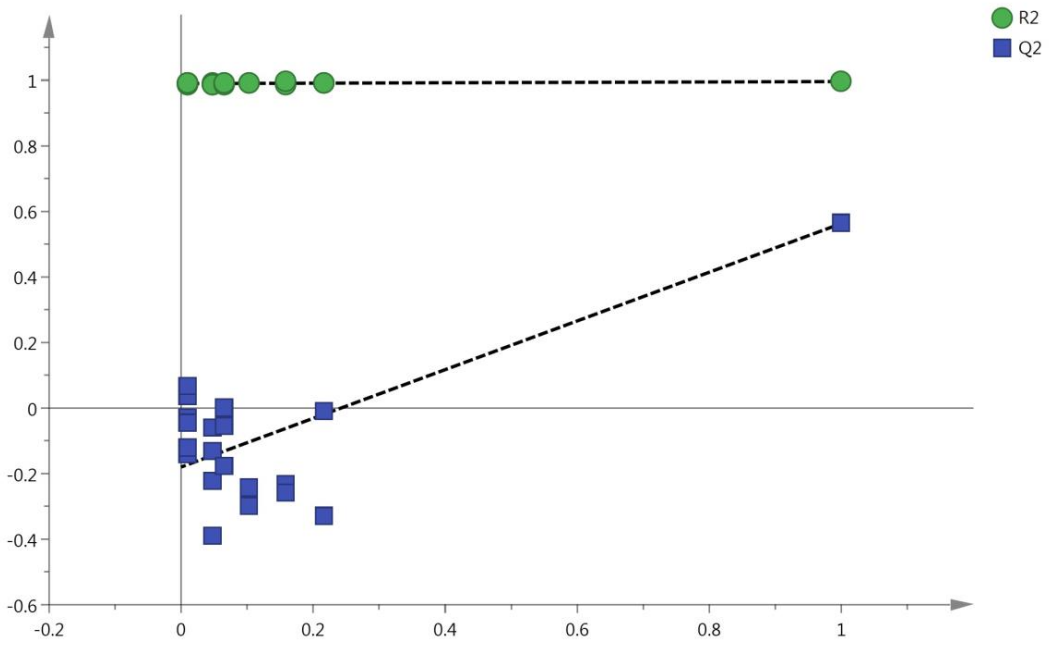
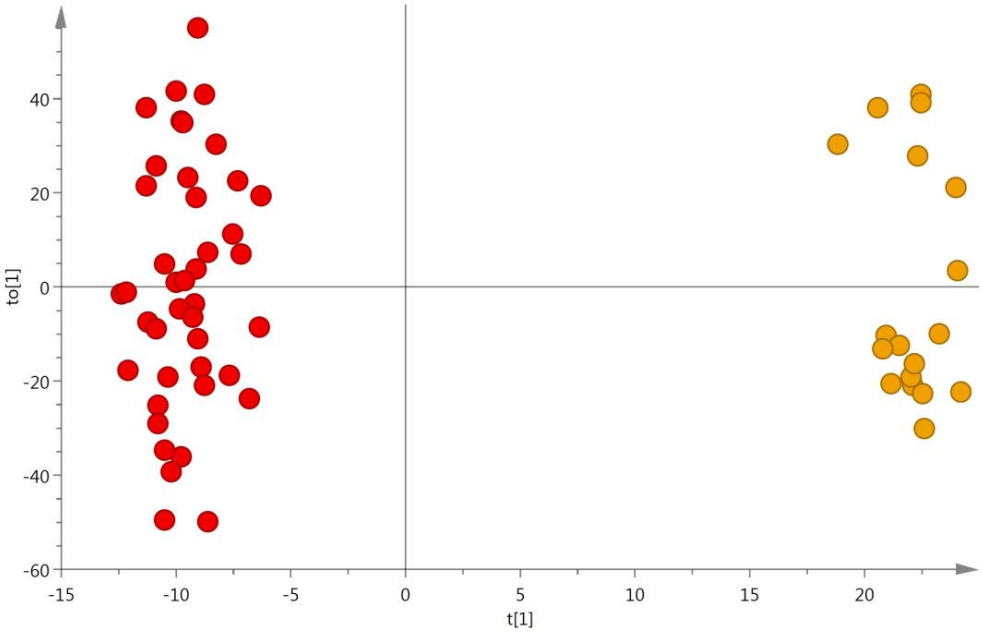


Figure 34: Permutation analysis with 20 permutations for OPLS DA of urine samples analysed with HILIC MS positive ionisation mode comparing symptomatic and asymptomatic carotid stenosis; $p = 4.492e^{-007}$.

Furthermore, comparison of urine from patients with embolising carotid disease with aneurysmal disease resulted in a favourably predictive OPLS DA model (Figure 35), which on permutation analysis was statistically highly significant (Figure 36). This was echoed by the comparison of embolising arterial disease with stenosing peripheral arterial disease (Figure 37 and Figure 38), again demonstrating convincing separation but with an overfitted R^2Y value. This model was also statistically significant.



R^2Y	0.990
Q^2	0.607
Principal components	4

Figure 35: OPLS DA (log transformed) showing separation of urine samples analysed with HILIC MS positive ionisation mode from patients with embolising carotid disease (red) and aneurysmal disease (orange).

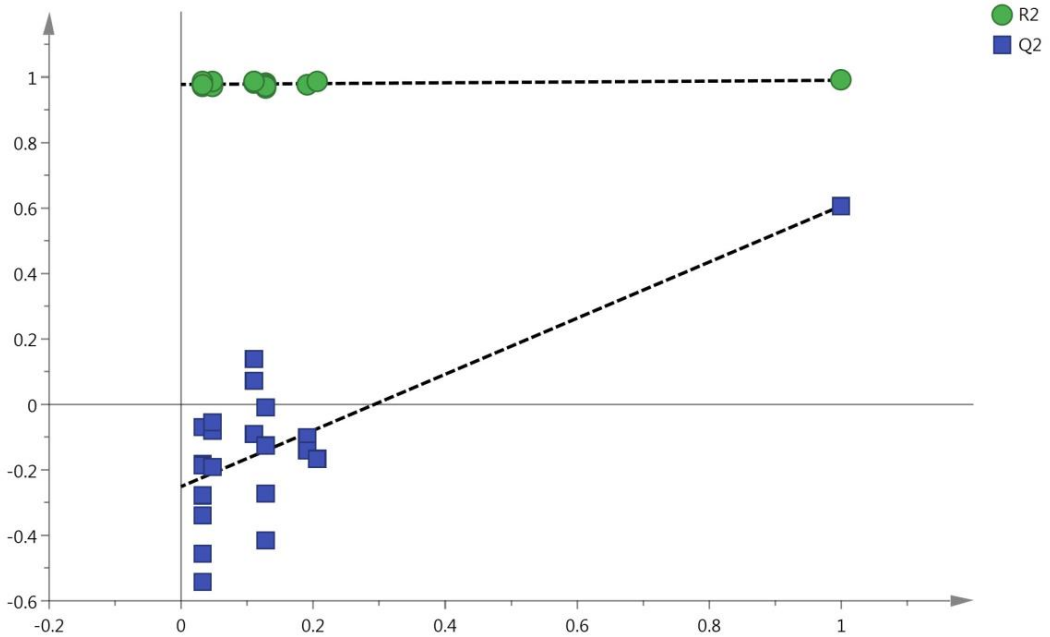
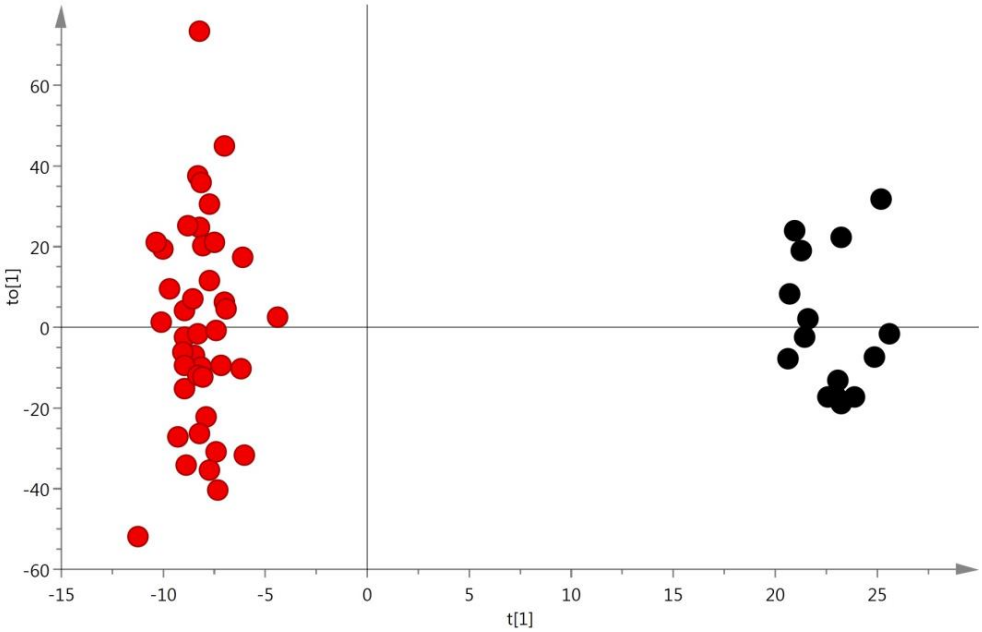


Figure 36: Permutation analysis (20 permutations) following OPLS DA of urine samples analysed with HILIC MS positive ionisation mode from patients with embolising (Group 1) and dilating (aneurysmal; Group 5) disease. The result is strongly significant with p = 1.79137e^{-007}.



R^2Y	0.990
Q^2	0.500
Principal components	4

Figure 37: OPLS DA (log transformed) separation urine samples analysed with HILIC MS positive ionisation mode from patients with embolising carotid disease (red) and peripheral arterial disease (black).

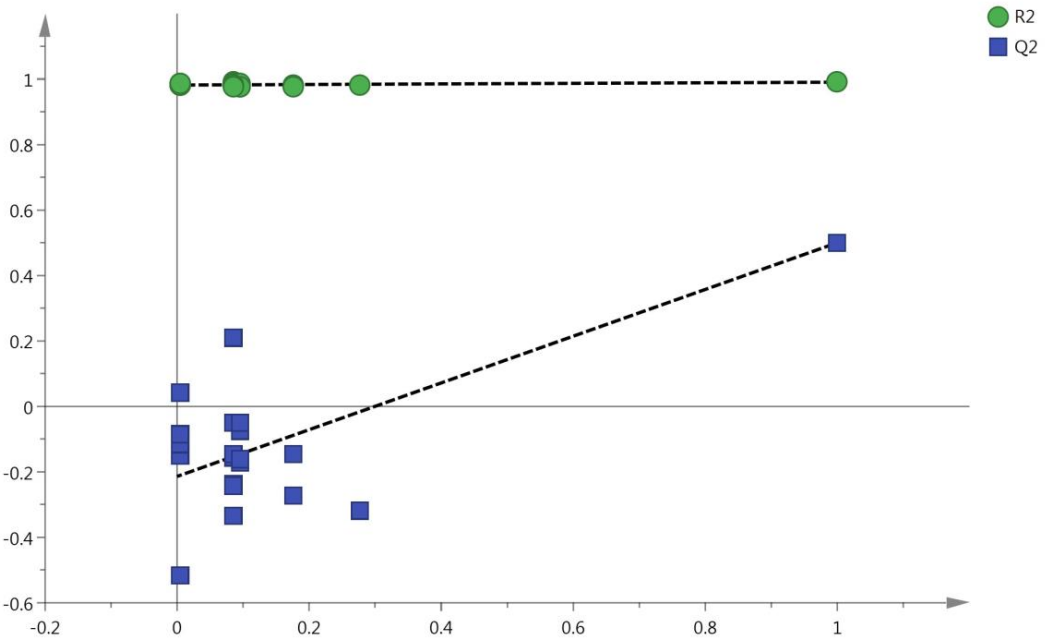


Figure 38: Permutation analysis (20 permutations) following OPLS DA of urine samples analysed with HILIC MS positive ionisation mode from patients with embolising (Group 1) and stenosing (Group 6) disease; p = 0.000128.

6.3 Urine ^{1}H-NMR

6.3.1 NMR urine buffer preparation

A 100ml conical flask and measuring cylinder was prepared by washing in demineralised water, LC MS water and then acetonitrile (each three times). The flask and cylinder were left in a 100 degree Celsius oven overnight to ensure all liquid had evaporated.

NMR urine buffer was prepared by dissolving g20.4 of KH_2PO_4 in 80ml of D_2O, to which was added 0.08g of TSP. A further 100mg of TSP and 13mg of NaN3 were dissolved in 10ml of D_2O, and added to the KH_2PO_4 solution. The mixture was shaken well, and pH adjusted to 7.4 through the addition of KOH pellets. Urine buffer was stored in a 4 degree Celsius fridge prior to sample preparation.

6.3.2 Sample preparation

Prior to preparation, samples were randomised using the randomisation function in Microsoft Excel for further preparation and analysis.

Urine frozen in 1ml aliquots at -80*C was retrieved and kept at room temperature to allow it to thaw (approximately 1 hour). Once defrosted, eppendorfs were centrifuged at 12000G and 4 degrees Celsius for five minutes. A 540 microliter volume of supernatant was added to a 5mm SampleJET NMR tube, to which 60 microliters of buffer was added. The SampleJet tubes were capped and sealed, then inverted multiple times to ensure mixing of urine and buffer.

Six quality control samples were prepared using pooled urine containing an equal proportion of each sample to be analysed.

6.2.3 Results: Urine 1H-NMR

PCA of all urine samples and QCs showed very tight clustering of QCs in the centre of all patient samples – indicating a reliable, high quality and stable analysis (Figure 39). No separation was seen between groups on PCA (Figure 40 and Figure 41), but PCA of all samples alone demonstrated one distinct outlier – again sample 60. This was excluded from subsequent OPLS DA analyses. UV scaling was used throughout, as is the norm with NMR multivariate analysis. Pareto scaling did not improve models.

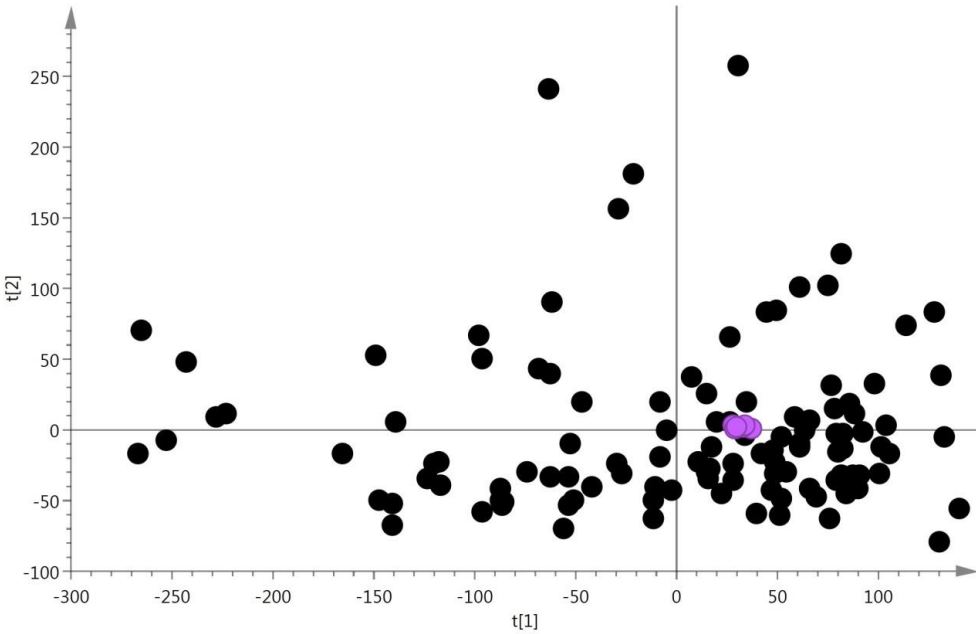


Figure 39: PCA showing distribution of urine samples (black) and QCs (purple) analysed with 1H-NMR.

9 principal components

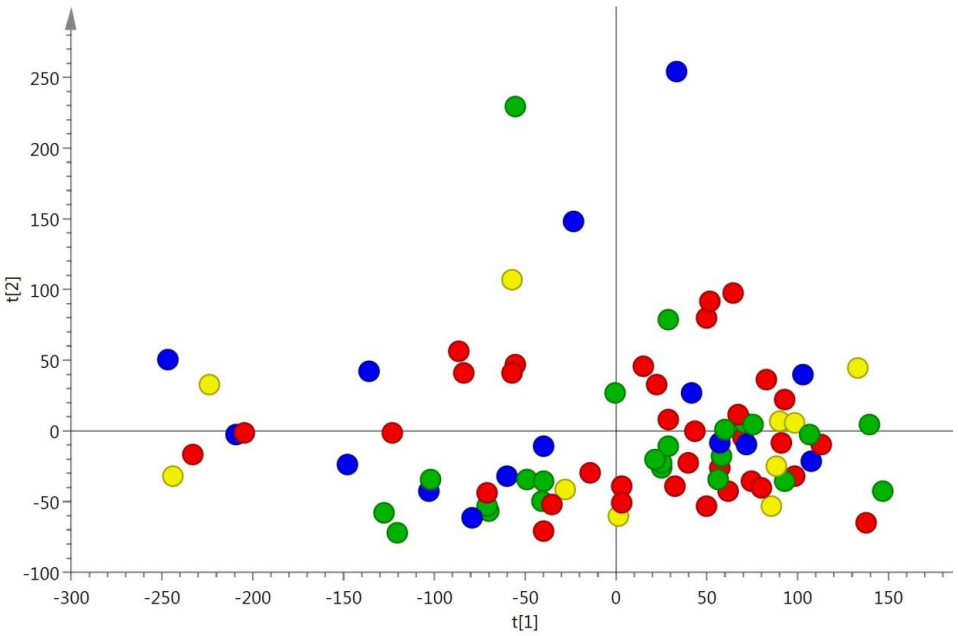


Figure 40: PCA showing distribution of urine samples analysed with 1H-NMR from patients with symptomatic >50% carotid atherosclerosis (red), non-carotid stroke/TIA (blue), asymptomatic >50% carotid atherosclerosis (green) and controls. The data is derived from 9 PCs.

7 principal components

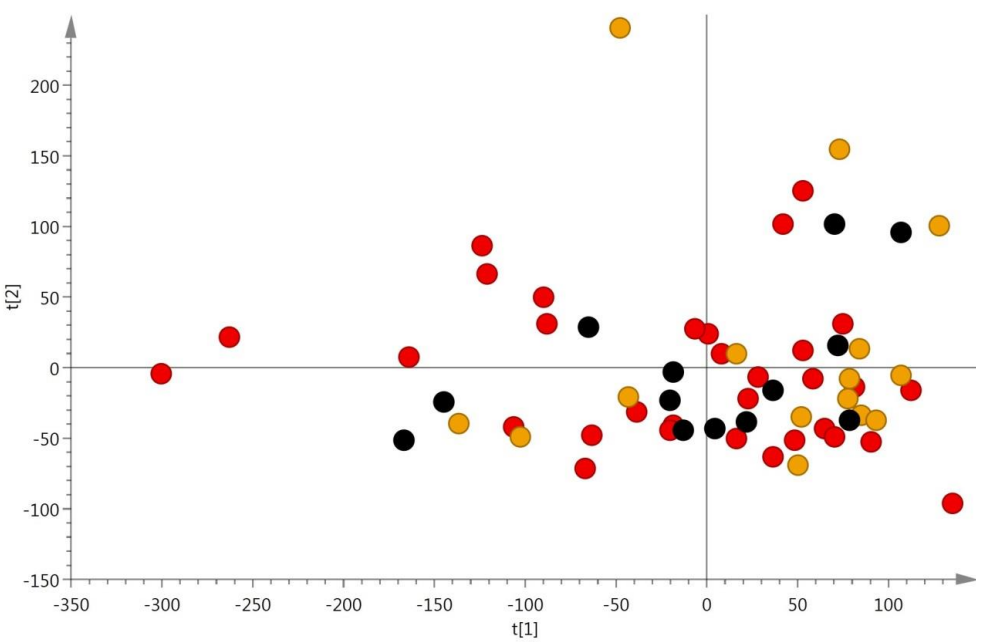
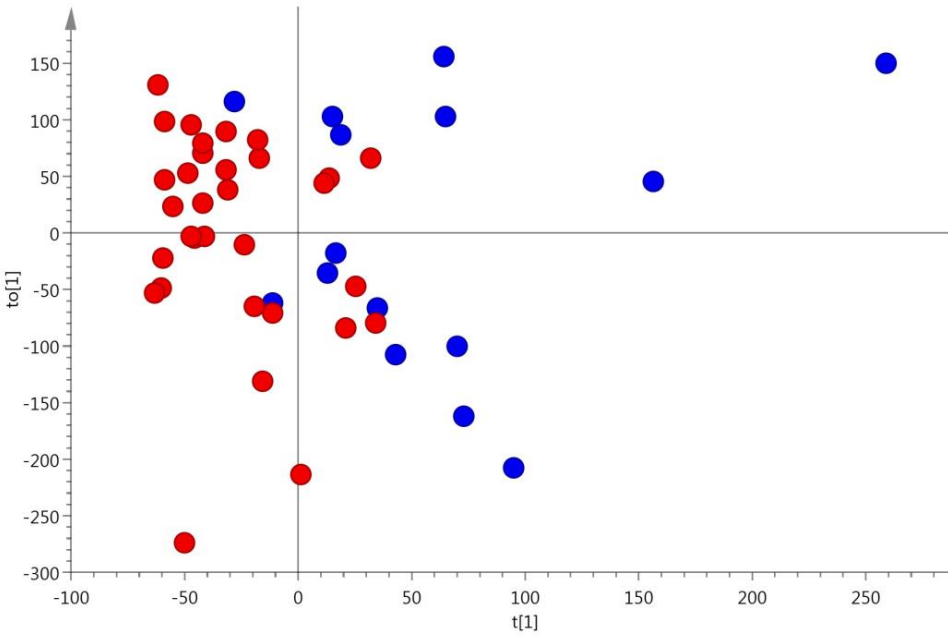


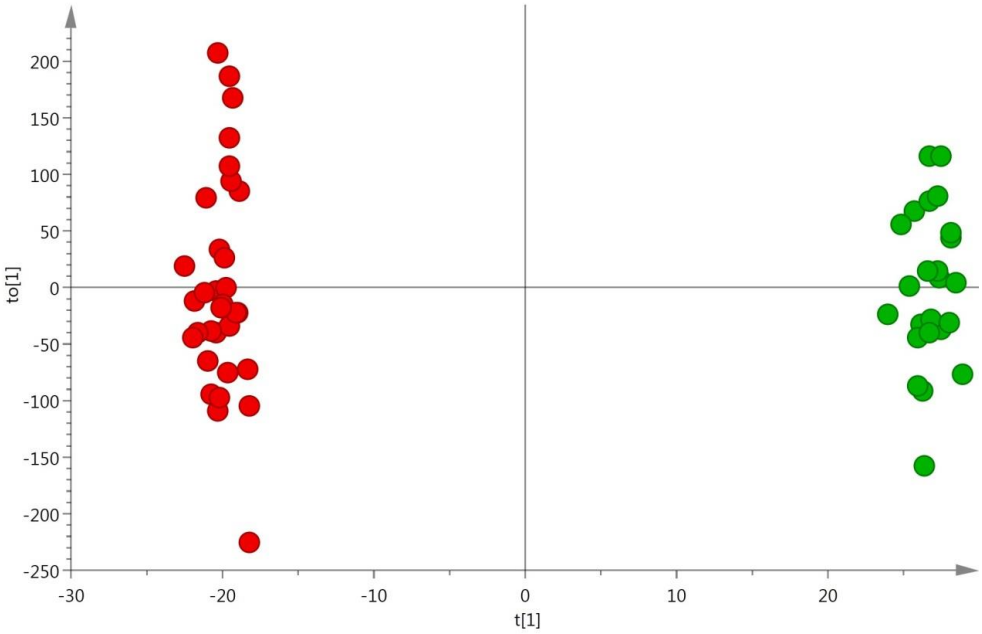
Figure 41: PCA showing distribution of urine samples analysed with 1H-NMR from patients with embolising carotid atherosclerosis (red), aneurysmal disease (orange) and peripheral arterial disease (black).



R^2Y	0.435
Q^2	-0.106
Principal components	2

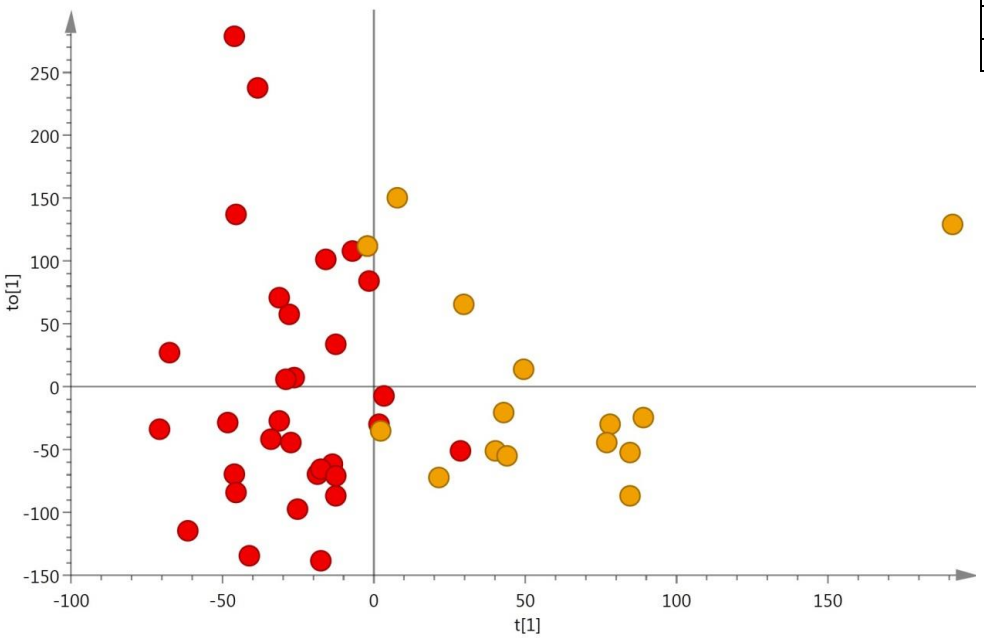
Figure 42: OPLS DA analysing NMR spectra of urine samples from patients with symptomatic carotid atherosclerosis (red) and non-carotid stroke (blue).

OPLS DA comparison of urine from patients with symptomatic carotid atherosclerosis and asymptomatic carotid atherosclerosis resulted in a non-predictive model, as denoted by the negative Q^2 value (Figure 42). Similarly, comparison of symptomatic and asymptomatic carotid atherosclerosis was also non-predictive (Figure 43). Weak separation was encountered between embolising carotid atherosclerosis and aneurysmal disease (Figure 44), and embolising carotid atherosclerosis and stenosing peripheral arterial disease (Figure 45).



R^2Y	0.988
Q^2	0.0752
Principal components	8

Figure 43: OPLS DA analysing NMR spectra of urine samples from patients with symptomatic carotid atherosclerosis (red) and asymptomatic carotid atherosclerosis (green).



R^2Y	0.592
Q^2	0.244

Figure 44: OPLS DA analysing NMR spectra of urine samples from patients with symptomatic carotid atherosclerosis (red) and aneurysmal disease (orange).

R^2Y	0.886
Q^2	0.155
Principal components	3

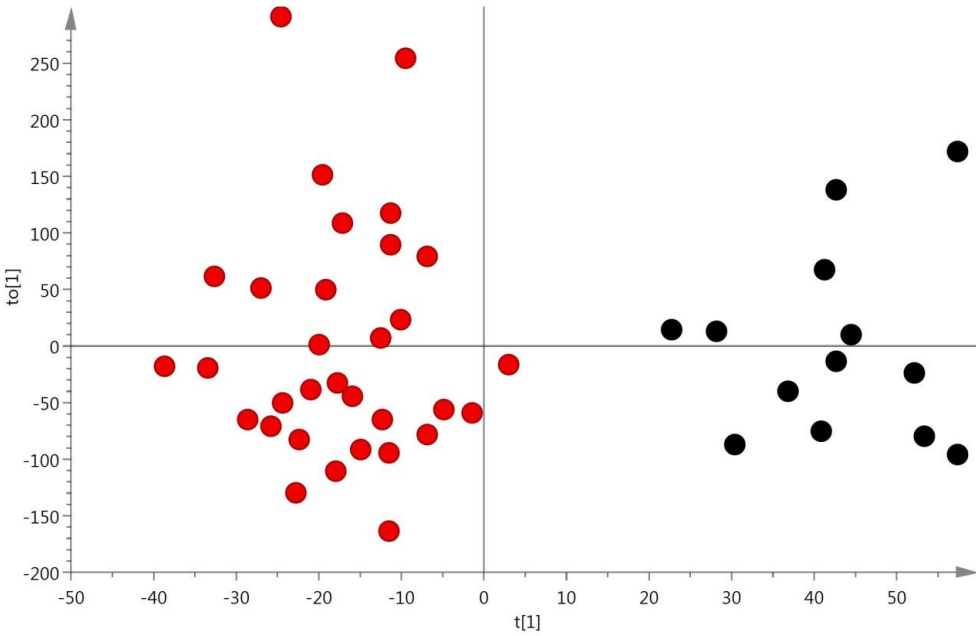


Figure 45: OPLS DA analysing NMR spectra of urine samples derived from patients with embolising carotid atherosclerosis (red) and stenosing peripheral arterial disease (black).

6.4 Metabolite Identification

The analysis yielding the strongest statistical models was selected for metabolite identification. This proved to be HILIC MS analysis of urine in the positive ionisation mode.

The S plots of significant OPLS DA models were generated in SIMCA. Each data point on an S plot represents a variable. The p axis represents variation between groups, and the p(corr) axis depicts consistency. The "tips" of the S plot therefore account for the most reliable and discriminating biomarkers.

Figure 46 represents the S plot of OPLS DA of urine in the HILIC MS positive ionisation mode comparing patients with symptomatic carotid atherosclerosis and asymptomatic carotid atherosclerosis. The red circles are the most

discriminating metabolites. Figure 47 demonstrates the S plot of embolising carotid and aneurysmal disease comparison, and Figure 48 shows the S plot of embolising carotid atherosclerosis versus peripheral arterial disease.

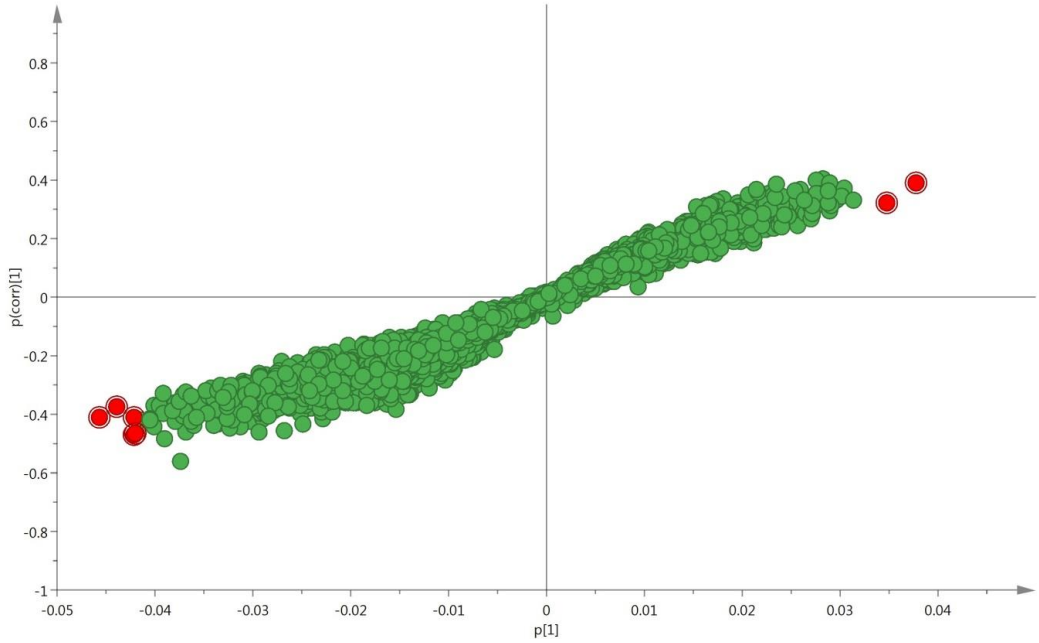


Figure 46 S plot generated from OPLS DA of urine analysed with HILIC MS in the positive ionisation mode from patients with symptomatic carotid atherosclerosis and asymptomatic carotid atherosclerosis

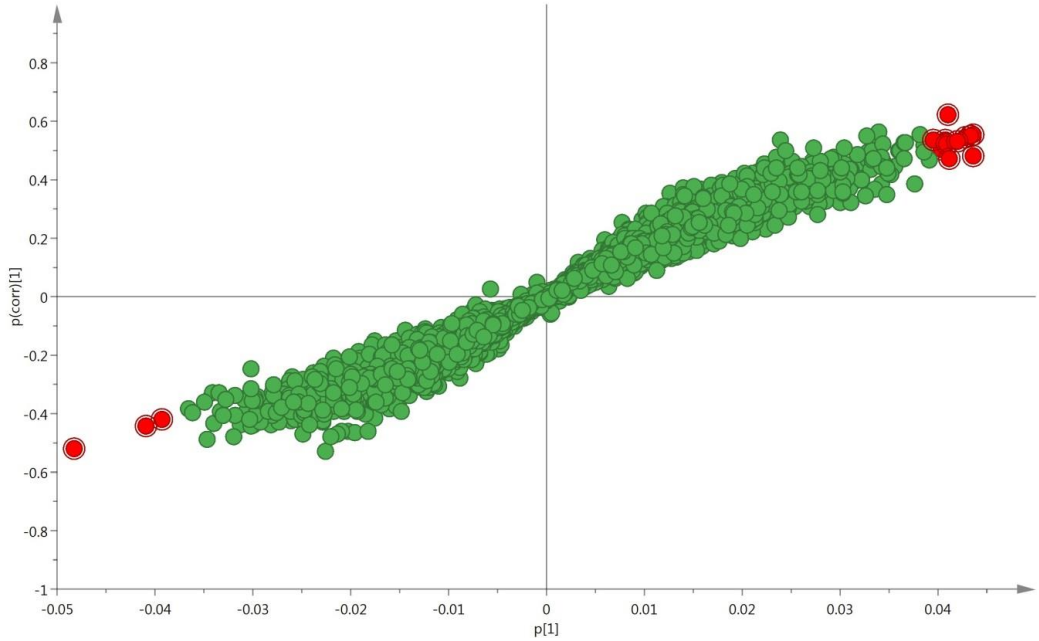


Figure 47 S plot generated from OPLS DA of urine analysed with HILIC MS in the positive ionisation mode from patients with embolising carotid disease and aneurysmal disease

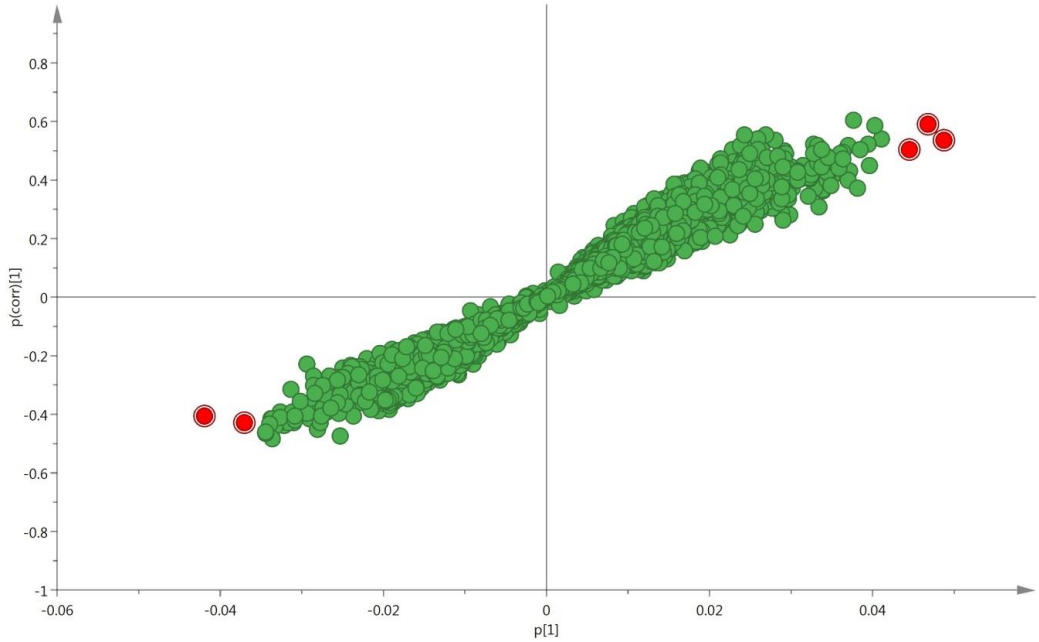


Figure 48 S plot generated from OPLS DA of urine analysed with HILIC MS in the positive ionisation mode from patients with embolising carotid disease and peripheral arterial disease

The highlighted discriminating variables were assessed and tabulated as shown in Table VI. The sample with the highest intensity signal for each variable was identified.

Table VI Adducts of discriminating metabolites

Comparison	m/z ratio	Retention time (min)	CV%	p	p(corr)	Relative increase	Sample
Group 1 vs Group 5	306.064	35.022	19.7285	0.0410094	0.623689	Group 5	P14U
Group 1 vs Group 5	643.008	35.023	24.6617	0.0436384	0.480811	Group 5	P14U
Group 1 vs Group 5	643.057	35.0245	10.7949	0.0394794	0.535176	Group 5	P14U
Group 1 vs Group 6	638.96	36.34	18.9545	-0.0370028	-0.427357	Group 1	3U
Group 1 vs Group 6	170.965	53.446	28.1746	0.0468661	0.592342	Group 6	P13U
Group 1 vs Group 5	562.93	56.7395	20.8328	-0.0482199	-0.520748	Group 1	74U
Group 1 vs Group 3	874.978	59.013	19.2126	0.0347251	0.322551	Group 3	19U
Group 1 vs Group 5	874.978	59.013	19.2126	0.0411489	0.471405	Group 5	19U
Group 1 vs Group 5	826.987	79.535	29.2148	0.0433635	0.551378	Group 5	P12U
Group 1 vs Group 6	826.987	79.535	29.2148	0.0487348	0.537729	Group 6	P12U
Group 1 vs Group 5	827.015	79.544	27.5743	0.0420143	0.531616	Group 5	A17U
Group 1 vs Group 6	827.015	79.544	27.5743	0.0444785	0.505102	Group 6	A17U
Group 1 vs Group 5	649.019	91.3535	29.2569	0.0435654	0.554331	Group 5	A16U
Group 1 vs Group 5	778.216	114.446	26.5512	0.0408778	0.523519	Group 5	A17U
Group 1 vs Group 5	444.079	203.659	18.6575	-0.0392469	-0.419671	Group 1	78U
Group 1 vs Group 3	705.839	204.554	15.3807	-0.0421514	-0.471397	Group 1	60U
Group 1 vs Group 3	718.058	206.706	14.9256	-0.0415242	-0.41956	Group 1	77U
Group 1 vs Group 6	432.357	274.377	27.0496	-0.0418705	-0.403748	Group 1	7U
Group 1 vs Group 3	769.809	295.202	10.1688	-0.0439087	-0.373	Group 1	60U
Group 1 vs Group 3	787.809	295.989	11.9322	-0.0419526	-0.466516	Group 1	63U
Group 1 vs Group 3	673.936	302.67	12.3514	-0.0456824	-0.409772	Group 1	63U
Group 1 vs Group 5	1167.35	327.306	26.0922	-0.0409917	-0.443194	Group 1	2U
Group 1 vs Group 5	247.048	337.536	11.2666	0.0428102	0.543858	Group 5	18U
Group 1 vs Group 5	475.046	338.805	24.4683	0.0407271	0.534706	Group 5	P14U
Group 1 vs Group 5	517.038	343.774	29.0611	0.0403155	0.503465	Group 5	A4U
Group 1 vs Group 3	773.076	372.118	19.9211	-0.0421496	-0.412028	Group 1	86U
Group 1 vs Group 3	627.214	393.648	23.5837	0.0378041	0.391852	Group 3	102U

In order to assign each metabolite, the spectrum from the sample with the highest intensity of each variable was examined. The following example illustrates the process, utilising the first variable (row) in Table VI.

The chromatogram from HILIC MS positive ionisation mode analysis of urine from participant P14 was examined in MassLynx (Figure 49).

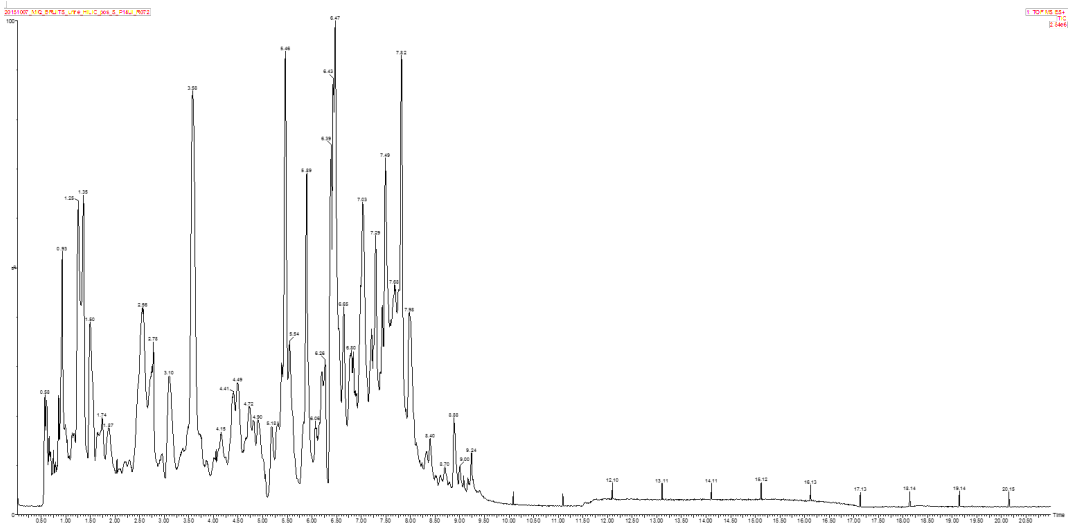


Figure 49 Chromatogram of HILIC MS (positive mode) analysis of urine from participant P14

Retention time was converted from minutes to seconds (35.022/60 = 0.5378). The chromatogram was searched for the mass/charge ratio of interest, in this case 306.064. The resulting chromatogram showing retention times for each moiety of this m/z ratio is shown in Figure 50.

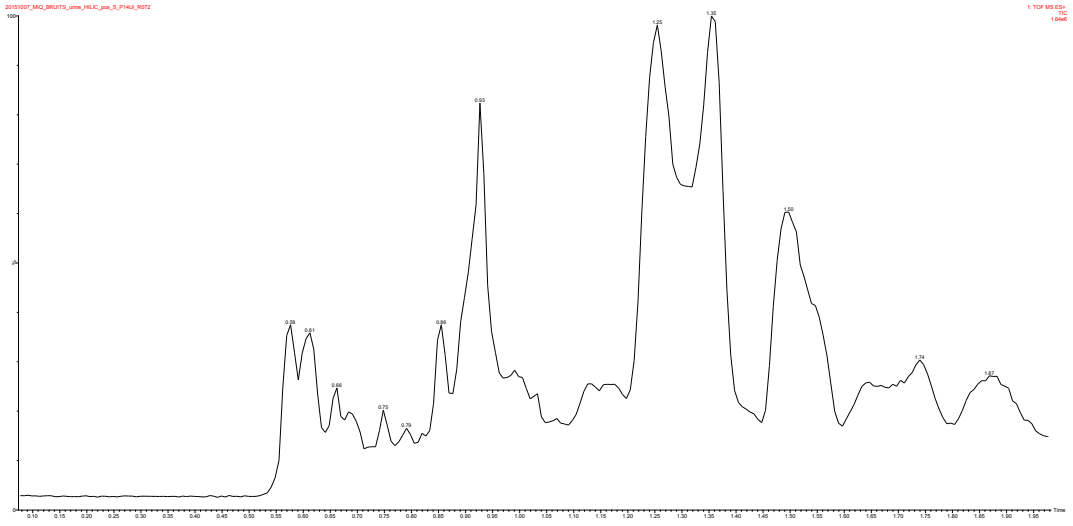


Figure 50 Chromatogram representing elution times of each moiety with m/z ratio 306.064

The peak corresponding to the appropriate retention time was identified, and the peaks on the MS spectrum were examined (Figure 51) to identify the m/z ratio moiety of interest, in this example 306.064, evident in Figure 51. The relationship of this peak with neighbouring dominant peaks and adducts was assessed to identify the parent ion (hydrogenated metabolite).

Certain relationships between adduct and parent ion commonly exist in this form of mass spectrometry analysis. However in certain cases it was not possible to identify the parent ion.

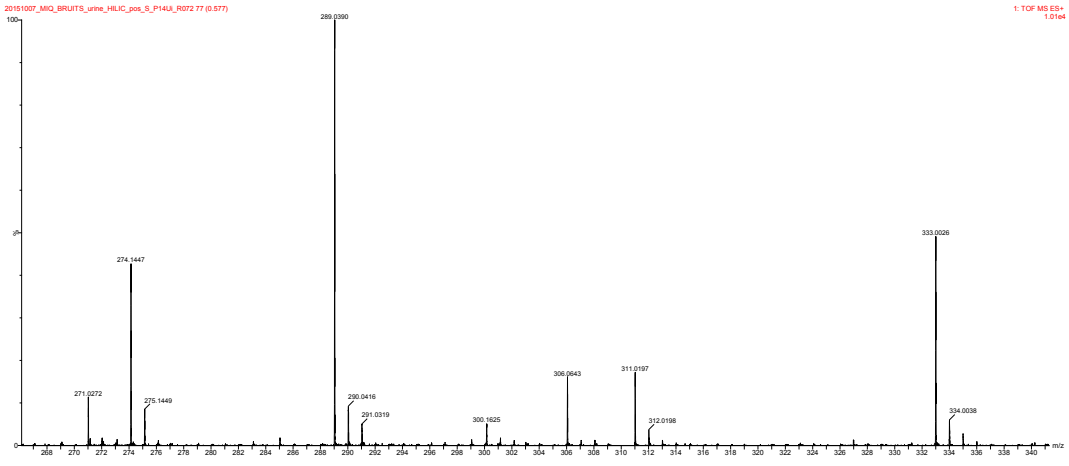


Figure 51 m/z ratios of moieties eluting at the desired retention time

Once the parent ion was known, a database search was performed to identify the responsible metabolite. Three databases were used: Human Metabolome Database, Metlin and Lipid Maps. If a metabolite was not listed on each of the databases, it was termed “unassigned”. Tables VII, VIII and IX show the results of metabolite identification.

Table VII Putative biomarkers discrminating between symptomatic (Group 1) and asymptomatic (Group 3) carotid atherosclerosis

Relative change	Retention time (s)	m/z ratio	Ion Type	Possible Metabolites
Higher in Symptomatic	204.554	705.839	[M+H}+	UNASSIGNED
Higher in Symptomatic	295.202	747.84	[M+H]+	UNASSIGNED
		769.809	[M+Na]+	
		787.809	isotope of [M+K]+	
Higher in Symptomatic	302.67	651.969	[M+H]+	UNASSIGNED
		673.936	[M+Na]+	
Higher in Symptomatic	372.118	773.076	[M+H]+	Guanosine triphosphate adenosine
Higher in Asymptomatic	393.648	302.197	[M+H]+	Nonanoyl carnitine Prolyl-tryptophan Dobutamine
		626.225	[2M+Na]+	
		627.214	Isotope of [2M+Na]	

Table VIII Putative biomarkers discriminating between symptomatic carotid atherosclerosis (Group 1) and aneurysmal disease (Group 5)

Relative change	Retention time (s)	m/z ratio	Ion type	Possible metabolites
Higher in Aneurysms	35.022	289.039	[M+H]+	5'-(3',4'-dihydroxyphenyl)-gamma-valerolactone sulfate
		306.064	[M+NH4]+	
		643.008	[2M+3Na-2H]+	
Higher in Aneurysms	79.544	381.038	[M+H]+	1-(naphthalen-2-yl)-2-[(6-nitro-1,3-benzothiazol-2-yl)sulfanyl]ethanone
		827.015	[2M+3Na-H]+	
Higher in Aneurysms	91.3535	649.019	[M+H]+	Unassigned
Higher in Aneurysms	114.446	739.216	[M+H]+	3-O-beta-D-Galactopyranosylproanthocyanidin A5' Malvidin 3-glucoside-5-(6''-malonylglucoside) Kaempferol 7-methyl ether 3-[3-hydroxy-3-methylglutaryl-(1->6)]-[apiosyl-(1->2)-galactoside] Apigenin 7-rhamnosyl-(1->6)-(4''-E-p-methoxycinnamoylglucoside)
		777.214	[M+K]+	
		778.216	isotope of [M+K]+	
Higher in Aneurysms	337.536	247.048	[M+H]+	L-alpha-Aspartyl-L-hydroxyproline

Table IX Putative biomarkers discriminating between embolising carotid (Group 1) and stenosing peripheral arterial disease (Group 6)

Relative change	Retention time (s)	m/z ratio	Ion type	Possible metabolites
Higher in symptomatic carotids	36.34	638.96	Uncertain	Unassigned
Higher in PAD		381.038	[M+H]+	1-(naphthalen-2-yl)-2-[(6-nitro-1,3-benzothiazol-2-yl)sulfanyl]ethanone
	79.544	827.015	[2M+3Na-H]+	
Higher in symptomatic carotids	274.377	432.357	[M+H]+	N-stearoyl phenylalanine
				OMDM-1/OMDM-2
				Peimine
				Zhebeinine
				Solanocardinol

6.5 Discussion

Global metabolic profiling techniques have been applied to urine samples to determine biomarkers of high-risk carotid disease, and find features distinguishing embolising disease from dilating and stenosing phenotypes of atherosclerosis. Following ^{1}H-NMR, HILIC MS and RP MS profiling, the strongest statistical models have stemmed from HILIC MS analysis, both positive and negative ionisation modes, while acknowledging the statistical overfitting encountered, denoted by relatively high values of R^2Y. A number of putative biomarkers have been identified when comparing urine from symptomatic carotid atherosclerosis with asymptomatic and other forms of arterial disease. These biomarkers span an array of chemical moieties, but certain compounds

bear a recognised association with neurological function and/or cardiovascular physiology.

6.5.1 Adducts pertaining to separation between symptomatic and asymptomatic carotid atherosclerosis

Guanosine triphosphate adenosine is a dinucleoside polyphosphate with known intra- and extra-cellular functions (124, 125). It has been isolated from cerebral nerve terminals and acts on P receptors found widely in the brain but also peripheral tissue including smooth muscle. It is postulated that GTP serves not only as a neurotransmitter, but has a neuroendocrine regulatory function (124, 125).

Prolyl-tryptophan is a dipeptide combination of proline and tryptophan and is a product of protein catabolism. It has not previously been isolated in human biofluids, but in rat brain high levels of Prolyl-tryptophan have been found to inhibit serotonin production and enhance the kynurenine pathway (126). Increased ratio of kynurenine:tryptophan has been studied in coronary artery disease, and linked to both inflammation, and the development of depressive symptoms (127). Decreased levels of prolyl-tryptophan in asymptomatic disease is therefore a scientifically credible possibility.

It could be postulated that these discriminating metabolites are a result of stroke/TIA as opposed to the presence of symptomatic carotid atherosclerosis. If this were the case, it would be expected that they would remain discriminatory

when comparing embolising carotid disease with peripheral arterial disease and aneurysmal disease, as none of these participants had suffered a recent neurological event. However, these putative metabolites did not retain their discriminating influence when comparing symptomatic carotid atherosclerosis with aneurysmal disease and peripheral arterial disease, as shown in Figures 52 and 53.

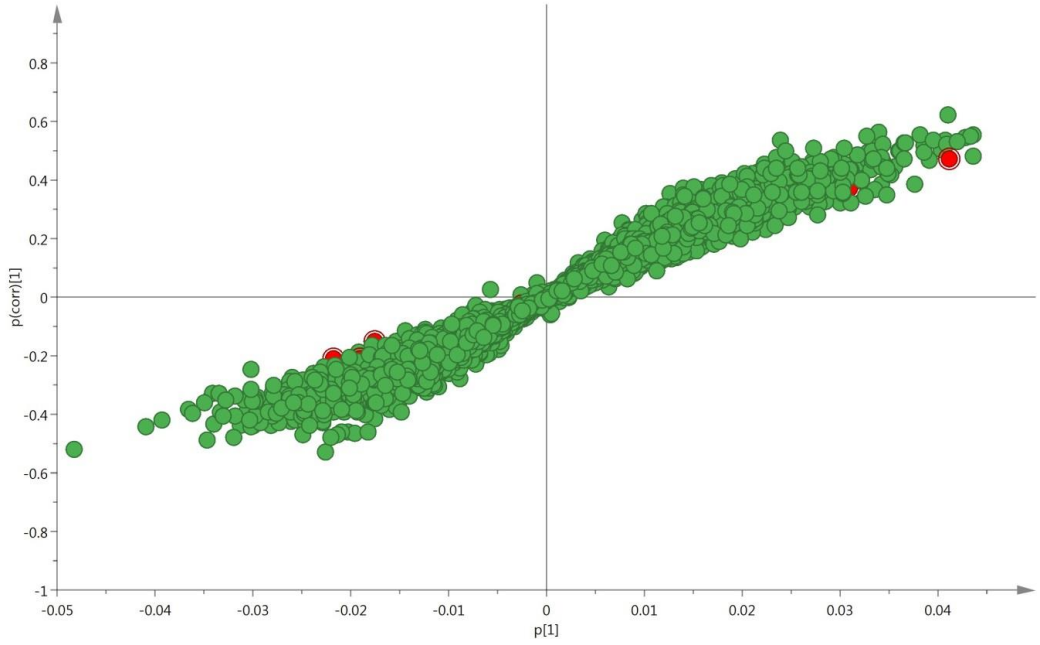


Figure 52 S plot generated from OPLS DA of embolising carotid disease compared with aneurysmal disease. The red circles indicate metabolites discriminating between symptomatic and asymptomatic carotid atherosclerosis.

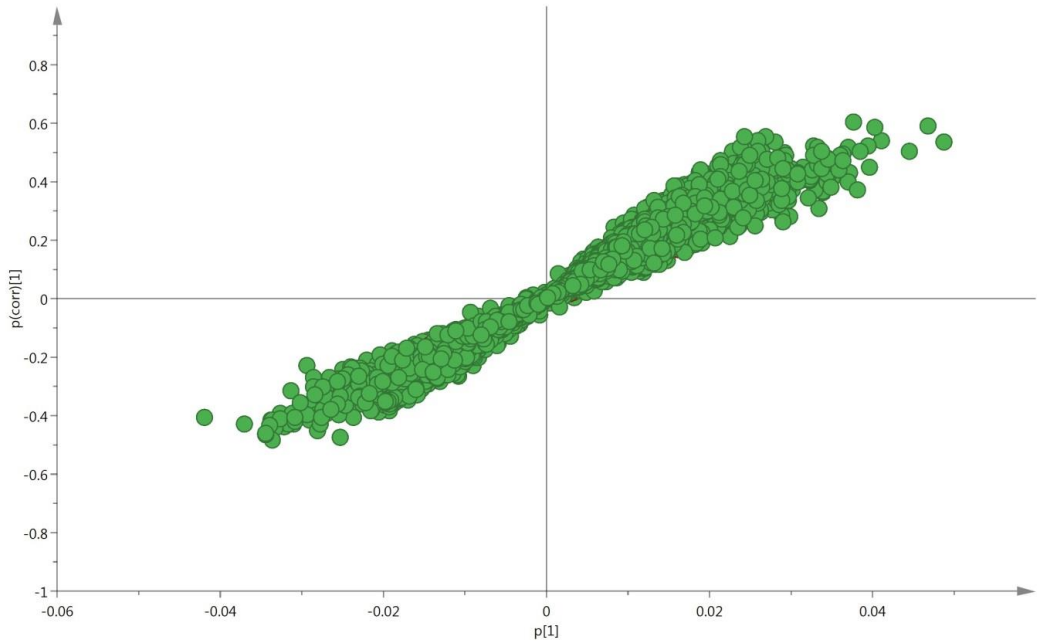


Figure 53 S plot generated from OPLS DA of embolising carotid disease compared with peripheral arterial disease. Metabolites discriminating between symptomatic and asymptomatic carotid atherosclerosis are masked and non discriminatory here.

Additionally, metabolites pertaining to cerebral injury (as opposed to symptomatic carotid atherosclerosis) are filtered twice, at the blood-brain barrier as well as the Bowman's capsule. This could make such metabolites less readily detectable beyond the acute event of brain injury.

6.5.2 Adducts pertaining to separation between embolising carotid disease and alternative phenotypes of peripheral arterial disease

One metabolite is consistently diminished in embolising carotid disease when compared with aneurysmal or peripheral arterial disease: 1-(naphthalen-2-yl)-2-[(6-nitro-1,3-benzothiazol-2-yl)sulfanyl]ethanone. Little is known or published regarding this entity, it is termed an "expected" metabolite as it is known to be found in humans, but has not yet been quantified. As it successfully discriminates embolising carotid from peripheral arterial disease as well as aneurysmal disease, it may be pertinent to further investigate its importance.

Another interesting finding is the possible role of OMDM1/OMDM2 in distinguishing between symptomatic carotid atherosclerosis and claudication. OMDM is a selective reuptake inhibitor of AEA, a long chain fatty acid. As described previously, Naccarato et al. used mass spectrometry to compare plasma concentrations of AEA in patients with hyper-acute stroke to well-matched healthy volunteers(116) and found that AEA was significantly elevated in stroke patients as compared to healthy controls (3.42 +/- 2.71 pmol/lipid mg vs. 1.81 +/- 1.53 pmol/lipid mg; $p<0.05$). Plasma AEA correlated positively with size of infarct, and degree of neurological impairment(116). Peripherally, AEA acts on CB2 receptors. It is uncertain here whether this finding is a consequence of stroke/TIA or embolising disease, but this metabolite did not discriminate between symptomatic stroke/TIA patients and those with aneurysmal disease.

Several metabolites are known to be found in urine (128), e.g. 5'-(3',4'-dihydroxyphenyl)-gamma-valerolactone sulfate a metabolite of tea and cocoa

synthesised by gut microflora (129), but their role in the pathogenesis of cardiovascular disease is undetermined. Further investigation would permit metabonomics to serve as the tool interpreting the complex interplay of genetic predisposition with both lifestyle choices and microbiomal environments to determine pathways leading to systemic disease.

6.5.3 Level of assignment

As the metabolites, where possible, have been assigned through database search, the "level of assignment" remains low. The highest level of assignment is achieved through MS/MS experimentation, where a purchased standard is fragmented and the resulting spectrum compared with that which is experimentally acquired to determine a chemical match.

Future Directions

The findings herein of urinary metabolites distinguish embolising, symptomatic carotid atherosclerosis from other phenotypes of arterial disease, including asymptomatic carotid atherosclerosis, is unprecedented. In keeping with the hypothesis-generating nature of global metabolic profiling, it opens the door to avenues of future clinically-relevant translational research.

Prior to embarking on further investigation, it would be scientifically and clinically important to validate the findings through a second cohort of patients, and may involve refining the design of this study to rectify certain challenges encountered.

Once validated, MS/MS experimentation would be necessitated, in order to maximise the level of assignment of discriminating metabolites. These would thereafter be subjected to targeted investigation.

Chapter 7: Metabolic profiling of serum

Serum is the most commonly interrogate biofluid in metabonomic analysis(130). In this study, the composition of serum serves as the bridge between pathophysiological processes within arterial tissue, and the resultant alteration in urine metabolome.

Alteration of the serum metabolome in arterial disease may be indicative of the risk factors leading to pathology, or indeed reflect the burden of disease presence.

Within cardiovascular disease, metabolic profiling of serum and/or has been performed to determine biomarkers of coronary artery disease(34), stroke(104, 105) and aneurysm presence(71, 74, 77). This chapter describes the use of serum metabonomics to delineate biomarkers of high-risk carotid stenosis, and features distinguishing embolising from dilating and stenosing atherosclerotic disease.

7.1 Serum lipid profiling mass spectrometry

7.1.1 Sample preparation

Serum frozen at -80*C was retrieved and kept at room temperature to allow it to thaw (typically 30 minutes to 1 hour). Once defrosted, 100 microliters of serum from each patient were transferred to a labelled 2ml Eppendorf tube and refrozen at -80*C until required.

For liquid-liquid extraction, a mixture of MTBE and Methanol was prepared as follows. A 1000ml glass flask and a 100ml glass measuring cylinder were washed with demineralised water, Chromasolve and Methanol and MTBE. In total, 600 ml of MTBE was measured and decanted into the flask, to which 200ml of Methanol was added. The mixture was sealed and chilled at -20*C overnight.

Samples were randomised using the randomisation function in Microsoft Excel, and all further preparation was performed in a randomised manner.

Aliquots of 100 microliter serum were permitted to defrost at room temperature (approximately 20 minutes). When thawed, 800 microliters of the chilled 3MTBE:1Methanol mixture was added to each serum aliquot. The suspension was subjected to intense vortexing for 1 minute with a multimixer, in order to maximise protein precipitation. Finally, the Eppendorfs were centrifuged at 4*C for 20 minutes at 16,000G, resulting in separation of the aqueous (lower) and organic layers.

From each Eppendorf, 700 microliters of the upper organic layer was decanted into a new labelled 2ml Eppendorf tube and left open in a fume cupboard overnight to allow evaporation of the organic solvents. The extracts were then stored at -40*C until further processing.

Pooled serum sample

A 100ml glass flask was washed three times each with demineralised water, Chromasolve and Methanol. Into this container, 300 microliters of each serum sample was decanted in order to create a pooled sample, which was then aliquoted in 100 microliter samples into 32 labelled 2ml Eppendorf tubes. To each aliquot, 800 microliters of the chilled 3MTBE:1Methanol mixture was added and then vortexed and centrifuged as described above. Again, 700 microliters of the organic layer was decanted into a new labelled 2ml Eppendorf, which was allowed to evaporate. The extracts were stored at -40*C until further processing.

Blank samples

Into 8 empty, labelled 2ml Eppendorf tubes, 800 microliters of the 3MTBE:1Methanol mixture were decanted. These aliquots were subjected to the same processing as were the serum-containing samples to determine the influence of the preparation process on metabolic profiling.

Reconstitution

Reconstitution solution was prepared as follows. A glass flask and measuring cylinder were washed with demineralised water, chromasolve, acetonitrile and isopropanol (each three times). In total, 60ml of reconstitution solution was prepared using isopropanol/acetonitrile/chromasolve in the ratio 2:1:1.

To each eppendorf containing lipid precipitant, pooled sample precipitant and blank samples, 120 microliters of reconstitution solution was added. The mixture was vortexed for 1 minute, then centrifuged at 20000G at 4 degrees

Celsius for 20 minutes. The supernatant (110 microliters) was decanted into preloaded LC MS inserts. Seven pooled samples were individually treated (quality controls), and the remaining 25 pooled samples were added to a glass vial.

Preparation of dilutions

Three dilutions were prepared using pooled sample and reconstitution solution:

- 1:2
- 1:3
- 1:6

Dilutions, quality control samples and test samples were stored overnight at 4 degrees Celsius prior to commencing MS experiments.

7.1.2 Mobile phase preparation for serum lipid profiling

Mobile Phase A:

The aqueous mobile phase was prepared using a mixture of acetonitrile and Chromasolve LC MS grade water. Containers and measuring cylinders were washed using Acetonitrile and LC MS grade water. To prepare 2.5 L, 1.5 L of acetonitrile was mixed with 1.0 L of water, to which 1.573 g of ammonium formate was weighed and added. A further 2.5ml of 0.1% formic was added. The mobile phase was mixed, and then sonicated for 20 minutes to aid mixing and promote degassing.

Mobile Phase B:

To prepare the organic mobile phase, containers and measuring cylinders were washed using a mixture of isopropanol and acetonitrile. To prepare 2.5 L, 2250 ml of isopropanol was added to 250ml of acetonitrile and 2.5 ml formic acid. The mixture was sonicated for 20 minutes to aide mixing and degassing.

Mobile phases were wrapped in aluminium foil and stored at room temperature overnight prior to MS analysis.

7.1.3 MS lipid profiling experiment

Individual components of the mass spectrometer source were cleaned using LC MS grade water and methanol to prevent contamination during analysis. The MS was then calibrated, and intensity optimised (injection volume and voltage) using pooled samples. The response to dilutions was tested to ensure accuracy. The MS was conditioned using quality control samples before started experimentation run. A quality control sample was analysed after every ten serum samples.

During automated sample running, a checklist was followed to ensure all aspects of data acquisition were progressing appropriately. This checklist is summarised in Appendix 3.

7.1.4 Results: Serum Lipid Profiling Mass Spectrometry Positive Ionisation Mode

PCA of samples, QCs, dilutions and blanks showed tight clustering of each group (Figure 54). However blanks were exerting a metabonomic influence implying the presence of a contaminant and these features were excluded. Furthermore, those features with coefficient of variance over 30% in the QC samples were excluded. Figure 55 and Figure 56 demonstrate PCA analysis of sample classes, where as expected, no visual separation was seen.

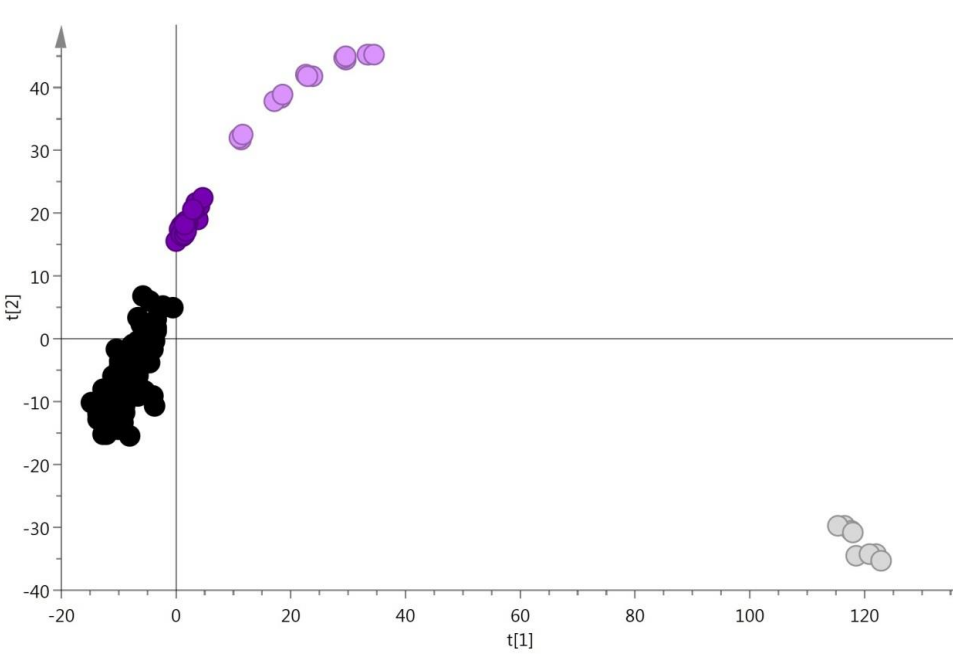


Figure 54: PCA showing separation of serum samples analysed with LP MS in the positive ionisation mode (black) from tightly clustered QCs (purple) and expected drift of subsequent dilutions (pink) from blanks (grey).

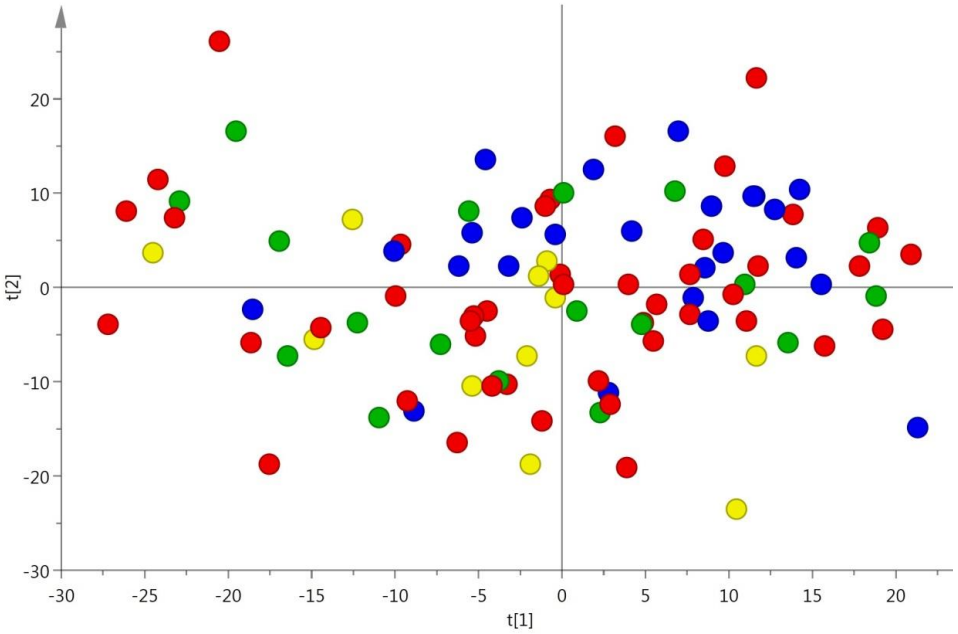


Figure 55: PCA showing distribution of serum analysed with LP MS in the positive ionisation mode from patients with symptomatic carotid stenosis (red), non carotid TIA/stroke (blue), asymptomatic carotid stenosis (green) and controls (yellow).

11 principal components

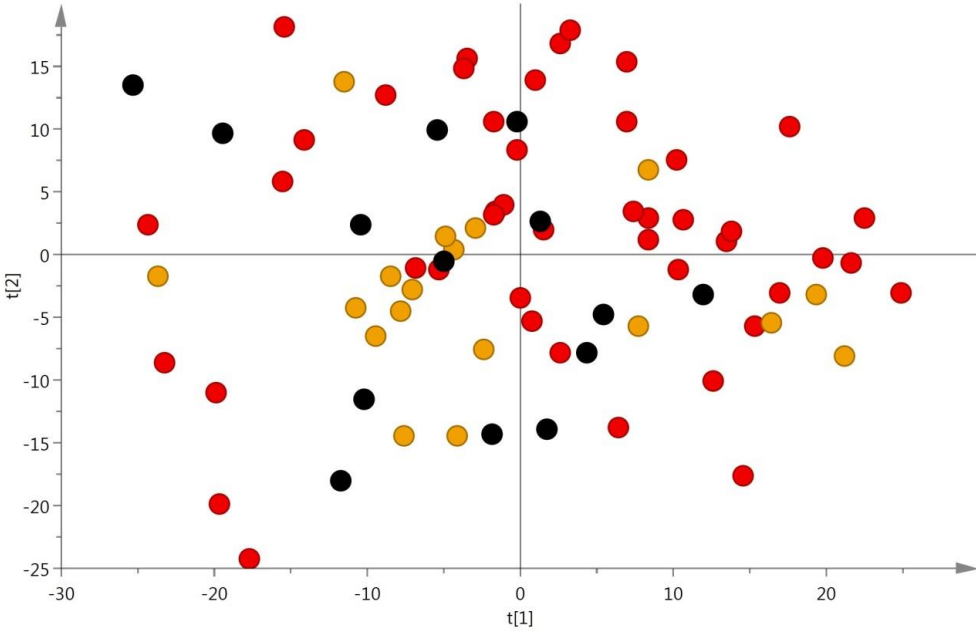
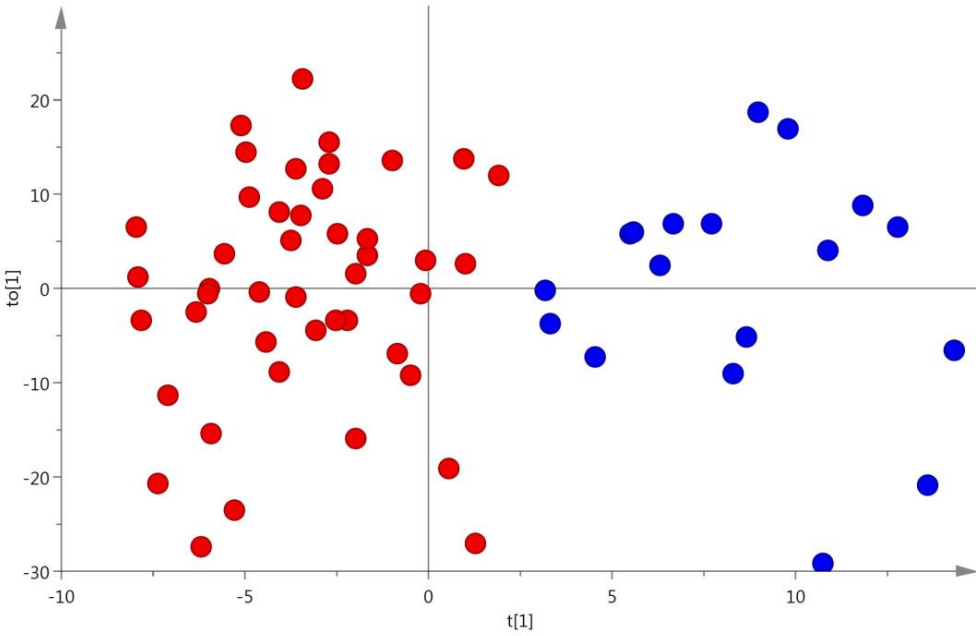


Figure 56: PCA showing distribution of serum samples analysed with LP MS in the positive ionisation mode from patients with embolising carotid stenosis (red), aneurysmal disease (orange) and peripheral arterial disease manifesting as claudication (black).

Data were log transformed for OPLS DA and this improved the predictive values of models. Overall comparisons however failed to show convincing separation. OPLS DA of symptomatic carotid disease compared with non-carotid stroke/TIA was non predictive (Figure 57). Very weak separation was encountered in the comparison of urine from patients with symptomatic and asymptomatic carotid atherosclerosis (Figure 58). OPLS DA comparison of embolising carotid atherosclerosis and aneurysmal disease was again non-predictive (Figure 59). Weak and highly overfitted separation was demonstrated with OPLS DA of urine from patients with embolising carotid stenosis compared with peripheral arterial disease (Figure 60).



R^2Y	0.781
Q^2	-0.227
Principal components	2

Figure 57: OPLS DA (log transformed) of lipid profiling MS (positive mode) analysis of serum analysed with LP MS in the positive ionisation mode from patients with symptomatic carotid stenosis (red) and non-carotid strokeTIA (blue).

R^2Y	0.902
Q^2	0.134
Principal components	3

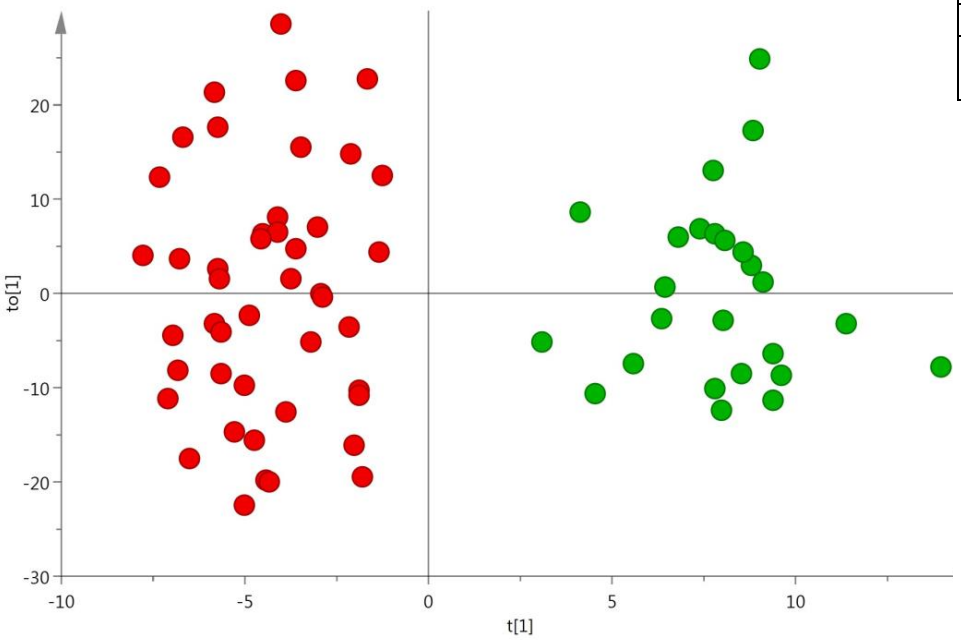


Figure 58: OPLS DA analysing log-transformed HILIC MS spectra of serum samples analysed with LP MS in the positive ionisation mode from patients with symptomatic carotid stenosis (red) and asymptomatic carotid stenosis (green).

R^2Y	0.728
Q^2	0.792
Principal components	2

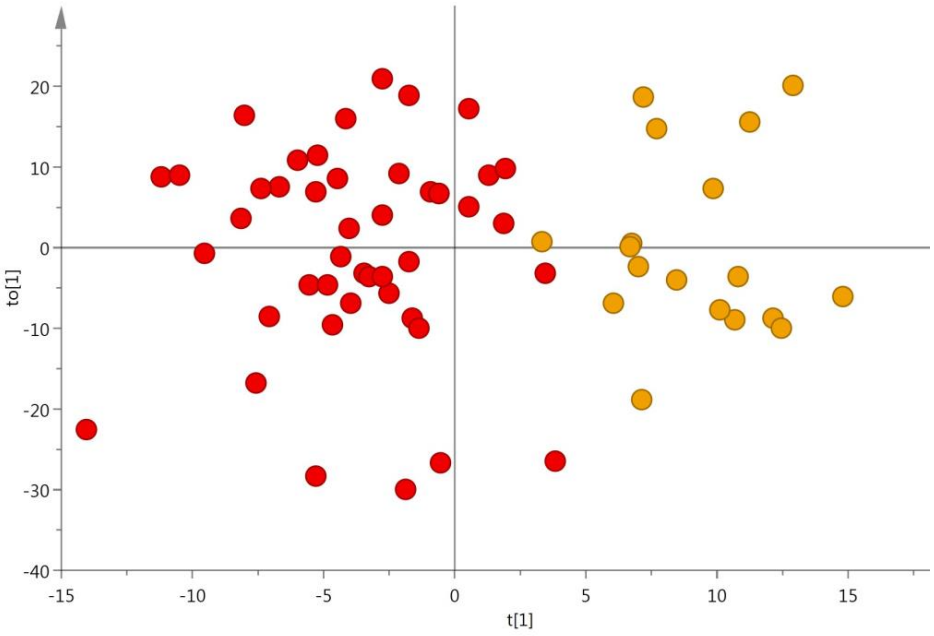


Figure 59: OPLS DA analysing log transformed Lipid Profiling MS spectra of serum samples analysed with LP MS in the positive ionisation mode from patients with embolising carotid atherosclerosis (red) and aneurysmal disease (orange).

R^2Y	0.808
Q^2	0.138
Principal components	2

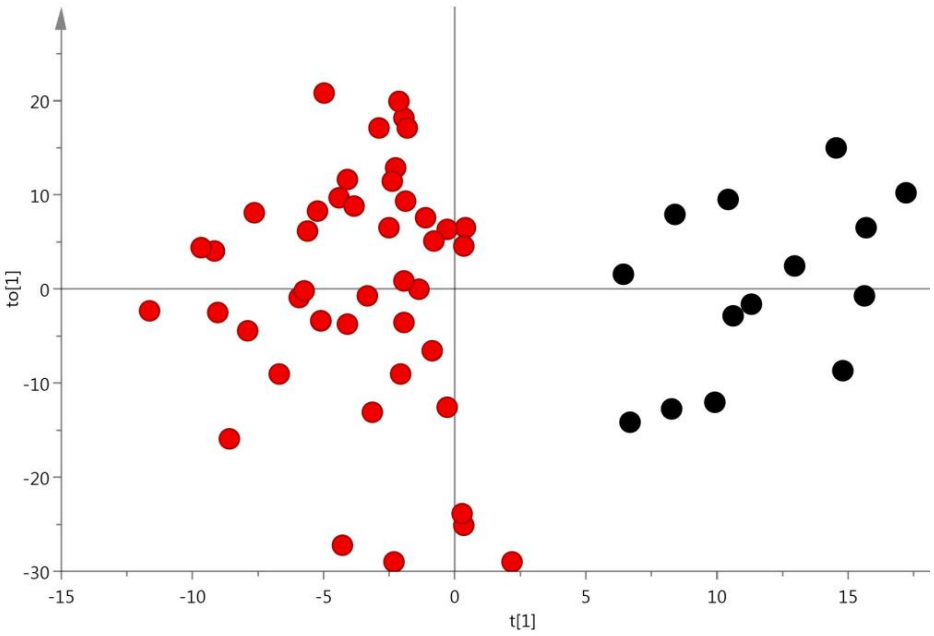


Figure 60: OPLS DA of Lipid Profiling MS spectra of serum samples analysed with LP MS in the positive ionisation mode from patients with embolising carotid atherosclerosis (red) and stenosing peripheral arterial disease (black).

7.1.5 Results: Serum Lipid Profiling Mass Spectrometry Negative Ionisation Mode

Spectroscopic data acquired for negative ionisation mode were processed similarly to positive ionisation mode. The results were also largely similar. No outliers were encountered on PCA, but blank features and those exhibition high coefficient of variance were excluded. Figure 61 shows the results of PCA of samples, QCs, dilutions and blanks. PCAs for separate classes of samples are shown in Figure 62 and Figure 63. Finally, Figures 64 to 67 demonstrate OPLS DA analysis to compare embolising carotid stenosis with other classes. Values of Q^2 obtained are weakly predictive at best and consistently below 0.20. Therefore metabolite identification was not indicated.

17 principal components

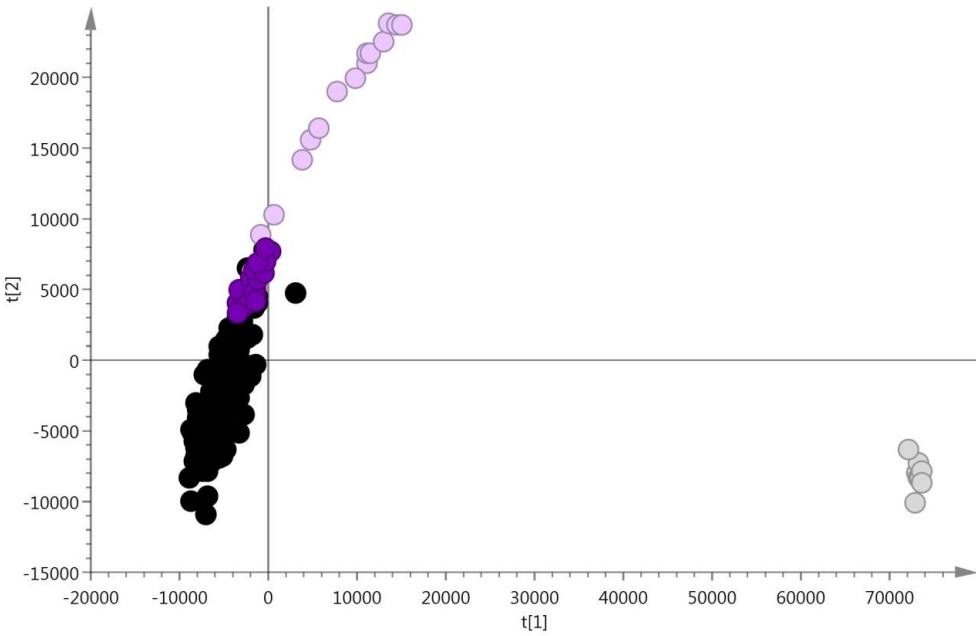


Figure 61: PCA showing distribution of serum samples analysed with LP MS in the negative ionisation mode (black), QCs (purple), dilutions (pink), and blanks (grey).

12 principal components

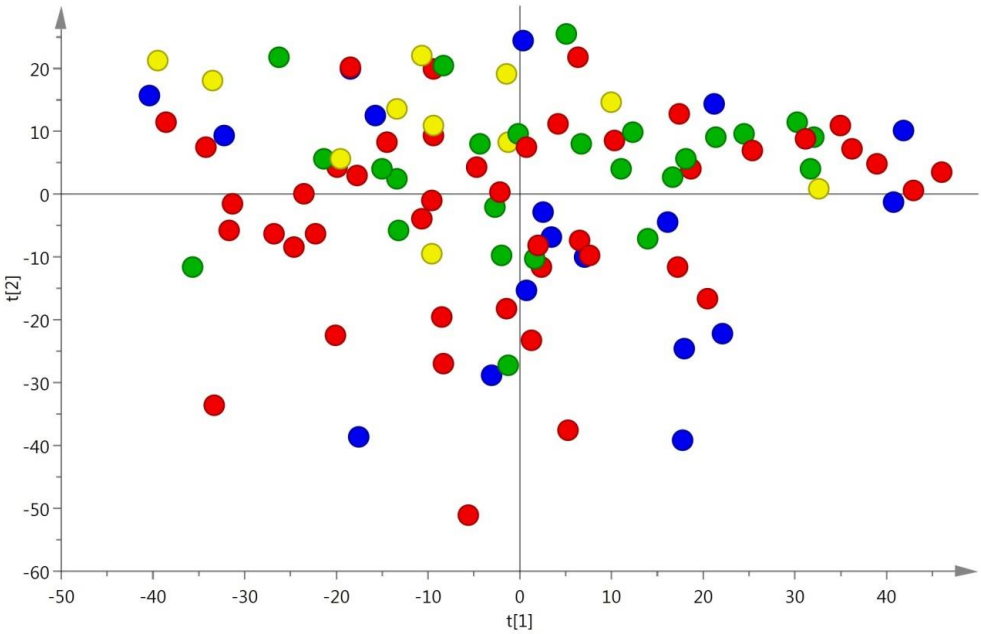


Figure 62: PCA showing distribution of serum samples analysed with LP MS in the negative ionisation mode from patients with >50% symptomatic carotid stenosis (red), non-carotid stroke/TIA (blue), asymptomatic >50% carotid stenosis (green), and controls (yellow).

10 principal components

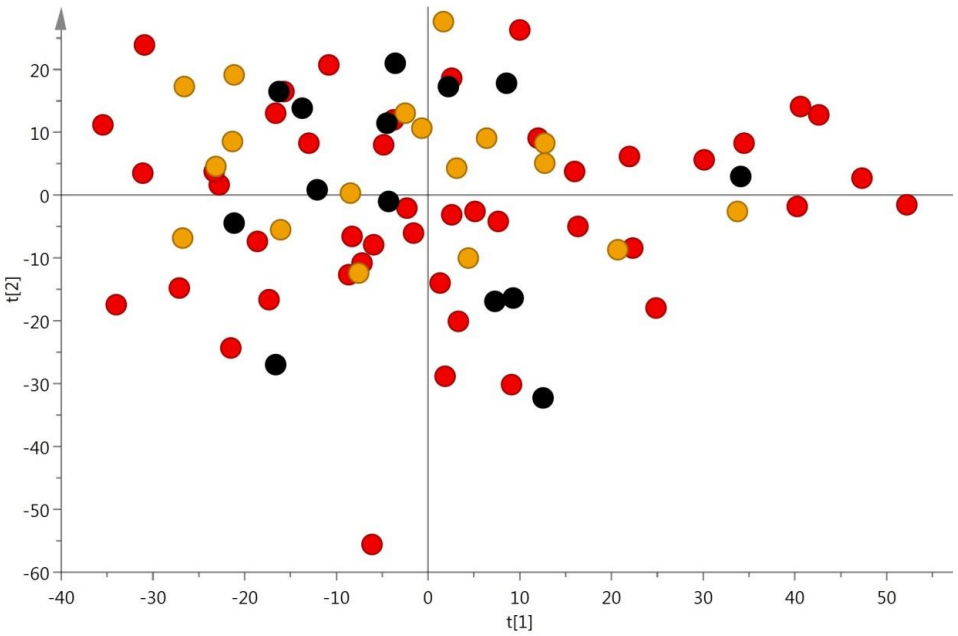


Figure 63: PCA showing distribution of serum samples analysed with LP MS in the negative ionisation mode from patients with symptomatic carotid atherosclerosis (red), aneurysmal disease (orange) and peripheral arterial disease (black).

R^2Y	0.849
Q^2	-0.127
Principal components	2

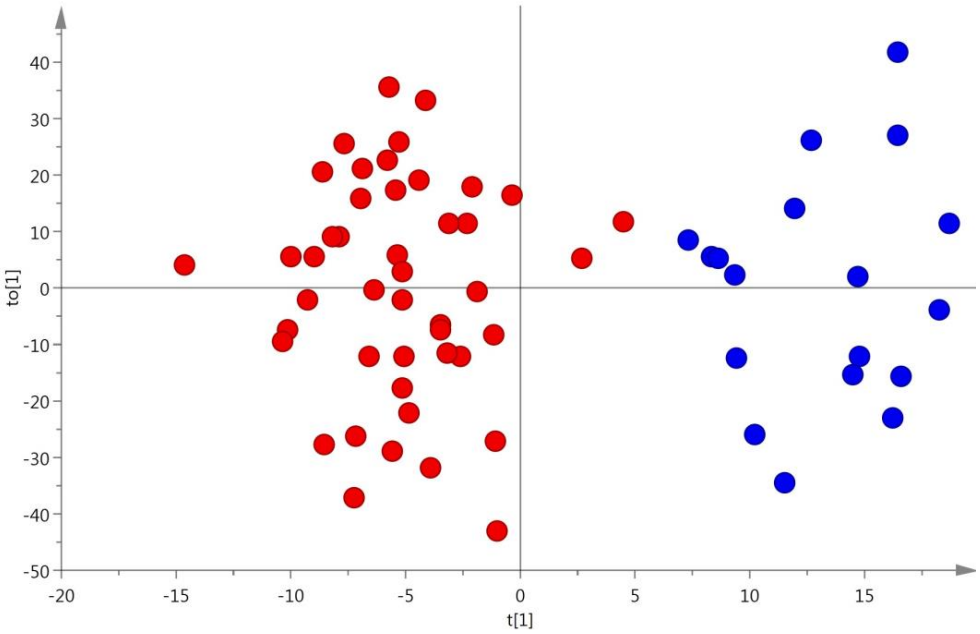
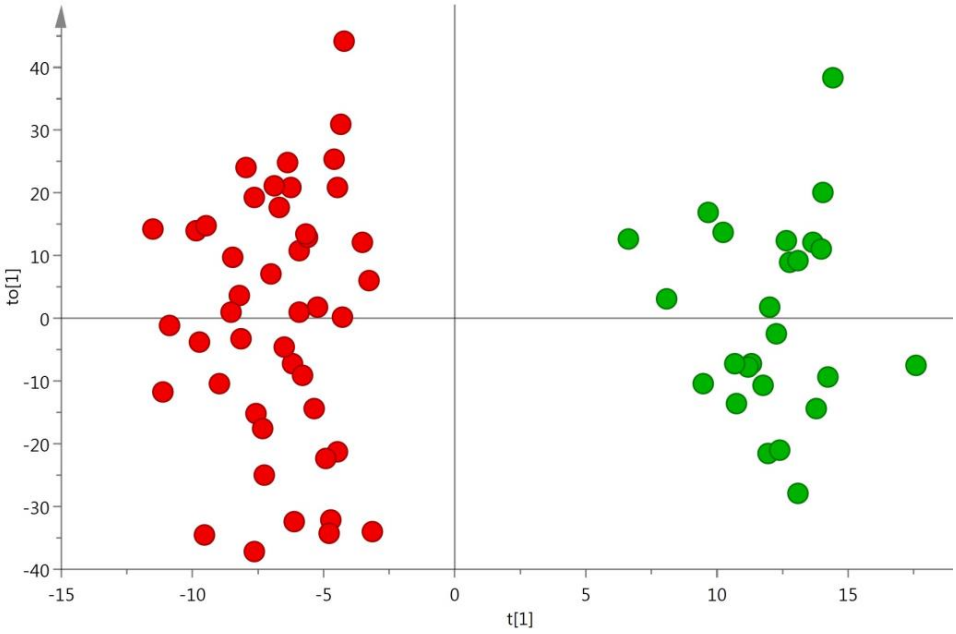
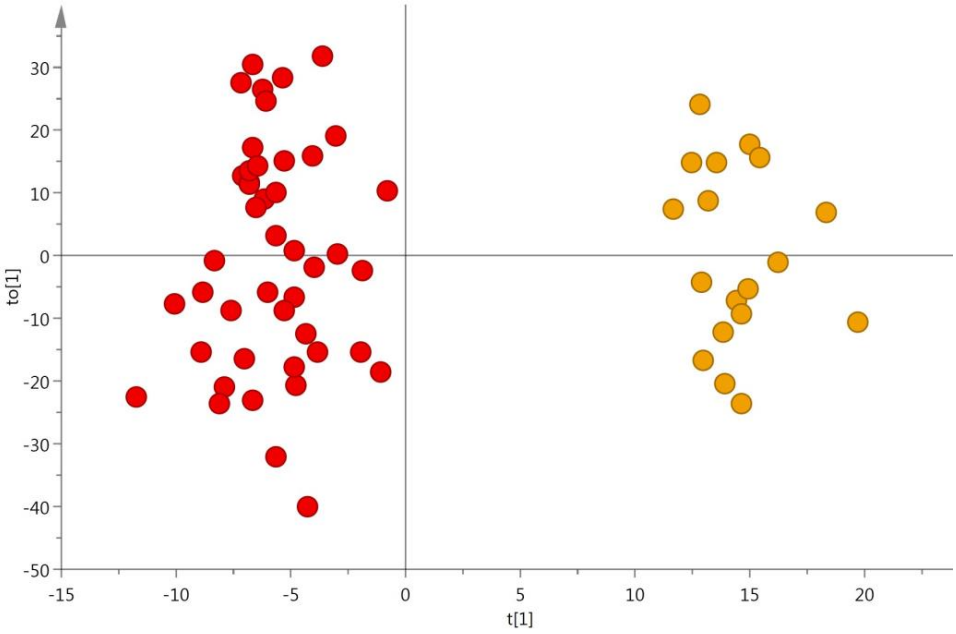


Figure 64: OPLS DA analysing lipid profiling MS negative ionisation mode spectra of serum derived from patients with symptomatic carotid atherosclerosis (red) and non-carotid stroke/TIA (blue).



R^2Y	0.946
Q^2	0.157
Principal components	3

Figure 65: OPLS DA analysing log-transformed lipid profiling MS negative mode spectra of serum samples from patients with symptomatic carotid stenosis (red) and asymptomatic carotid stenosis (green).



R^2Y	0.948
Q^2	0.197
Principal components	3

Figure 66: OPLS DA of log transformed lipid profiling MS negative mode spectra of serum from patients with embolising carotid disease (red) and aneurysmal disease (orange).

R^2Y	0.991
Q^2	0.194
Principal components	4

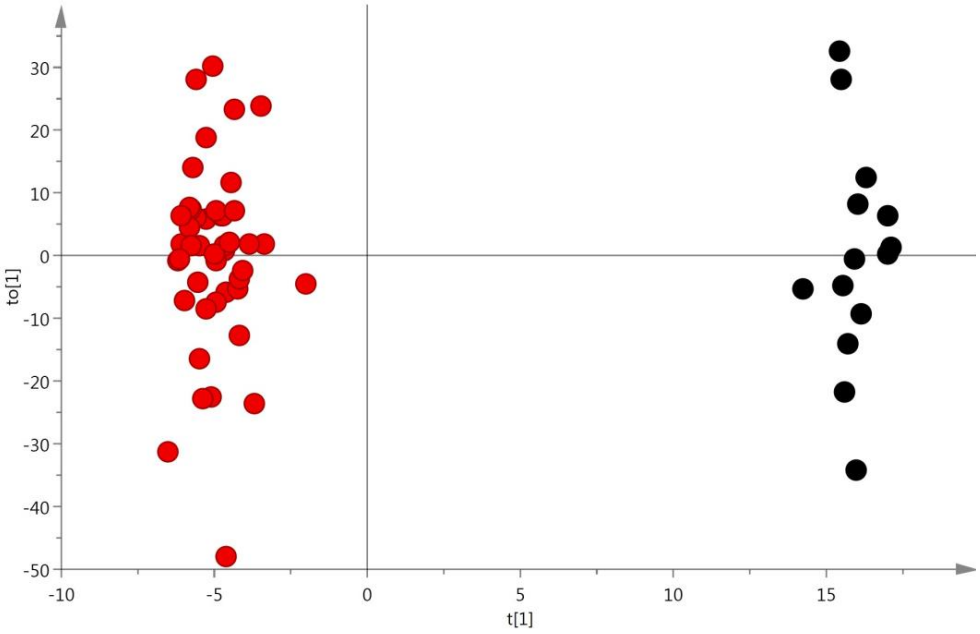


Figure 67: OPLS DA of log transformed lipid profiling spectra of serum analysed with LP MS in the negative ionisation mode from patients with embolising carotid disease (red) and peripheral arterial disease (black).

7.2 Serum HILIC mass spectrometry

7.2.1 Sample preparation: solid phase extraction (SPE)

Serum samples were randomised for preparation and HILIC MS analysis. One-hundred microliter aliquots of serum from each patient together with pooled serum from all patients were allowed to thaw at room temperature over one hour. Once defrosted, 100uL of pooled serum was placed in six 2ml eppendorfs. All serum samples were centrifuged for one minute at 4*C and 14000G.

Following the randomised order, 90uL from each sample was placed in an individual well of a 96-well Ostroplate, containing a cartridge to remove proteins

and lipids from the serum. Six wells were used to contain an equivalent volume of pooled serum.

A 1% solution of formic acid and acetonitrile was prepared by adding 1ml of formic acid to 100ml of acetonitrile, using stringent precautions to prevent contamination. To each serum well, 270uL of the acetonitrile solution was added resulting in a serum:solvent ratio of 1:3.

Vacuum pressure at 15 was applied for 10 minutes to each ostroplate and the filtrate (devoid of protein and lipid) was collected in a 96-well MS plate.

The Quality Control (QC) sample was created by siphoning 50uL from each filtered serum well to create a second pool of all samples. Five wells in each MS well plate were filled with 500uL of the QC. Dilutions of the QC were created in the ratios 1:2, 1:3, 1:4, 1:6 and 1:8.

7.2.2 Preparation of Mobile Phases for serum HILIC MS

Mobile Phase A

Two batches of 2.5L Mobile Phase A were created. Mobile Phase containers and measuring cylinders were washed repeatedly with LC MS grade water and Acetonitrile. To prepare 2.5L of Mobile Phase A, 1.927g of ammonium acetate was dissolved in 125ml of LC MS grade water with 2.5ml of formic acid. The mixture was sonicated to degas and aid dissolution of the salt. The mixture was

made up to 2.5L through the addition of acetonitrile in increasing volumes from 200ml to 975ml. The final mixture was sonicated for 45 minutes.

Mobile Phase B

To prepare the aqueous Mobile Phase B, 1250ml LC MS water was used to dissolve 1.927g of ammonium acetate. A further 2.5ml of formic acid was added. This mixture was sonicated prior to the addition of 1250ml acetonitrile.

Prepared mobile phases were stored at room temperature overnight prior to commencing HILIC analysis.

7.2.3 Results: HILIC MS Serum Positive Ionisation Mode

Initial PCA of all samples was utilised to exclude blank features, and those features with a coefficient of variance over 30% in QC samples. The subsequent PCA of samples, QCs, dilutions and blanks is depicted in Figure 68.

7 principal components

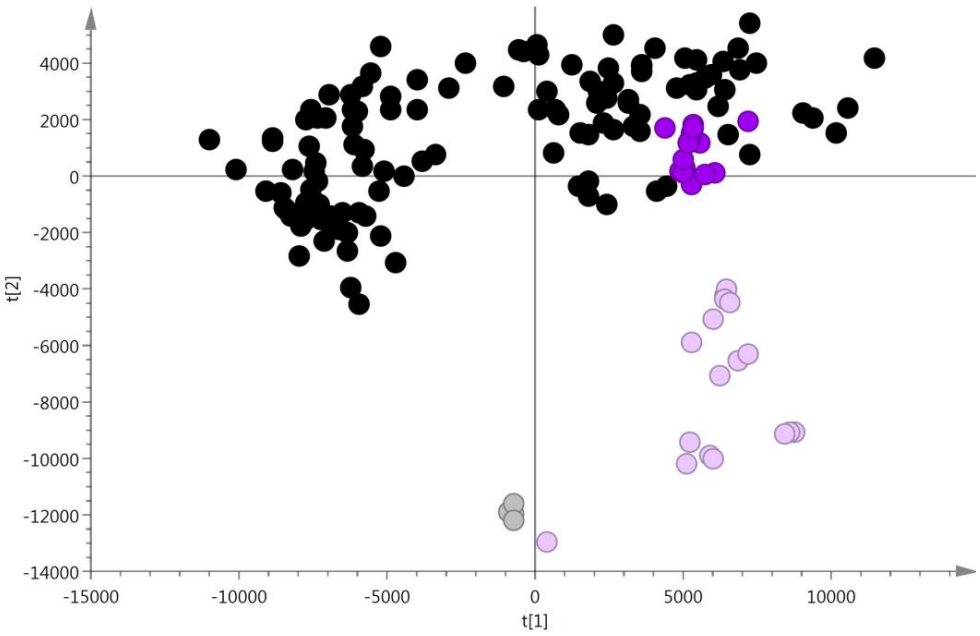


Figure 68: PCA (log transformed) showing all serum samples analysed with HILIC MS in the positive ionisation mode (black), QCs (purple), dilutions (pink) and blanks (grey).

As no separation was encountered on PCA of individual classes, OPLS DA was selected to enhance separation. Despite exclusion of outliers and features with inconsistent levels, no OPLS DA model was of sufficient strength to enable metabolite identification (Figure 69 to 72).

R^2Y	0.485
Q^2	0.0358
Principal components	2

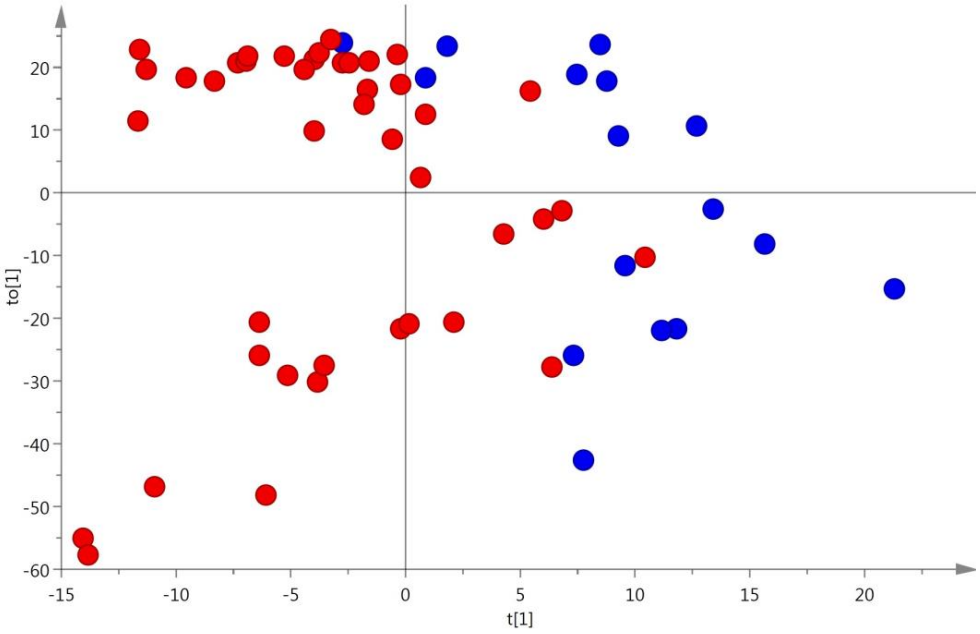


Figure 69: OPLS DA (log transformed) of serum analysed with HILIC MS in the positive ionisation mode from patients with symptomatic carotid stenosis (red) and non-carotid stroke/TIA.

R^2Y	0.779
Q^2	-0.0604
Principal components	3

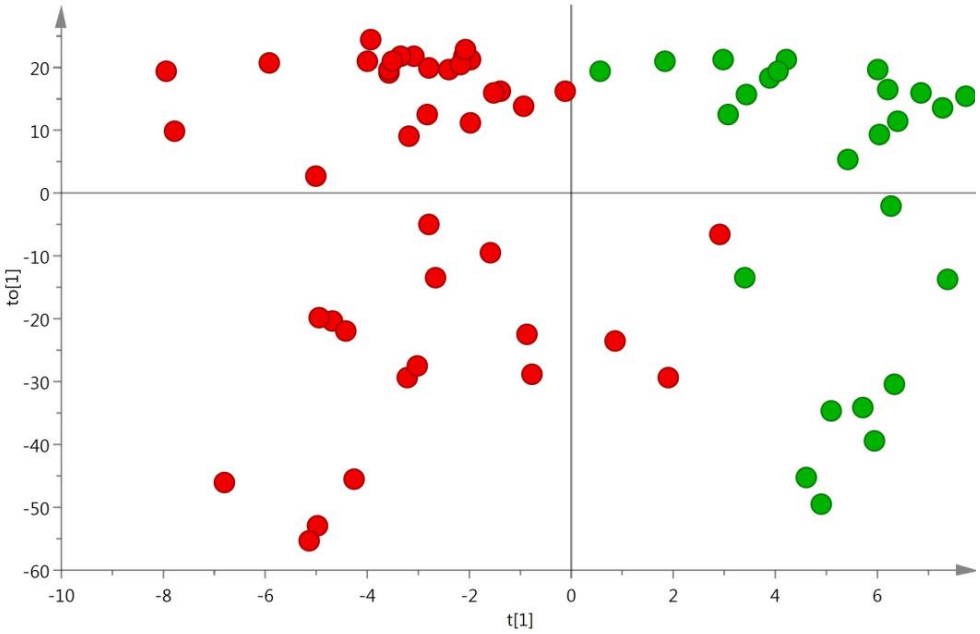


Figure 70: OPLS DA (log transformed) of serum analysed with HILIC MS in the positive ionisation mode from patients with symptomatic carotid stenosis (red) and asymptomatic carotid stenosis (green).

R^2Y	0.727
Q^2	0.0908
Principal components	3

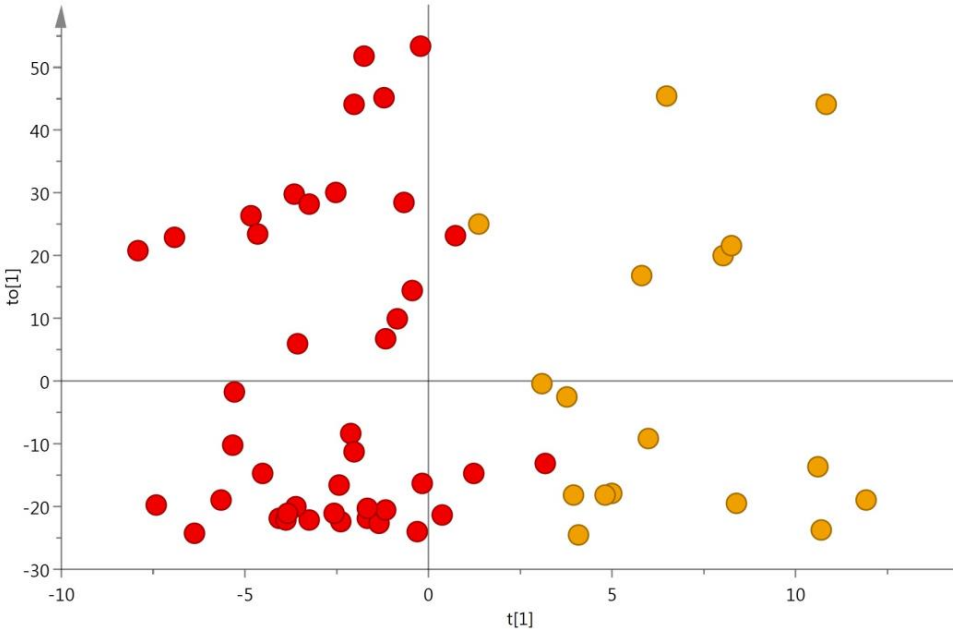


Figure 71: OPLS DA (log transformed) of serum analysed with HILIC MS in the positive ionisation mode from patients with embolising carotid disease and dilating aneurysmal disease (orange).

R^2Y	0.417
Q^2	0.0595
Principal components	2

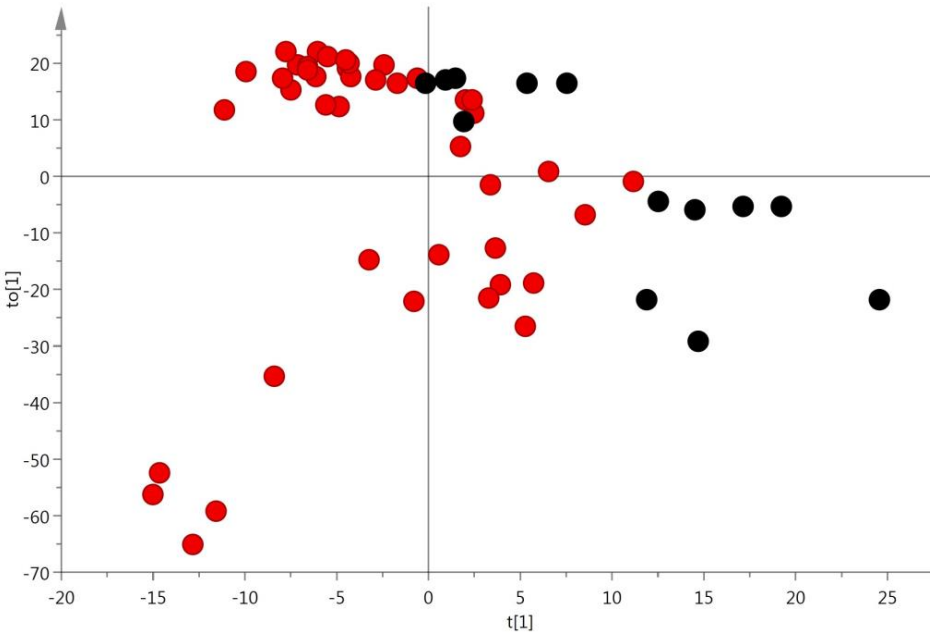


Figure 72: OPLS DA (log transformed) of serum analysed with HILIC MS in the positive ionisation mode from patients with embolising carotid disease (red) and dilating aneurysmal disease (black).

7.2.4 Results: HILIC MS Serum Negative Ionisation Mode

Negative mode analysis did not improve the HILIC MS Serum positive mode findings. PCA of samples, QCs and blanks showed tight clustering (Figure 73). Figures 74 to 77 portray the results of multivariate statistical analysis. No model was suitable or of sufficient strength to merit metabolite identification.

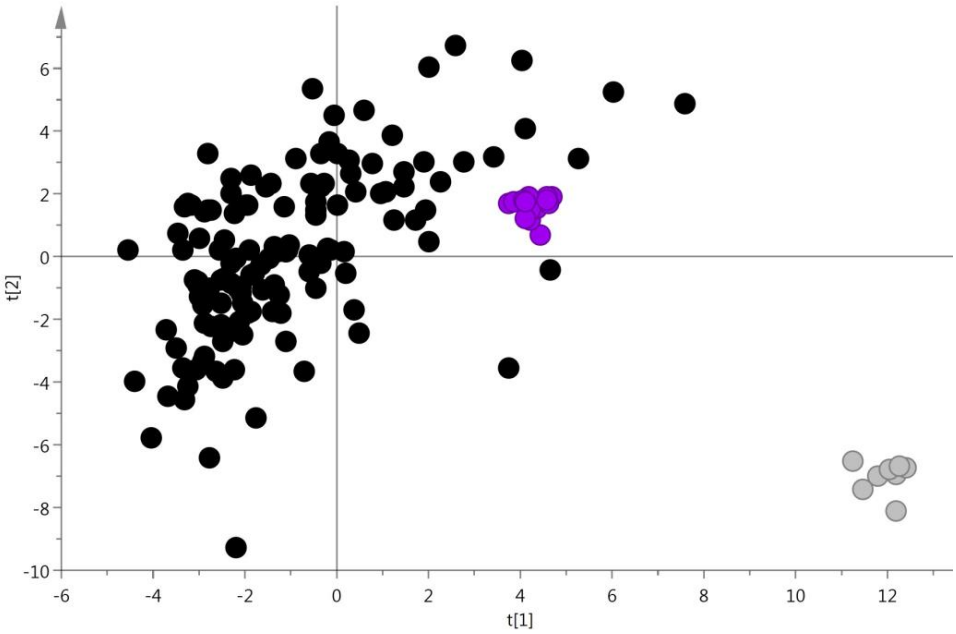


Figure 73: PCA (log transformed) of serum samples analysed with HILIC MS in the negative ionisation mode (black), QCs (purple), and blanks (grey). The data is derived from 24 principal components.

R^2Y	0.371
Q^2	0.0483
Principal components	1

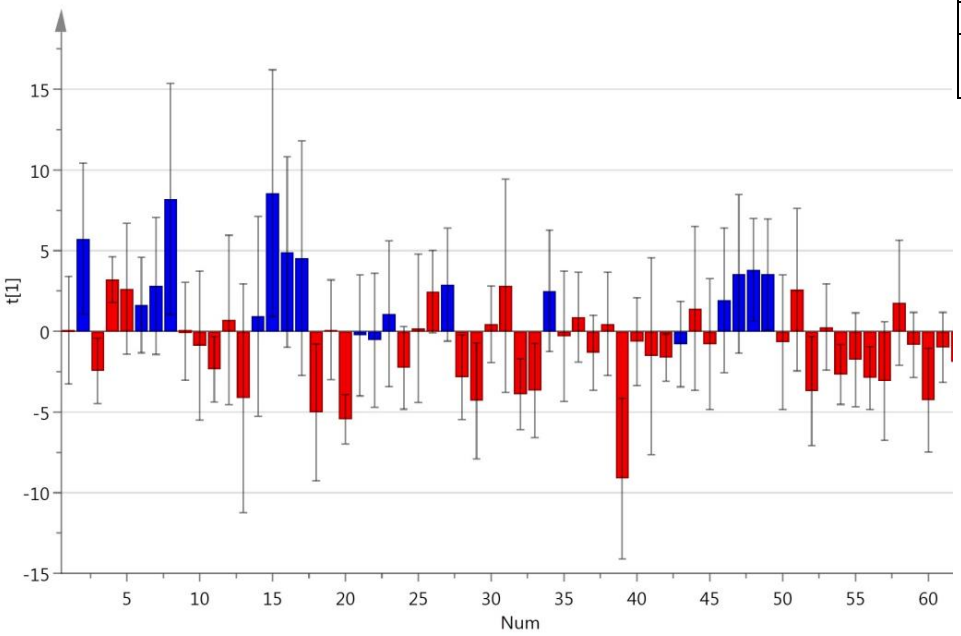


Figure 74: OPLS DA (log transformed) column plot of serum analysed with HILIC MS in the negative ionisation mode from patients with symptomatic carotid stenosis (red) and non-carotid stroke/TIA (blue).

R^2Y	0.684
Q^2	-0.089
Principal components	2

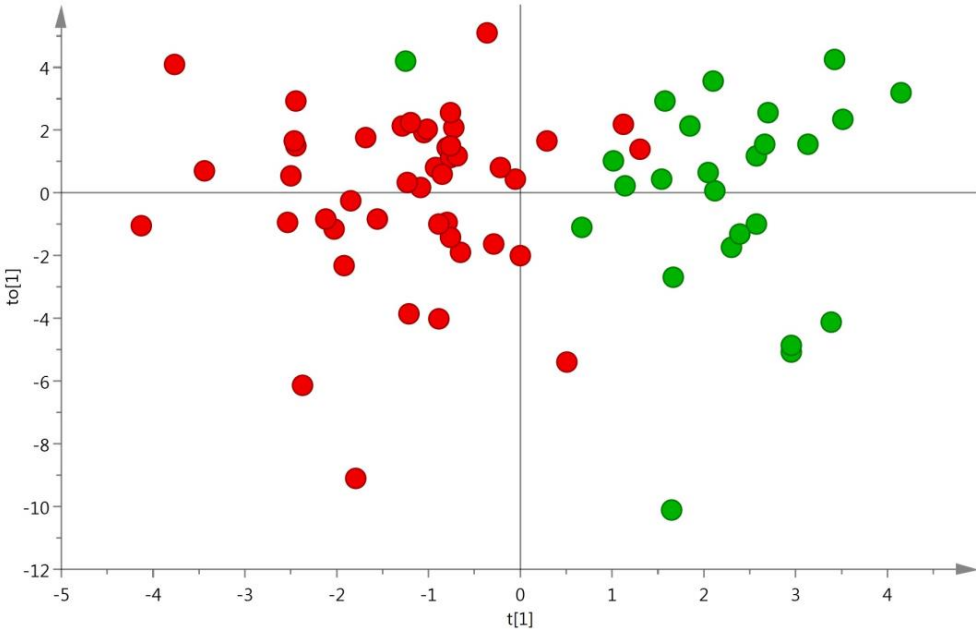
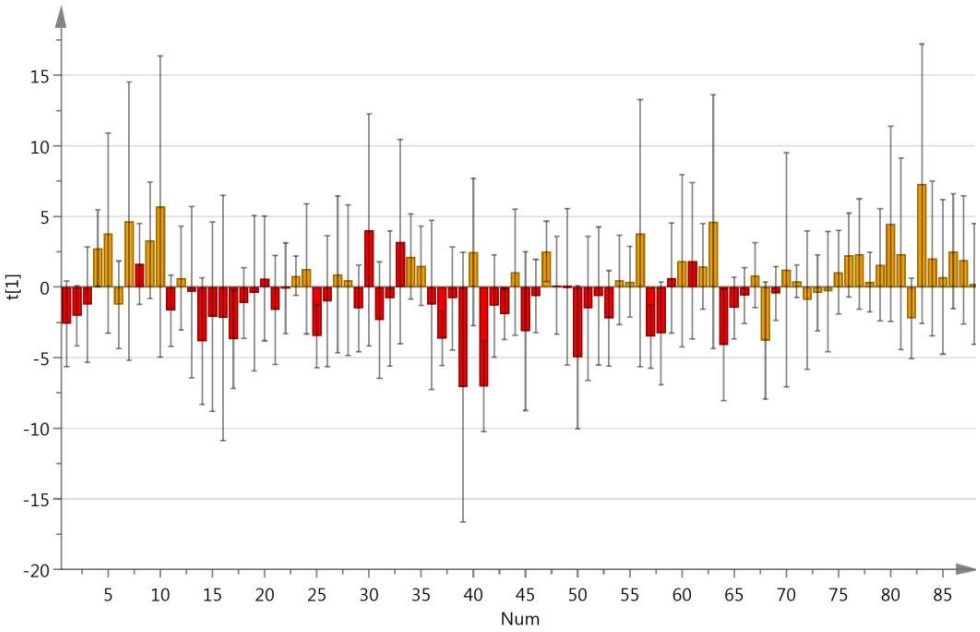
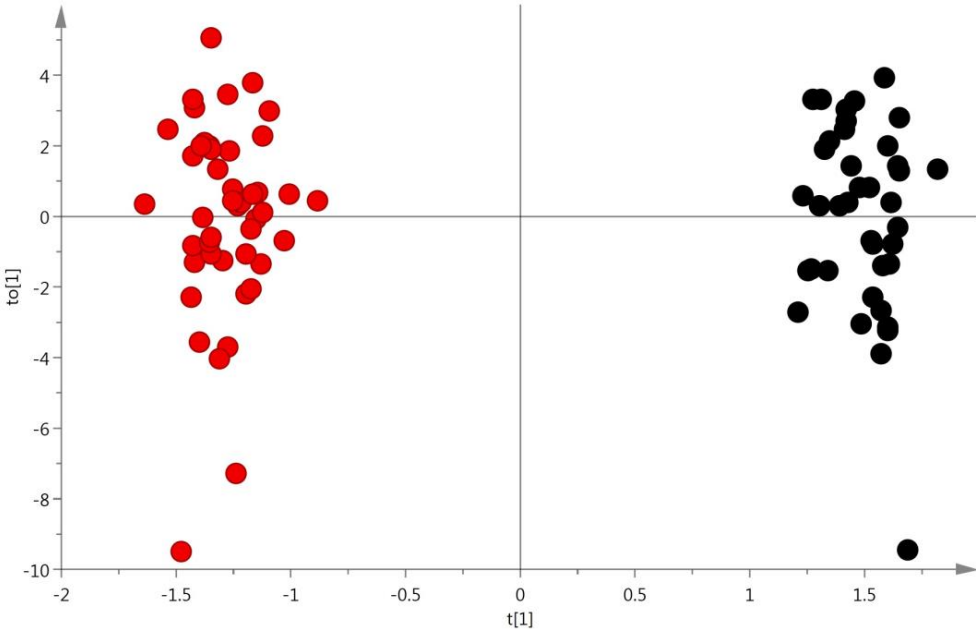


Figure 75: OPLS DA (log transformed) of serum analysed with HILIC MS in the negative ionisation mode from patients with symptomatic carotid stenosis and non-carotid stroke/TIA.



R^2Y	0.364
Q^2	-0.0259
Principal components	1

Figure 76: OPLS DA (log transformed) column plot of serum analysed with HILIC MS in the negative ionisation mode from patients with embolising symptomatic carotid stenosis (red) and dilating aneurysmal disease (orange).



R^2Y	0.989
Q^2	0.116
Principal components	9

Figure 77: OPLS DA (log transformed) of serum analysed with HILIC MS in the negative ionisation mode from patients with embolising carotid stenosis (red) and stenosing peripheral arterial disease (black).

7.3 Serum 1H-NMR

1H-NMR lacks the sensitivity of MS analysis, and it is uncommon to find statistically significant results using NMR when MS has not been successful. However, as a significant proportion of preceding work within atherosclerotic metabonomics has been performed using NMR, the analysis was completed.

7.3.1 NMR serum buffer preparation

A 100ml conical flask and measuring cylinder was prepared by washing in demineralised water, LC MS water and then acetonitrile (each three times). The flask and cylinder were left in a 100 degree Celsius oven overnight to ensure all washing liquid had evaporated.

NMR serum buffer was prepared by dissolving 1.064g of NaH_2PO_4 in 76ml of Chromasolve LC MS grade water, to which was added 0.08g of TSP. The mixture was shaken until the powder dissolved. A 4% solution of NaN_3 was added at a volume of 1ml to kill bacteria, followed by 20ml of D_2O. The volume was adjusted to 100ml by addition of water. The pH (originally 5.4) was adjusted by adding NaOH, and overshot correct with 1M HCl solution to achieve a pH of 7.4. Buffer was stored in a 4 degree Celsius fridge prior to sample preparation.

7.3.2 Sample preparation

Prior to preparation, samples were randomised using the randomisation function in Microsoft Excel for further preparation and analysis.

Serum frozen at -80*C was retrieved and kept at room temperature to allow it to thaw (typically 30 minutes to 1 hour). Once defrosted, 400 microliters of serum from each patient were transferred to a labelled 2ml Eppendorf tube and refrozen at -80*C until required. Six quality control samples were prepared using pooled serum containing an equal proportion of each sample to be analysed

Serum samples were defrosted, and centrifuged at 12000G at 4 degrees Celsius for five minutes. A 350 microliter volume of supernatant was added to a new labelled eppendorf, to which 350 microliters of chilled NMR serum buffer was added. The serum/buffer mixture was again centrifuged at 12000G at 4 degrees Celsius for five minutes, and 600 microliters of the supernatant was added to a 5mm SampleJet NMR tube. Each tube was capped and sealed using POM ball. SampleJet tubes were stored at 4 degrees Celsius overnight prior to NMR analysis.

7.3.3 Results: Serum ^{1}H-NMR CPMG Normalised Spectra

Initial multivariate data analysis was performed using UV scaling. However results were found to be considerably sharper with the utilisation of Pareto scaling, therefore Pareto scaling was employed as the scaling method of choice. Figure 78 shows tight clustering of QCs centred on samples on PCA analysis indicating a high quality analysis. One outlier is evidenced (sample P13; Group 6) and this was excluded from the subsequent analysis of claudicants.

21 principal components

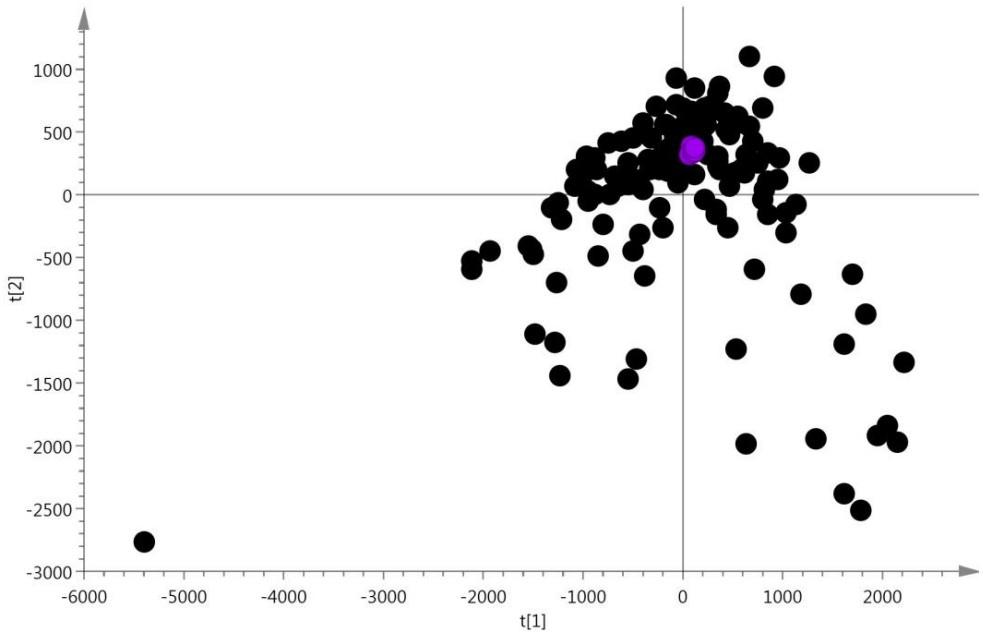
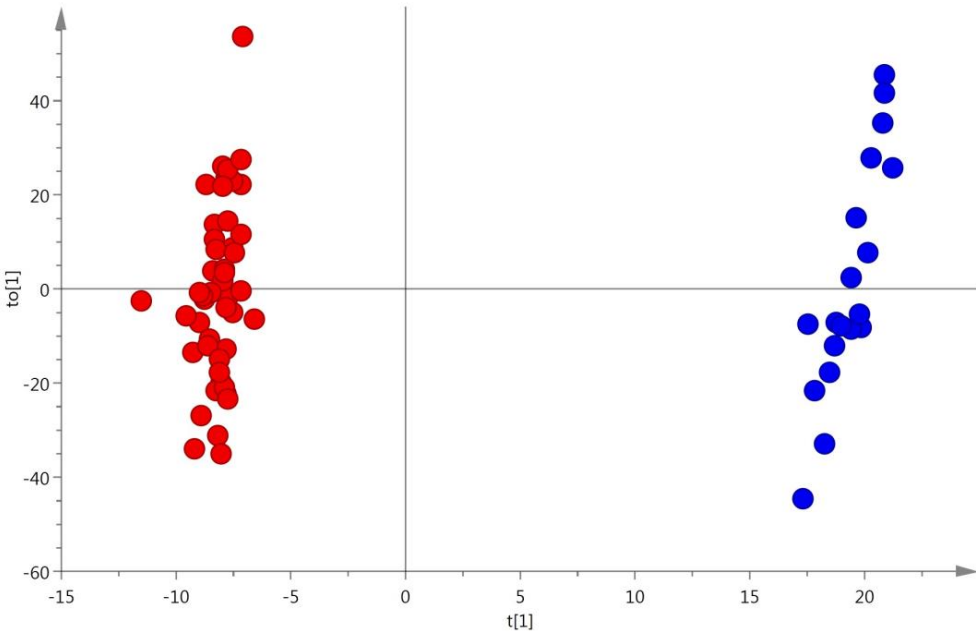


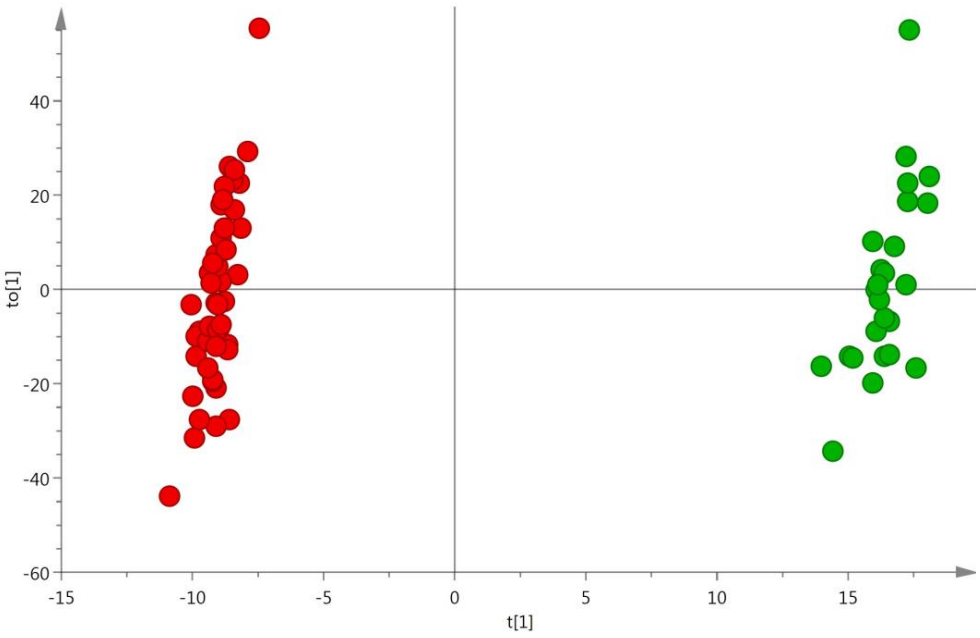
Figure 78: PCA of all serum 1H-NMR analysis (black) and QCs (purple). The QCs are tightly clustered. The data is derived from 21 principal components.

OPLS DA analysis of the carotid/stroke subgroups were not found to be predictive (Figure 79 and Figure 80). This was expected following the weak models obtained from MS analysis of serum.



R^2Y	0.892
Q^2	0.118
Principal components	8

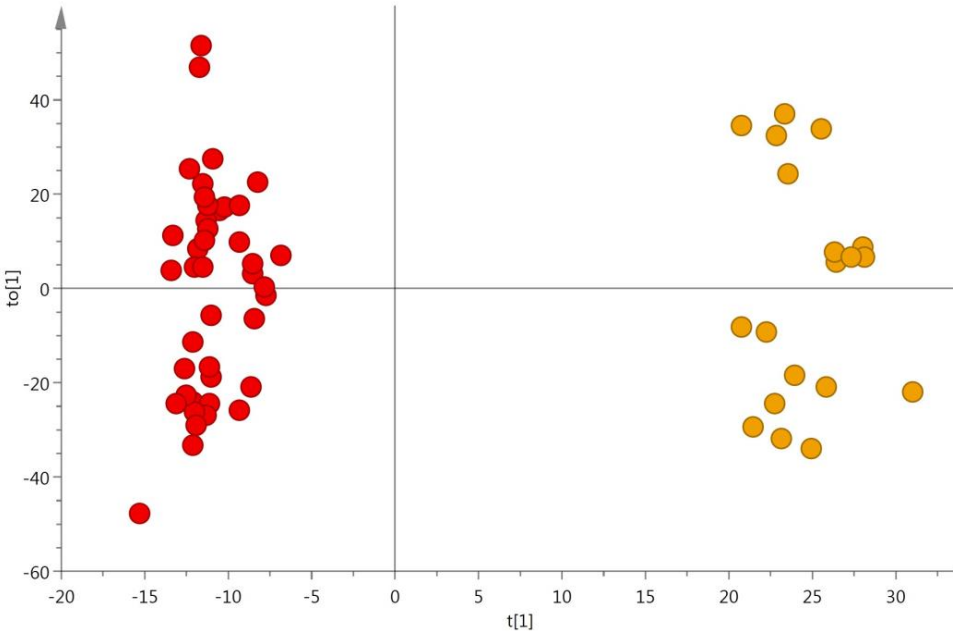
Figure 79: OPLS DA of serum 1H-NMR analysis from patients with symptomatic carotid stenosis (red) and non-carotid stroke/TIA (blue).



R^2Y	0.893
Q^2	0.0741
Principal components	7

Figure 80: OPLS DA (log transformed) of serum 1H-NMR analysis from patients with symptomatic carotid stenosis (red) and asymptomatic carotid stenosis (green).

However, OPLS DA comparing serum from patients with embolising carotid disease and aneurysmal disease yielded markedly significant results. The model suggests high predictive value (Q^2 = 0.843; Figure 81) and with a narrow difference in Q^2 and R^2Y, is considered well-fitted. Permutation analysis (20 permutations) is shown in Figure 82. The CV-ANOVA value was highly significant ($p = 1.38751e^{-010}$).



R^2Y	0.983
Q^2	0.843
Principal components	2

Figure 81: OPLS DA (log transformed) comparing serum analysed with 1H-NMR from patients with embolising carotid disease (red) and aneurysmal disease (orange).

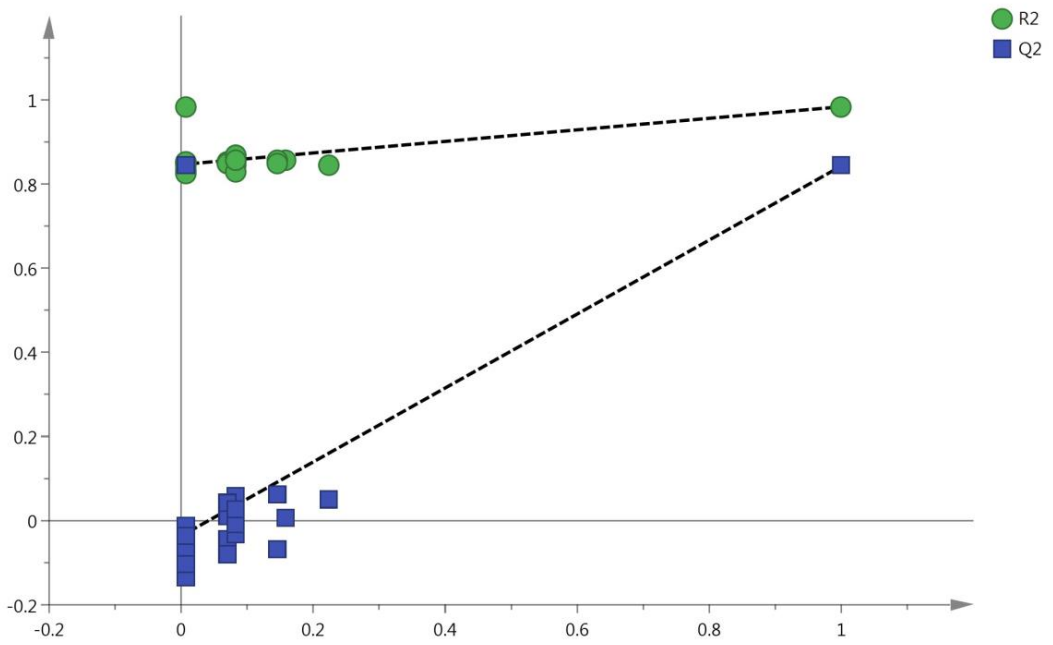
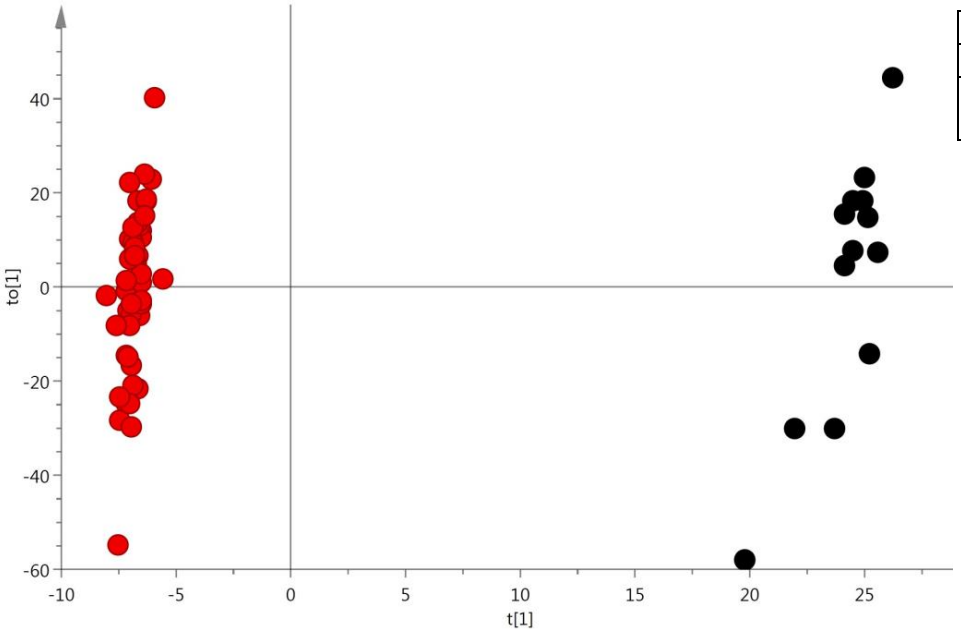


Figure 82: Permutation analysis (20 permutations) for OPLS DA comparing 1H-NMR analysis of serum from patients with embolising and aneurysmal disease. The model is statistically significant with p = $1.38751e^{-010}$.

The final OPLS DA comparison of embolising carotid disease with peripheral stenosing arterial disease was not predictive of class (Figure 83).

1H-NMR CPMG data was also examined with unnormalised spectra, however these results were substantially weaker and did not add any value to the current analysis (data not shown).



R^2Y	0.885
Q^2	-0.0307
Principal components	4

Figure 83: OPLS DA (log transformed) comparing serum from patients with embolisng carotid disease (red) and peripheral arterial stenosing disease (black).

7.4 Influence of time since symptom-onset on serum risk stratification of carotid atherosclerosis

The highest risk of stroke recurrence following a carotid event is known to occur within two-weeks of symptom onset. To determine whether this influenced the lack of separation encountered when comparing carotid subgroups, patients with clinically significant symptomatic carotid atherosclerosis (n=46) were stratified into two further subgroups: patients recruited within two weeks of symptom onset, and those recruited following two weeks of symptom onset (Table X). OPLS DA comparison of serum was then performed for each metabonomic platform. No separation was seen based on time of recruitment (data not shown).

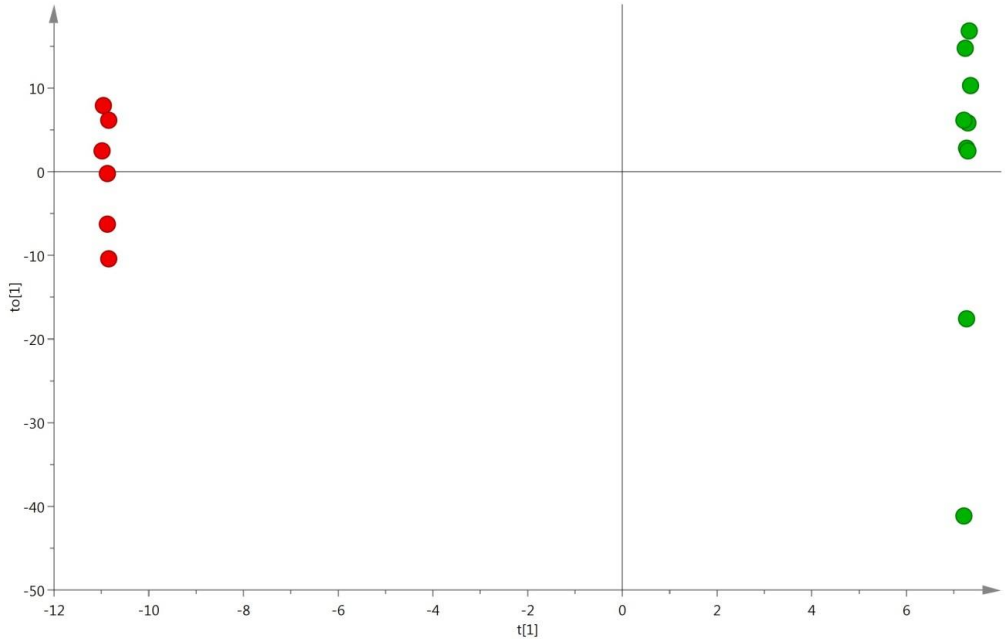
7.5 Influence of ECST-2 risk stratification metabonomic findings

In total, 45 of 46 participants with symptomatic carotid atherosclerosis had sufficient data availability to permit formal European Carotid Surgery Trial-2 risk scoring (Table X). To determine whether risk-scoring influenced metabolic separation, serum analysed with both MS platforms in both ionisation modes from participants with an ECST-2 risk score of 5 was compared with serum from participants with an ECST-2 risk score of over 15 (Figures 84-87). Models still failed to achieve sufficient strength to merit metabolite identification.

Table X ECST-2 risk scores and time since recruitment for participants with symptomatic carotid stenosis

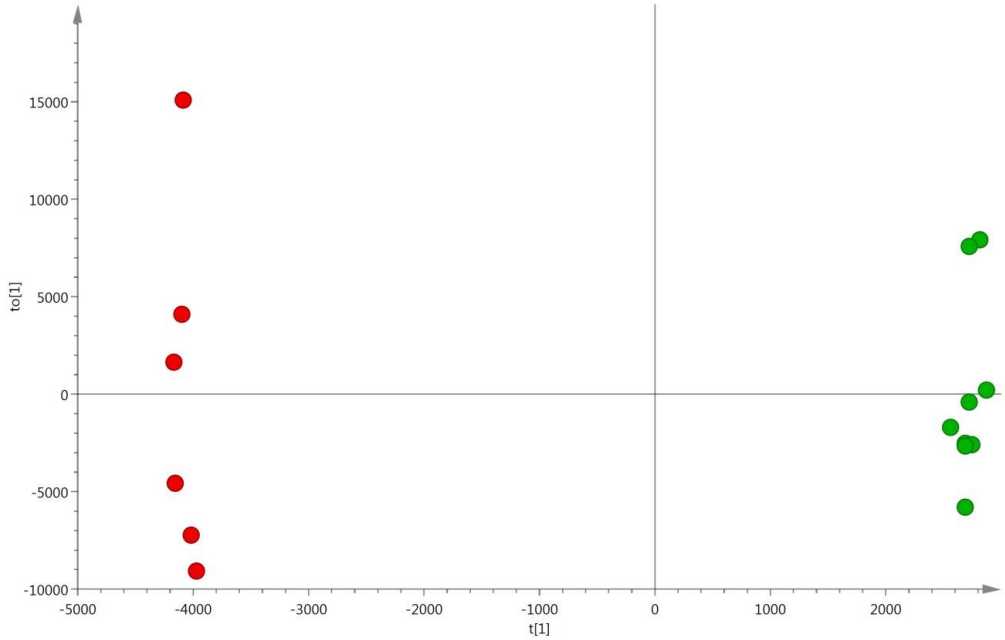
Number	Age	ECST2 SCORE	Days since neurological event
17	77	5	61
31	59	5	0
32	78	5	14
49	83	5	182
66	62	5	62
93	42	5	2
95	79	5	115
7	58	6	89
8	62	6	45
9	68	6	2
80	55	6	1
29	53	7	13
64	66	7	2
79	65	7	9
85	80	7	3
94	66	7	5
101	78	7	6
22	84	8	125
69	70	8	72
55	62	9	5
75	54	9	3
3	90	10	0
23	81	10	3
39	82	10	24
44	86	10	16
2	71	11	1

Number	Age	ECST2 SCORE	Days since neurological event
78	65	11	7
1	62	12	74
86	85	12	2
98	71	12	3
51	67	13	2
58	80	13	2
60	52	13	0
65	86	13	2
88	81	13	8
37	70	14	5
100	78	14	5
21	75	16	3
38	68	16	4
45	85	17	0
11	83	18	17
59	81	20	7
76	77	23	2
5	75	26	4
6	91	26	2
50	73		



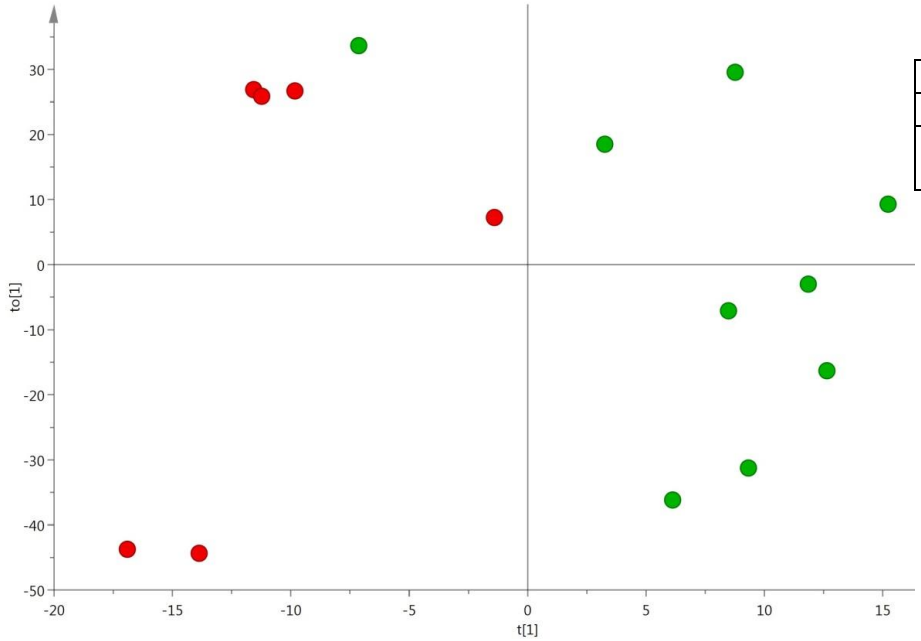
R^2Y	1
Q^2	0.378
Principal components	5

Figure 84 OPLS DA comparison of serum from symptomatic carotid patients with ECST-2 risk score of 5 (red circles and ECST-2 risk score >15 (green circles) analysed with LP MS (positive ionisation mode)



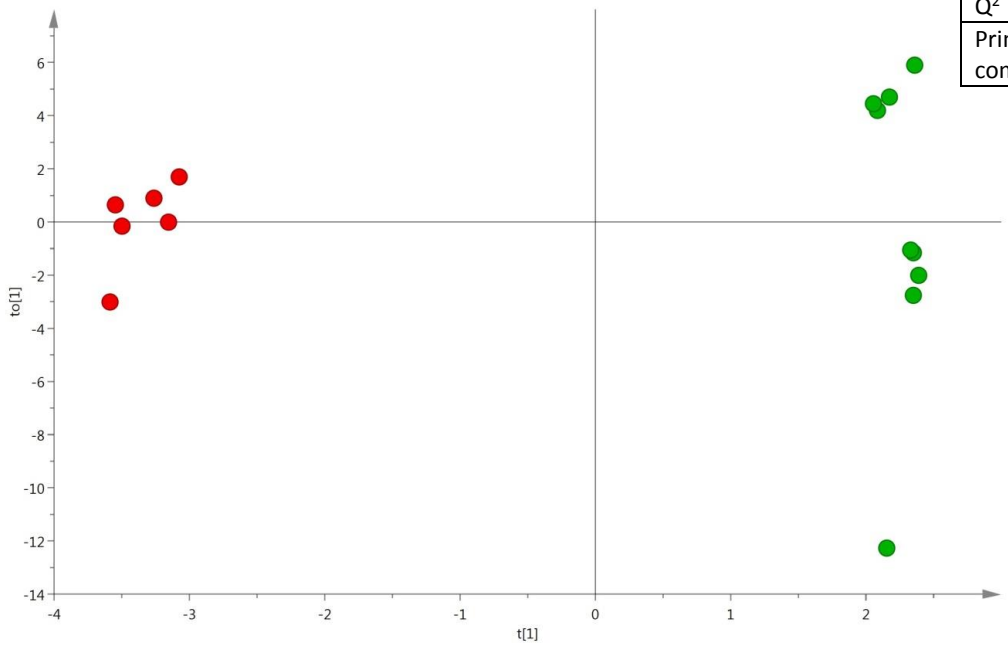
R^2Y	0.999
Q^2	0.0813
Principal components	5

Figure 85 OPLS DA comparison of serum from symptomatic carotid patients with ECST-2 risk score of 5 (red circles) and ECST-2 risk score >15 (green circles) analysed with LP MS (negative ionisation mode)



R^2Y	0.717
Q^2	-0.0371
Principal components	2

Figure 86 OPLS DA comparison of serum from symptomatic carotid patients with ECST-2 risk score of 5 (red circles) and ECST-2 risk score >15 (green circles) analysed with HILIC MS (positive ionisation mode)



R^2Y	0.997
Q^2	0.145
Principal components	4

Figure 87 OPLS DA comparison of serum from symptomatic carotid patients with ECST-2 risk score of 5 (red circles) and ECST-2 risk score >15 (green circles) analysed with HILIC MS (negative ionisation mode)

7.6 Discussion

Data models comparing metabonomic spectra of serum from patients with symptomatic carotid stenosis, non-carotid stroke/TIA and asymptomatic carotid stenosis were not found to be predictive of class identity. If indeed serum metabolic profiling of symptomatic carotid stenosis is not distinguishable from asymptomatic stenosis, markers of recent stroke should still be identifiable, resulting in a significant difference between asymptomatic and symptomatic carotid stenosis, whether or not the difference stems from the plaque. This was the outcome of the detailed systematic review of the metabolic profile of ischaemic stroke detailed in Chapter 3.

The four published metabonomic studies of ischaemic stroke do however differ markedly in their aims and design to this study. BRUITS focuses on the discovery of biomarkers of high-risk carotid stenosis, and not stroke in general. As a result, salient distinctions in study design may account for the discrepancy encountered.

For the purpose of this study, all patients with symptomatic carotid stenosis fulfilling specified inclusion criteria were included, with no distinctions between stroke and TIA, unlike previous ischaemic stroke metabonomic studies that either excluded TIAs, or incorporated TIAs as controls.

Secondly, the symptomatic period was retained as six months in keeping with guideline definitions of symptomatic carotid stenosis. However, previous studies recruited participants and collected samples within 72 hours (and often within 6 hours) of symptom onset. Given the nature of the blood-brain barrier, it is conceivable that biological markers stemming from brain injury become undetectable beyond this time.

Clinical studies of carotid stenosis provide consistent evidence that the highest rates of stroke recurrence secondary to symptomatic carotid stenosis occur in the first two weeks following index neurological event. To determine whether this translates into metabolic variation, serum from Group 1 patients (symptomatic carotid stenosis) that had been recruited within two weeks of symptoms were compared with patients that were recruited beyond two weeks. No class separation was achieved.

The underlying assumption driving the recruitment of patients into Group 1 was that patients with recently symptomatic carotid disease are 1) high-risk of further neurological events and 2) are symptomatic due to embolising atherosclerosis. To test whether the metabolic profile was influenced by formal risk scoring, the ECST-2 score was calculated for each patient in Group 1 (Table X). Of the 46 participants in this groups, sufficient data was available for 45 to permit ECST-2 score calculation. Group 1 serum was then stratified by risk score to determine whether this influenced the metabolic profile. Again, no separation was achieved, but given the small number of samples in each sub-category, this may well be Type II Error.

It is generally accepted that MS has higher sensitivity than NMR. Despite this generalisation, serum from patients with aneurysmal disease showed distinct metabolic disparities from patients with embolising carotid disease, a finding not registered through MS analysis. This could have been explained by the signal to noise ratio of these techniques: MS being more sensitive can also incorporate greater noise, therefore masking clinically significant features. In reality, the separation was artefact; a consequence of pre-processing of data.

Although disappointing, lessons learned from the results of global metabolic profiling of serum will arm future metabonomic studies in this domain.

Chapter 8: Final Discussion and Conclusion

This is the first study to demonstrate discriminating urinary biomarkers pertaining to embolising carotid stenosis. These biomarkers span a host of chemical moieties: fatty acids, nucleotides, dipeptides, amino acids and products of carbohydrate metabolism. It sheds new light on the importance of interrogating urine to unravel pathways of complex disease, an underexplored area in cardiovascular research.

Overall, PCA models of samples vs QCs for each of the analyses demonstrate the work conducted to be of high technical quality. This is ascertained by tight clustering of QCs (and sequential "peripheralisation" of diluted samples in MS analysis).

OPLS DA models constructed from spectral analysis of urine are stronger than those of serum. Urine is not subjected to homeostatic regulation, and is particularly valuable in revealing polar metabolites that undergo rapid clearance. In keeping with this, HILIC MS of urine produced stronger models than RP MS of urine.

Clinically, the non-invasive nature of urine collection is an advantage, and repeated sampling is also not an issue. However, urine was surprisingly more challenging to obtain from patients than blood, and this is reflected in the smaller number of urine samples obtained as compared with serum. There are a number of contributing factors:

- Elderly patients, particularly male patients, find it difficult to provide "on demand" urine samples, and especially those with prostatic enlargement.
- Stroke can affect continence.
- Even with normal bladder function, impaired limb coordination makes urine collection a challenge for some stroke patients.
- Urine was not collected from catheterised patients as bacterial colonisation of catheters and catheter bags results in an altered urine metabolome.

Despite the broad metabolic fingerprinting approach, clear separation of classes in serum studies has not yet been encountered. In the duration of this study, there has been significant expansion of cardiovascular metabolic research, however no published study has focused on risk stratification of carotid atherosclerosis, or circulating metabolite differentiation between regional atherosclerosis. There are a number of reasons why this may have been unsuccessful to date.

The asymptomatic burden within symptomatic carotid atherosclerosis: Many patients with symptomatic carotid atherosclerosis have a significant asymptomatic disease presence contralaterally, which may exert its own metabolic influence, thereby masking signals from the symptomatic plaque. Furthermore, there is frequently disease presence within the external carotid artery, the presence of embolic phenomena from which are not considered to be symptomatic events.

Time elapsed between symptoms and sample collection: Symptomatic patients were defined as those that had suffered a focal ischaemic neurological event in the preceding six months, as this is the definition of symptomatic carotid atherosclerosis. The systematic review of stroke studies that forms the introduction of this thesis demonstrates that all published studies of metabolic profiling in stroke involved sample collection within hours of symptoms. In line with European and US guidelines regarding carotid intervention – that it should be achieved within one to two weeks of the index symptomatic event – a repeat analysis was attempted including only those Group 1 patients that were recruited within two weeks of symptom onset, and comparing these patients to Group 2 (non carotid stroke) and Group 3 (asymptomatic carotid atherosclerosis). No separation was achieved on PCA, and OPLS DA models were weakened.

Distinction between stroke and TIA: This study focuses on the metabolic characteristics of embolising carotid disease, not stroke in general. Despite this distinction, comparison of symptomatic and asymptomatic disease could also highlight markers of stroke in general. Metabolically, TIA and stroke have been shown to be distinct entities. Furthermore, international contested changes in the definition of stroke and TIA centering upon radiological presence or absence of infarction could not be assessed within this study, as asymptomatic patients do not routinely undergo brain imaging. If newer definitions were applied, it could have resulted in regrouping of patients. Additionally, both stroke and TIA can result from carotid embolism, but carotid embolic CVA has a much higher risk of stroke recurrence than carotid TIA, implying a difference in plaque behaviour.

Fasting: Lipid models of serum were non discriminatory. Collection of fasting samples from all patients was not feasible, and therefore not attempted. Fasting alters the lipid composition of serum, and oral intake influences the metabolome.

Phase of study: This is in essence a Phase 4 diagnostic study, in that consecutive patients meeting inclusion criteria were incorporated into the project. The control group was therefore poorly matched in terms of age, gender, comorbidities and medications. Preceding metabonomic publications of peripheral vascular diseases and stroke are predominantly Phase 1 studies with stringent exclusion criteria. Although malignancy functioned as an exclusion criterion, as patients with cancer seldom undergo carotid intervention (even for symptomatic disease), and patients with atrial fibrillation were also not included (as this serves as a confounder for stroke aetiology), other strict exclusion criteria were not imposed. Diabetes, hypertension, chronic kidney disease can all alter the metabolome, but serve as important risk factors for atherosclerosis.

Drug modulation: Statin therapy can exert a metabolic impact and together with homeostatic mechanisms, reverse potential metabolic aberrance resulting from atherosclerotic disease. A large proportion of included patients were prescribed statins, which may again mask metabolic consequences of disease. One question for future metabolic studies is why some optimally treated patients continue to suffer cardiovascular events.

Assumption of low risk in asymptomatic atherosclerosis: The long-standing controversy within carotid research is the frequent assumption that

asymptomatic atherosclerosis represents low-risk disease. Conversely, symptomatic atherosclerosis is deemed high risk, but stroke recurrence following a symptomatic carotid event (although higher-risk than other stroke aetiologies) occurs in 2-30% of patients. BRUITS is a pilot study, incorporating relatively small numbers of patients per group. To our knowledge, no patient suffered a recurrent cerebrovascular event during the course of the study (though this would be modulated by carotid endarterectomy), and it is conceivable that there was no major difference between the risk of recurrence between symptomatic and asymptomatic groups. ECST-2 score profiling of the symptomatic group revealed a poor spread of risk scores, insufficient to permit meaningful sub-stratification of metabolic phenotypes. Most patients had a moderate risk of recurrence.

Possibility of silent infarction: Asymptomatic patients with carotid atherosclerosis did not undergo brain imaging to determine the presence of silent infarction. Although according to UK definitions, this would not be considered a stroke or TIA, it could still imply a high risk carotid lesion.

Cross prevalence of disease types: Patients included in the symptomatic carotid group, claudicant group and aneurysm group were not scanned for the presence of other atherosclerotic disease – presence in the symptomatic carotid group and claudicant group was determined based on symptoms alone.

The design of future metabonomic studies pertaining to carotid atherosclerosis should be modified to take these considerations into account. Firstly, it would appear that classifying patients based on symptom status alone is insufficient.

Means of stratifying risk in symptomatic carotid atherosclerosis include PET imaging, but this is expensive and logistically challenging as it is not offered at the same site as the Charing Cross HASU. Risk scoring may be more valuable, e.g. with the ECST-2 risk scoring tool. Subsequent stratification of symptomatic carotid atherosclerosis patients based upon ECST-2 scores showed that in reality, the majority of patients were low to moderate risk, as evidenced by the fact that no patient included in this study suffered a recurrent neurological event. Alternative methods may include transcranial Doppler assessment to document evidence of embolic phenomena, but again this is time consuming and highly operator-dependent, and not necessarily specific for embolising carotid atherosclerosis.

Narrowing the window of recruitment following a neurological event may be beneficial. Metabonomics is a time-dependent discipline, and homeostatic return to normal parameters of serum may obliterate early signals. Repeat sampling to reduce variance and permit patients to serve as their own controls would allow temporal trends in pertinent biomarkers to emerge, and further guide research regarding the optimum time-point for sample collection.

Furthermore, distinguishing between stroke and TIA may seem like categorisation based on effect rather than the underlying vascular pathology, but stroke secondary to carotid atherosclerosis has a significantly higher chance of recurrence as compared with an initial carotid TIA. There is an implied distinction in plaque behaviour.

The aim remains to better risk stratify carotid atherosclerosis permitting a personalised approach to enhance patient care and reduce the burden of stroke. Both the positive and negative findings of BRUITS serve to advance knowledge in this domain, leading to new research building upon and learning from the outcomes presented in this thesis.

III. Future Work

Chapter 9: Further projects in progress

With consideration to the results and limitations of BRUITS, I have designed and secured grant funding for three subsequent metabonomic studies of vascular disease encompassing stroke (MBRACE), peripheral arterial disease (MetaboPlasty), and aneurysmal disease (MEASURE AAA). These projects have been funded through awards from the Circulation Foundation, The Royal College of Surgeons of England, The Dunhill Medical Trust, Rosetrees Trust, Graham-Dixon Charitable Trust and Masons Medical Research Foundation. The following sections contain abbreviated protocols for each study.

10.1 MBRACE: Metabolic Biomarker Responses in Acute Cerebral Events

Introduction

Clinical Background and Importance

Stroke is a leading cause of death and disability worldwide (131). A diagnosis of "stroke" encompasses many possible aetiologies, broadly divided into ischaemic or haemorrhagic subtypes. The majority are ischaemic (85%) (132) and may result from cardioembolism; large artery thromboembolism, dissection or occlusion; cerebral small vessel disease; or even vasculitis. Delineating the causative mechanism is often not possible at initial presentation, and stroke remains a clinical diagnosis.

The advent of thrombolysis for acute ischaemic stroke has revolutionised management strategy in the immediate aftermath of a cerebrovascular accident,

enabling rapid reperfusion of ischaemic brain tissue with the potential to reverse disability resulting from stroke (133) (134). Guidelines advise administration of thrombolytic therapy within 4.5 hours of onset of ischaemic stroke (132). After this time, the ischaemic territory is unlikely to recover, and the risk of iatrogenic bleeding caused by thrombolytic therapy exceeds the likelihood of benefit to the patient. Thus patients with "wake-up" strokes, are frequently unable to undergo thrombolysis, as the time of onset of stroke is not known (135).

Hyper Acute Stroke Units have been shown to improve outcomes for stroke patients (136). In such a unit, the aim is to deliver thrombolytic therapy within thirty minutes of patient arrival, which in turn requires rapid patient assessment and investigation, multi-disciplinary decision-making and communication.

Central to this process, and the primary investigation required, is an unenhanced CT head to ensure that a patient with a haemorrhagic stroke does not receive thrombolytic therapy. Beyond exclusion of intracranial bleeding, CT head within hours of the onset of stroke is often not of sufficient sensitivity to definitively image acute cerebral infarction, and the decision must frequently be based on clinical grounds. The need for rapidity precludes the use of diagnostic MRI in most patients prior to the administration of thrombolysis, and depending on the patient's circumstances, MRI may not be feasible or possible. As a result of this inevitable diagnostic uncertainty, patients may be erroneously be administered thrombolytic therapy, or denied it when required, resulting in permanent disability(136).

There is a pressing clinical need to determine biomarkers of hyperacute stroke subtypes in order to categorise and age the infarct, and to better direct treatment and diagnostic investigations. The ultimate aim is to develop a point-of-care test that can differentiate accurately between ischaemic and haemorrhagic stroke, which could deliver the possibility of prehospital thrombolysis administration.

Methodological Background and Existing Knowledge

Metabonomics is a hypothesis-generating approach, and carries the advantage of analysing metabolites produced following the pathological process that has led to stroke, providing an up-to-date molecular picture of acute pathology.

Proton nuclear magnetic resonance spectroscopy (1H-NMR) and mass spectrometry (MS) techniques have proven suitable analytical approaches for metabonomic study of cardiovascular disease(137). A significant proof-of-principle study has identified that the metabonomic analysis of plasma can be used to predict the future risk of cardiovascular events and improve risk stratification in patients with suspected with coronary artery disease (138). The knowledge of blood brain barrier dysfunction, endothelial cell death and cytosolic content release during acute stroke has been utilized in a separate study, proving that a protein biomarker panel can improve the diagnosis of acute stroke taken from peripheral blood samples (100). We have previously demonstrated metabonomic differentiation of symptomatic and asymptomatic carotid plaque(139). The extension of metabonomic research to acute stroke is required to identify highly sensitive biomarkers for stroke of all causes, building in mechanisms to reduce confounding and enable clinical application.

Early studies have demonstrated the feasibility of metabonomics within stroke research and are summarised in Chapter 3.3.

Existing metabonomic untargeted studies investigating biomarkers diagnostic of stroke compared stroke patients with healthy volunteers or patients attending for routine outpatient appointments – as such they are Phase 1 and 2 diagnostic studies(101), and patient groups are often not matched for vital characteristics, or stringent exclusion criteria imply that findings may not be translated into real-world practice. Risk factors such as diabetes, hypertension, dyslipidaemia and arterial disease confer distinct metabolic profiles; it is not known whether groups matched for such characteristics would retain the significant metabolic distinctions implied by Phase 1 studies, particularly given the small number of participants included. Furthermore, investigators did not consider the use of pertinent medications, such as statins. Additionally, the small number of study subjects coupled with the large number of metabolite variables generated by spectroscopic techniques leaves significant room for statistical error. Our study design takes account of these limitations, through the recruitment of 300 subjects, as well as attempting to match groups based on cardiovascular risk factors.

Aims

1. To identify metabolic biomarkers differentiating haemorrhagic from ischaemic stroke
2. To correlate biomarkers of stroke with clinical and radiological severity
3. To determine the variability of significant biomarkers over time

Project Design

Setting

Imperial College Healthcare NHS Trust and specifically Charing Cross Hospital serves as the regional Hyper Acute Stroke Unit (HASU) and recently ranked as the leading HASU in the UK. The Academic Section of Vascular Surgery at Imperial College London, based at Charing Cross Hospital, is thus ideally suited for subject recruitment.

Subjects

300 patients will be recruited into the following groups:

1. Patients presenting within 6 hours of onset of ischaemic stroke

   Subgroups:

   - Large artery atherosclerosis (including symptomatic carotid stenosis)
   - Lacunar strokes
   - Cardioembolic strokes

2. Patients presenting within 6 hours of a haemorrhagic stroke

   Subgroups:

- Intraparenchymal haemorrhage
- Subarachnoid haemorrhage

3. Thrombolysis-call patients whose final diagnosis is non-stroke (control group)

Inclusion criteria include:

1. Adult patients presenting to hospital within 6 hours of onset of neurological symptoms
2. Age over 50 years

Exclusion criteria include:

1. Active malignancy
2. Preceding stroke or thrombolysis within six months
3. Inability to undergo informed consent
4. Status elipticus
5. Sepsis
6. Pre-menopausal women

We will match groups for the following criteria:

- Age
- BMI
- Smoking history
- Alcohol intake

- Hypertension
- Dyslipidaemia
- Diabetes
- Previous stroke/TIA
- Atrial fibrillation
- Antiplatelet therapy
- Anticoagulation therapy
- Statin us

Justification of sample size

It is not possible to power a metabonomics study. This is because there are thousands of potential variables (metabolites). In this circumstance, we are reliant upon expertise (and Professor Holmes is an internationally-renowned leader in metabonomic research) and lessons drawn from published literature. In this regard, we are confident that our subject size is appropriate.

Methodology & Data Analysis

Recognition of biomarkers of stroke subtype

Blood will be collected from each consenting patient prior to the administration of any medication, and in particular thrombolytic therapy (if given). Blood is routinely collected within minutes of the thrombolysis-call patient's arrival to the emergency department. We propose the additional collection of 12ml for research purposes as part of the same needlestick. This minimises patient discomfort, as addition needling is not required, and minimises time diverted to research as it adds mere seconds to the necessary clinical procedure.

Serum will be extracted from centrifuged blood and stored at -80 degrees Celsius prior to batch analysis with H1-NMR and Ultra-Pressure-Liquid-Chromatography-MS (UPLC MS).

Computational multivariate statistical analyses (such as Principal Component Analysis and Partial Least Square Analysis) will be employed to determine differences between metabolites in each subgroup. Datasets from patients with acute ischaemic stroke will be compared with those from patients with haemorrhagic stroke, and non-stroke, in order to determine biomarker signatures of stroke subtypes.

Further experiments will then be performed in order to determine absolute quantification of statistically significant metabolites.

Validation

One hundred fifty further patients will be recruited in an identical manner to that described. Serum samples will be collected for validation of identified significant biomarkers.

Correlation of biomarkers with size of infarct

All patients with ischaemic stroke undergo CT +/-MRI as part of routine inpatient investigation. We will compare serum biomarkers at presentation with volume of infarct in patients that have and have not received thrombolysis.

Temporal trends in biomarker signatures

To determine the time-dependence of statistically significant biomarkers, additional blood samples will be collected from 10 patients in each of the recruited subgroups at the following time intervals from onset of symptoms: 6

hours, 12 hours, 24 hours and 48 hours. Quantification analyses will again be performed to determine how the biomarker concentrations change with time from onset of stroke. Clinical evaluation scores will also be obtained in order to determine the variability of biomarker concentrations with early prognosis.

Translational Potential for Patient Care

Recognition of biomarker signatures of hyperacute stroke subtypes would provide the following clinical benefits:

1. Differentiation of stroke, 'stroke mimics', and 'stroke chameleons' to guide rapid decision making and permit individualised risk scores in the context of thrombolysis administration
2. The possibility of pre-hospital thrombolysis administration following diagnosis through a point of care test
3. The possibility to age the onset of stroke, enabling thrombolysis for appropriate "wake-up" stroke patients
4. Early diagnosis of stroke subtype to guide further acute management (e.g. need for urgent neurovascular diagnostics, out-of-hours carotid imaging and therefore early carotid endarterectomy)
5. Reduced exposure to unnecessary radiation in the form of CT and cost of neuro-imaging where biomarker testing results in a diagnosis of non-stroke
6. Reduced unnecessary admissions to Hyper Acute Stroke Units

Successful completion of this study would be followed by a clinical trial using targeted techniques to ensure significant metabolites (in conjunction with

established clinical tools) are of adequate sensitivity and specificity for clinical practice.

Timeline

This project is planned over 3 years. The Gantt Chart (Figure 89) depicts phases of research.

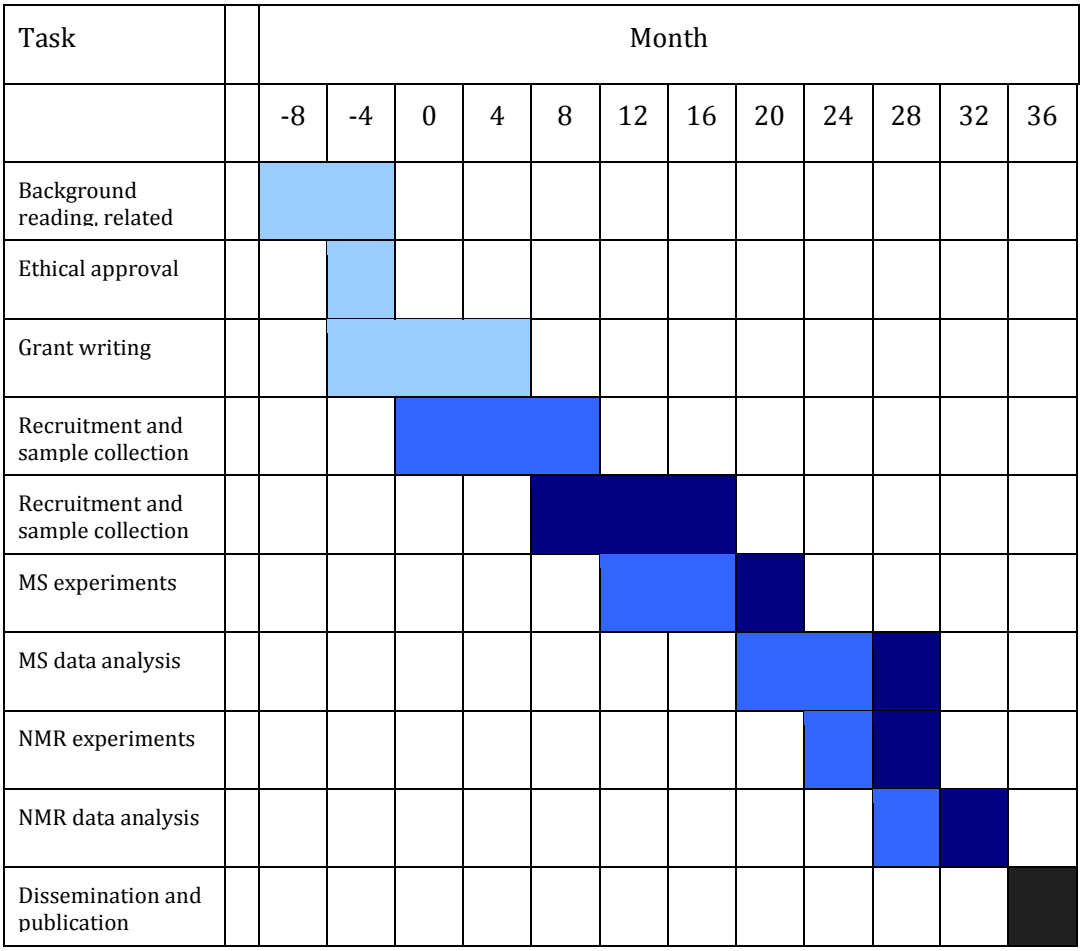


Figure 87: Gantt Chart depicting phases of work for M-BRACE

10.2 MetaboPlasty: Altering the metabolome of claudicants through endovascular intervention.

Clinical Background

Intermittent claudication (IC) is the most frequent manifestation of lower limb peripheral arterial disease (PAD) and the prevalence increases with age, up to around 7% of the population over the age of 70(140). The Trans-Atlantic Inter-Society Consensus Document on Management of PAD (TASC II) defines IC as lower limb muscle discomfort reproducibly produced by exercise and relieved within 10 minutes of rest(140). IC severely limits quality of life(141) and is an independent predictor of cardiovascular and all-cause mortality, independent of other atherosclerotic risk factors(142).

After diagnosis of IC, The National Institute for Health and Clinical Excellence (NICE) recommends risk factor modification and supervised exercises classes in the first instance(143). NICE state that if this does not achieve "satisfactory improvement in symptoms" and if the lesion is suitable, patients should be offered angioplasty(143). When TASC II was published in 2007, it was recommended that TASC type A and B lesions should be treated primarily with endovascular methods and that, where appropriate, the generally longer/more complex TASC C and D lesions should be treated surgically(140). There is now evidence that endovascular methods can be used to treat TASC type C and D lesions with similar patency rates(144, 145) and improvements in ankle brachial pressure index (ABPI)(145) as is seen with surgical repair. This evidence, combined with the increasing prevalence of IC with age and the ageing

population, suggest endovascular intervention for IC is likely to become more widely used.

Despite the defined eligibility criteria for angioplasty and efforts to predict which patients with IC are most likely to respond to endovascular treatment(146, 147), 22% of patients still experience IC(148) following angioplasty. There is a need therefore, to accurately predict the likelihood of clinical and radiological success after endovascular procedures in patients with IC.

Methodological Background

Two metabonomics studies of patients with IC have been performed to date. The first looked at the effect of exercise on NMR metabolic profile in 14 patients with IC(149). The IC group demonstrated predominantly anaerobic ATP generation and with antioxidant administration in the form of vitamin C and E, this oxidative stress was dampened(149). Furthermore, this effect was significantly less marked when investigated with traditional biochemical methods. The second study, generated a metabonomic profile, to predict near-term death (less than 8 months) in IC patients(150).

To our knowledge, the influence of endovascular intervention on the metabonomic profile of claudicants has not been established.

Aims

1. To determine the change in serum and urine metabolic profiles in patients with IC following angioplasty

2. To determine metabolic biomarkers which may predict the functional success of angioplasty in patients with IC
3. To establish potential metabolic pharmacological targets in patients with IC

Hypotheses

- That the serum and urine metabolic profile is significantly different before and after angioplasty in patients with IC
- That serum biomarkers exist that predict good clinical response to angioplasty in patients with IC
- That there are metabolic therapeutic targets in patients with IC

Methods

Clinical Setting & Patient Selection

Imperial College NHS Healthcare Trust incorporates a tertiary referral Vascular Surgery centre. Furthermore we have acquired ethical approval to set up a Biobank for blood, urine and tissue from patients with vascular diseases, for metabonomic analysis. This busy centre, based across Charing Cross and St Mary's Hospitals, is therefore ideal to recruit patients with IC awaiting angioplasty for metabonomic studies.

We aim to recruit 20 patients with IC as defined by the TASC II consensus(140) who are awaiting peripheral angioplasty and able to give informed consent.

Exclusion criteria are as follows:

- Age <50 years
- Pre-menopausal women
- Active malignancy
- Critical ischaemia (rest pain/tissue loss/gangrene)

Angioplasty waiting lists will be reviewed for suitable patients who will be invited to attend The Academic Section of Vascular Surgery at Charing Cross Hospital. Subject to written informed consent, the following baseline evaluation and metabonomic profiling shall be conducted within the 7 days before their scheduled angioplasty.

Baseline evaluation

Baseline patient characteristics will be recorded, including:

- Age
- Gender
- Height
- Weight
- Past medical history
- Drug history
- Family history (with particular regard to cardiovascular disease)
- Alcohol intake
- Smoking history
- Duplex findings

- ABPI – with a preceding 10 minutes of bed rest in a semi-recumbent position, so the patient's breathing is comfortable. Any differences in systolic arm blood pressure of greater than 20 mmHg will be referred for further investigation.

The participants will be asked to fill the following forms:

- The Edinburgh Claudication Questionnaire – a validated patient questionnaire for the diagnosis of IC(151)
- The Charing Cross Intermittent Claudication Questionnaire – a validated measure of quality of life in IC(152)
- General Health Measure – the EQ-5D is a validated questionnaire to measure perceived general help in a wide range of conditions(153)
- Quality of life questionnaire

Metabonomic profiling

Overnight fasting blood (12ml) will be collected from each patient, and serum extracted and stored in 1ml aliquots at -80 degrees Celsius. One fasting urine sample will also be requested and again stored in 1ml aliquots at -80 degrees Celsius. Both serum and urine will be subjected to batch metabonomic analysis using ^{1}H-NMR and Ultra Performance LC MS. Data will be mathematically modelled using chemometric software including SIMCA-P 12.0.1 (Umetrics® Sweden) and MATLAB R-2011 (Mathworks™). LC MS data will be processed using MarkerLynx™ Application Manager and XCMS software before importing to statistical software (SIMCA-P). Additional experiments will be performed for identification of specific metabolites (biomarkers) for example Tandem mass

spectrometry (MS/MS) experiments and 2 dimensional NMR. Baseline ABPIs will also be measured.

Angioplasty procedure

Two hours post-angioplasty, repeat blood and urine samples will be obtained and processed for NMR and LC MS as above.

Follow-up evaluation

The patients will be invited to return at another date 6-weeks post-angioplasty. At this time, repeat fasted blood and urine samples will be taken for ^{1}H-NMR and LC MS analysis. ABPIs and the baseline questionnaires will be repeated. Attempts will be made to correlate changes in metabonomic profiles with those with changes in quality of life score after angioplasty. The protocol is summarised in Figure 90 and project plan timescale in Figure 91.

Translational Value

This work intends to establish the change in the metabolic profile in patients with IC before and after angioplasty. This may allow:

- Increased understanding of the metabolic processes involved in the development of IC and how angioplasty affects these processes
- Prediction of which patients with IC are most likely to respond to angioplasty
- Avoidance of unnecessary complications in patients who may not benefit from angioplasty
- Increased potential to meet the growing demand for angioplasty by selecting the most suitable patients

- Awareness of potential therapeutic targets in patients with IC

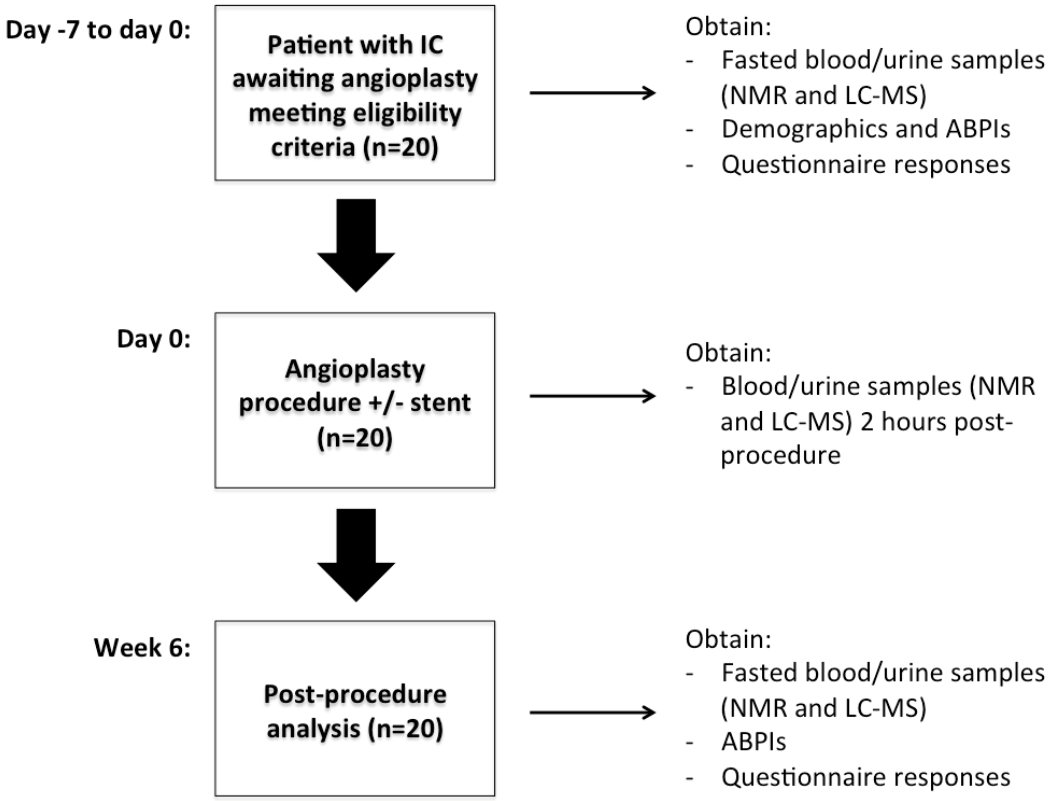


Figure 88: Metaboplasty project protocol

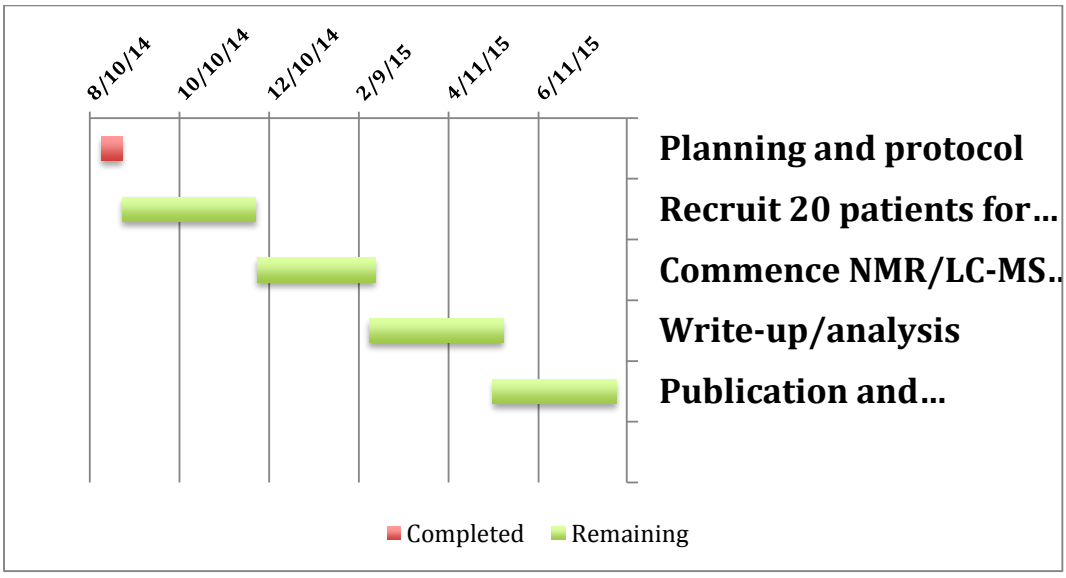


Figure 89: Gantt Chart for Metaboplasty

10.3 MEASURE AAA: MEtabonomic Analysis of Serum & URine to Evaluate Abdominal Aortic Aneurysms

Background

Ruptured abdominal aortic aneurysm (AAA) currently accounts for an estimated 4000 deaths per year in England and Wales(154). A single screening ultrasound scan to detect the presence of AAA in men can significantly reduce rupture-induced mortality. Therefore the Department of Health has introduced the National Health Service Abdominal Aortic Aneurysm Screening Program (NAAASP) that screens all consenting men for AAA at age 65 using an ultrasound scan(155).

Screening is carried out across ~40 sites in England. If an aneurysm is discovered that is as yet under 5.5cm, the patient is offered regular surveillance until such time that the aneurysm exceeds the 5.5cm threshold for treatment (when the risk of mortality from rupture outweighs the risk of elective surgery(156)). There is no treatment alternative to surgical intervention, and optimal medical treatment to prevent or slow progression of aneurysms remains uncertain(157).

Ultrasound scans carry certain limitations in their estimation of aneurysm size. These include operator bias, inter- and intra- observer reliability(158), particularly for suprarenal aneurysms, and impedance of view secondary to large body habitus. MR and CT are expensive substitutes, the former time

consuming, and the latter a source of radiation exposure and potential contrast-related complications. At present there is no feasible substitute to ultrasound as a screening tool.

At Imperial College Healthcare NHS Trust, 100 screening scans are offered each week across 3 sites; unfortunately attendance is averaged at 50-55%.

Methodological Background

A small metabonomic study of fasting patients with small (<5.5cm diameter; n=15), large (>5.5cm diameter; n=15) abdominal aortic aneurysms as well as normal controls (n=11) has shown clear separation of plasma samples when analysed with LC MS(71). Lipid-based metabolites implicated in the development and progression of aneurysms have also been identified. We wish to expand on the methodology by incorporating NMR (particularly valuable for lipid-based metabolite identification and quantification) and combining with Ultra Performance LC MS in a non-targeted approach for biomarker discovery. Secondly, we aim to employ a targeted approach to verify the metabolites discovered by Ciborowski et al. in a UK-based population.

Hypothesis

We hypothesise that the presence of an aortic aneurysm harbours a distinct metabolic profile, permitting recognition of biomarkers that vary with aneurysmal size.

Aims

1. To identify differential metabolic profiles for normal and aneurysmal aorta.
2. To determine biomarkers associated with large AAA.
3. To verify metabolites identified as potential biomarkers in smaller, non-UK studies.
4. To identify potential metabolic pharmacotherapeutic targets to prevent aortic dilatation.

Methodology

Research setting

Imperial College NHS Healthcare Trust is a tertiary referral centre for Vascular Surgery, and a recognised site for AAA screening. We have acquired ethical approval to obtain blood, urine and tissue samples from consenting patients with aneurysmal disease for biobank storage and subsequent metabonomic analysis (reference East Midlands NRES Committee 13/EM/0011).

The NIHR-MRC Phenome Centre also based at Imperial College Healthcare NHS Trust and led by Imperial College London has expanded capabilities in metabolic phenotyping, with enhanced opportunities in biomarker research within the omic disciplines. Collaboration between the Academic Section of Vascular Surgery and Section of Computational and Systems Biology has resulted in high impact publications stemming from translational research in this domain.

The Academic Section of Vascular Surgery has an established track record in aneurysm research as evidenced by randomised control trials including the UK Small Aneurysm Trial, and EVAR-1 & -2. Imperial College Healthcare NHS Trust

serves as a screening centre for the National Abdominal Aortic Aneurysm Screening Program (NAAASP). We are thus ideally suited for patient recruitment in aortic metabonomic studies.

Patient recruitment and data collection

Patients undergoing screening scans will be provided with written information regarding the research study, on the day of their screening scan appointment, as well as a verbal explanation detailing the purpose and process of research. The patient will be given the opportunity to ask questions or discuss with a member of their family and/or their GP.

Participation in this study subjects the participant to a short interview in which past and present medical problems are determined as well as family history and current medications.

Inclusion criteria will vary with the particular experiment being undertaken and are detailed in each sub-section below. General inclusion criteria are as follows:

1. Male gender
2. Age > 65 years
3. Ultrasound scan (or other imaging) at Imperial College Healthcare NHS Trust that determines the presence or absence of an abdominal aortic aneurysm
4. Capacity to consent for research.

Exclusion criteria are as follows:

1. Age <65 years
2. Inability to undergo informed consent.
3. Symptomatic aneurysms/ruptures
4. Active malignancy

Subject to written informed consent, 100 patients will be recruited and divided into the following three categories:

Group 1: Normal aorta (n=34)

Group 2: Early aneurysmal disease (aortic diameter between 3cm and 5.5cm) (n=33)

Group 3: Advanced aneurysmal disease (aortic diameter greater than or equal to 5.5cm) (n=33)

Baseline patient characteristics will be recorded. These include age, BMI, presence of AAA and maximal diameter, medical history, drug history, family history, alcohol and smoking history.

Sample collection and metabonomic profiling

Blood will be collected from each patient, centrifuged, and serum extracted and stored at -80 degrees Celsius. One urine sample will also be requested and again stored at -80 degrees Celsius. Both serum and urine will be subjected to batch metabonomic analysis using ^{1}H-NMR and Ultra Performance LC MS. Data will be mathematically modelled using chemometric software including SIMCA-P 12.0.1 (Umetrics® Sweden) and MATLAB R-2011 (Mathworks™). LC MS data will be

processed using MarkerLynx™ Application Manager and XCMS software before importing to statistical software (SIMCA-P). Additional experiments will be performed for identification of specific metabolites (biomarkers) for example Tandem mass spectrometry (MS/MS) experiments and 2 dimensional NMR.

Translational value

Recognition of metabolic biomarkers of aneurysmal disease will permit:

1. Personalised risk estimate for patients undergoing aortic aneurysm screening.
2. Improved efficiency of the ultrasound screening program through exclusion of normal aorta by blood/urine testing.
3. Increased knowledge of biochemical/pathological pathways leading to the development of aneurysmal disease.
4. Awareness of potential pharmacotherapeutic targets and a means of monitoring arterial wall stabilisation therapy.

Cost

Global metabonomic profiling costs £50 per sample. Therefore for 200 samples from 100 patients (one serum and urine sample per patient), the cost for analysis of this study is £10,000.

Dissemination

The findings of this study will be disseminated through peer-reviewed publications, and national and international presentations.

IV. References

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V. Appendices

Appendix 1: Data collection proforma

Serum	Urine	Blood	Plaque	P	A	C

ARTERIAL PATIENT PROFORMA FOR METABONOMIC RESEARCH

Name	DOB	Hosp no.
Contact	Gender	Handedness
	Height	Weight

CAROTID ☐ **ANEURYSM ☐** **PVD ☐**

CAROTID

Asymptomatic ☐					**Symptomatic ☐**		
Previous carotid events & details					TIA ☐	CVA ☐	Amaurosis ☐
					Date:		
					Side of body:		
					Symptoms:		
					Thrombolysis:		
Carotid Duplex					**Brain Imaging (CT/MRI)**		
Date:							
RIGHT	CCA	ICA	ECA	V			
Characteristics:							
LEFT	CCA	ICA	ECA	V			
Characteristics:							
Notes:							
CEA ☐			**Date:**		**Complication:**		

ANEURYSM

Asymptomatic ☐	**Symptomatic ☐**	**Leak ☐**	**Rupture ☐**
Location:			
Previous intervention:			
Duplex	Date:	**CTA**	Date:
Max Size:		Max Size:	
Notes:		Notes:	

PERIPHERAL ARTERIAL DISEASE

RIGHT	**LEFT**
Previous angioplasty:	Previous angioplasty:
Previous surgery & indication:	Previous surgery & indication:
Current symptoms:	Current symptoms:
Tissue loss:	Tissue loss:

ADDITIONAL COMORBIDITIES

Atrial Fibrillation	
Diabetes (1/2)	
Hypertension	
Ischaemic Heart Disease & Details	
Hypercholesterolaemia	
Renal Impairment	
Neurological Disorder	
Psychiatric History	
Connective Tissue Disorder	
Inflammatory Disorder	
Infection?	
Others:	

MEDICATIONS Please state if any recently started, e.g. antiplatelets on admission post-event

Drug	**Dose**

FAMILY/SOCIAL

Family history:
Smoking history:
Alcohol intake (units per week):

BLOODS

Basic				**Date**
WCC	Ly	Ne	Ma	
Urea	Creatinine	CRP	ESR	
Lipids				**Date**
Total Chol	HDL	LDL	HDL ratio	TG

NOTES

Appendix 2: Baseline patient data

No.	Group	Age	Days since event	Sex	Statin	Anti-Platelet therapy	HTN	IHD	DM	Smoke	BMI	WCC	Ur	Cr	CRP	ESR	CHOL	TRIG	HDL	LDL	CHOL:HDL
1	1	62	74	M	ATORVA	A	1	0	0	1	24.2	8	3.7	73	14	50	4.3	2.49	0.8	2.37	5.38
2	1	71	1	M	ATORVA	A	1	1	1	0	30.8	4.7	10.5	181	1.3	19	5.3	1.64	0.92	3.63	5.76
3	1	90	0	M	ATORVA	C	0	0	0	1	24.5	4.8	5.8	90	1.1	7	3.3	1.5	0.87	1.75	3.79
4	3	85	1	M	ATORVA	A	0	0	0	1	22.9	6.3	8.1	97	6	54	4.8	0.81	1.14	3.29	4.21
5	1	75	4	M	SIMVA	A	1	1	1	1	30.4	6.4	7.6	165	1.1	25	3.2	1.31	1	1.6	3.2
6	1	91	2	M	0	D	1	1	0	0	30.4	10.7	8.6	174	2.2	12	5	1.94	1.02	3	4.9
7	1	58	89	M	SIMVA	C	1	0	0	1	27.4	9.4	5.5	77	3.8	13	3.4	1.47	0.98	1.75	3.47
8	1	62	45	M	SIMVA	C	1	0	0	1	19.7	6.4	3.8	95	3.8	21	5.8	1.21	2.74	2.51	2.12
9	1	68	2	F	ATORVA	A	1	0	0	0	30.1	8.8	16.4	161	0.5		5.4	1.05	1.8	3.12	3
10	2	80	3	M	ATORVA	A + C	1	1	1	1	24.1	9.1	11.1	120	4.9	21					
11	1	83	17	F	SIMVA	A + W	1	1	0	1	22	6.3	5.4	55	7.3	11	6.2	1.14	1.79	3.89	3.46
12	2	56	16	F	0	A	0	0	0	1	33	8.7	5.5	64	5	8	6.1	2.15	1.65	3.47	3.7
13	3	65		M	SIMVA	A	1	1	1	1	28.1	5.5	8.5	88	12.8	38	3	1.53	1.19	1.11	2.52
14	2	69	2191	M	ATORVA	A + C	1	1	0	1	30.4	5.4	5.8	129	2.2	12	4.4	1.59	1.65	2.03	2.67
15	2	83	5	M	ATORVA	A	1	1	0	0	22.5	9	16.5	410	0.6		5.9	1.72	1.2	3.92	4.92
16	3	71	487	F	ATORVA	C	1	1	1	0	31.2	7.4	8.9	89	0.9	25	3.2	0.91	1.27	1.52	2.52
17	1	77	61	M	SIMVA	C	0	0	0	1	30.6	5.8	4.5	103	10.1	7	3.6	1.14	0.83	2.25	4.34
18	3	66	242	M	ATORVA	C	1	1	0	0	30.5	8.6	5.9	95	0.6	7	3.7	1.16	1.09	2.08	3.39
19	4	53		F	0	0	0	0	0	0	19.8	5.4	4.2	69	0.2	8	4.2	0.8	1.64	2.2	2.56
20	0	69	117	M	ATORVA	W	1	1	0	0	33	10.1	6.5	86	8.4	5	3.3	1.2	1.04	1.71	3.17
21	1	75	3	F	ATORVA	A	1	1	0	1	20.5	5.3	4.7	63	0.2	8	4.8	0.73	1.46	3.01	3.29
22	1	84	125	M	SIMVA	A	1	0	0	1	23.6	7.7	6.8	94	3.6	14	2.9	0.77	1.14	1.41	2.54

No.	Group	Age	Days since event	Sex	Statin	Anti-Platelet therapy	HTN	IHD	DM	Smoke	BMI	WCC	Ur	Cr	CRP	ESR	CHOL	TRIG	HDL	LDL	CHOL:HDL
23	1	81	3	M	SIMVA	A	1	0	0	1	18.7	7.6	7.7	128			3.89			2.19	
24	2	42	4	M	0	W	1	0	0	0	29.4	10.1	6.2	112	0.5	4	3.8	0.89	1.23	2.17	3.09
25	2	55	5	M	ATORVA	A	1	0	1	0	24.2	7.9	11.5	139	8.4	17	4	1.38	1	2.37	4
26	4	51		M	0	0	0	0	0	1		8.6	7	75	0.6	5	5.8	1.29	1.35	3.86	4.3
27	2	76	2	F	ATORVA	A + C	1	0	1	0	22	6.1	4.2	61	0.4	8	2.9	0.78	0.85	1.7	3.41
28	2	58	3	M	0	A	1	0	0	1	20.5	5.9	2.2	64	7.1	5	4	1.29	1.13	2.28	3.54
29	1	53	13	M	SIMVA	A	0	0	0	1	30.2	10.3	8.9	69	1.9	3	5	5.53	0.74	HIGH	6.76
30	4	62		F	0	A	1	1	0	0	24.2	5.7	6	63	0.7		5.8	1.24			
31	1	59	0	M	0	0	0	0	0	0	20.4	9.7	5	97	0.7		6.6	0.88	1.49	4.71	4.43
32	1	78	14	F	0	A + C	0	0	0	1	18.5	6.8	8.1	95							
33	2	73	1	F	0	0	1	0	0	0	23.6	7.2	10.1	116	3.5	8	5.1	0.83	1.82	2.9	2.8
34	2	55	4	F	ATORVA	A	0	0	0	1	28.6	7.2	4.8	58	4.9	18	4.7	0.68	1.35	3.04	3.48
35	2	79	0	F	SIMVA	A	1	0	1	0	33.9	6.5	9.3	100			4.1	0.75			
36	4	46		M	0	0	1	0	0	0	29.6	5.2	5.3	142	3.3	8	7.2	1.1	1.1	5.6	6.55
37	1	70	5	M	ATORVA	A	0	0	0	1		7	6.9	73	0.9	7	5.6	2.29	1.22	3.34	4.59
38	1	68	4	M	ATORVA	A	1	0	0	1	27.5	4.4	5.5	79	3.3	8	5.5	1.05	1.51	3.51	3.64
39	1	82	24	M	SIMVA	C	1	1	0	1	26.7	8.2	6.6	81	159.8	66	3.6	1.2	0.93	2.12	3.87
40	4	53		F	0	0	0	0	0	0	20.6	3.9	4.6	66	2	29	6.4	1.04	1.6	4.33	4
41	2	59	10	F	0	A	1	0	0	0	27.9	7.4	4	88	2.6	7	6.9	1.05	1.42	5	4.86
42	0	90	2	M	0	A + C	0	0	0	1	23.5	7.3	4.4	67	0.5	34	4.1	0.83	1.38	2.34	2.97
43	3	67		M	SIMVA	C	1	1	0	1	24.3	6	5.9	95	0.5		3.6	1.06	1.51	1.61	2.38
44	1	86	16	M	0	A	0	0	0	0	22.5	6.1	5.7	68	3.4	9	5.4	0.53	1.85	3.31	2.92
45	1	85	0	M	0	0	1	0	1	1	26	8.2	8.3	106	1.9		5.2	1.59	1.28	3.2	4.06
46	4	59		M	0	A	0	0	0	0	20.8	7.8	5.3	74	0.4	8	5.9	1.26	1.33	4	4.44
47	3	74		F	ATORVA	A	1	0	1	1	19.8	8.7	6.4	95	1	30	2.6	1.02	1.17	0.97	2.22
48	3	76		M	SIMVA	W	0	0	0	0	24	7.8	5.7	80		5	3.7	1.44	1.41	1.64	2.62
49	1	83	182	M	SIMVA	C	0	0	1	1	27	9.8	4.5	79	3.2	11	3.9	1.13	1.28	2.11	3.05
50	1	73		F	ATORVA	A + C	1	1	0			6.9	5.1	86	0.4	24	5.3	2.17	1.11	3.2	4.77

No.	Group	Age	Days since event	Sex	Statin	Anti-Platelet therapy	HTN	IHD	DM	Smoke	BMI	WCC	Ur	Cr	CRP	ESR	CHOL	TRIG	HDL	LDL	CHOL:HDL
51	1	67	2	M	ATORVA	A	1	1	1	1	26.9	9.7	4.7	97	2.3	13	6.3	1.98	1.07	4.33	5.89
52	0	61	31	M	ATORVA	W	1	1	0	0	36.3	11.6	4.7	78	18.9	5	6.4	1.25	1.21	4.62	5.29
53	0	77	4	M	ATORVA	A	1	0	1	1	26.1	4.7	11.4	134	4.8		5	1.63	0.99	3.27	5.05
54	2	82	4	M	ATORVA	A	1	0	1	0	26.9	4.5	5.8	65	2.1	5	3.6	1.03	1.07	2.06	3.36
55	1	62	5	F	ATORVA	A	1	1	1	1	22.9	6.5	2.3	69	0.4	15	3	2.54	0.87	0.98	3.45
56	3	88		M	ATORVA	C	1	1	0	1	24.5	8.6	10.2	152	13.8	34	3.9	1.03	1.03	2.4	3.79
57	3	79		M	0	0	0	0	0	1	24.7	6.2	6.7	98	1.5	8	4.7	2.13	1.4	2.33	3.36
58	1	80	2	M	ATORVA	A	1	1	1	1	28.3	4.7	10.1	139	0.9	18	2.8	1.09	1.05	1.25	2.67
59	1	81	7	M	SIMVA	A	1	0	0	0	30.3	5.1	5.3	86	1.3	7	2.8	1.05	0.9	1.42	3.11
60	1	52	0	M	SIMVA	A	1	0	1	1	22.4	8.4	1.8	56	0.1	8	2.9	1.02	1.48	0.96	1.96
61	4	65	1096	M	0	C	1	0	0	1	24.6	10.9	7	107	1.4	14	3.8	0.9	1.23	2.16	3.09
62	3	68	278	F	ATORVA	C	0	0	0	1	20.8	5.4	7	64	0.4	5	5.3	0.92	1.57	3.31	3.38
63	4	62		F	SIMVA	0	1	0	0	1		7.1	3.7	62	1.8	32	4.9	0.98	2.05	2.4	2.39
64	1	66	2	F	0	A	1	0	0	1	32.2	13.2	11.4	130	2.8		5.6	1.83	1.32	3.45	4.24
65	1	86	2	F	0	A	1	0	0	1	28.6	9.9	8.4	106	4.2	21	7.6	1.4	1.86	5.1	4.09
66	1	62	62	M	ATORVA	A + C	1	1	0	1	24.7	11.1	4.1	67	1						
67	2	65	2	M	ATORVA	A + C	1	1	0	1	24.9	5.2	6	87	1	16	3.3	0.84	1.02	1.9	3.24
68	3	51		M	ATORVA	A + C	1	0	0	1	35.9	9.7	5.6	80	3	8	4	3.61	0.79	1.57	5.06
69	1	70	72	M	ATORVA	0	1	0	0	1	26.8	14.8	6.8	115	9.5	24	4	1.53	0.9	2.4	4.44
70	2	76	2	M	SIMVA	A	1	0	1	1	30.8	9.1	7.8	102	3.8		4.7	2.29	1.1	2.56	4.27
71	2	53	1	F	SIMVA	A	1	0	1	1	41.8	7.8	16.5	132	7.1	15	5.5	2.78	1.01	3.23	5.45
72	2	76	6	F	ATORVA	A	1	0	0	1	29	8.4	8.8	73	3.8	25	6.7	1.06	1.36	4.86	4.93
73	2	72	67	M	ATORVA	A + C	0	0	0	1	22.5	10.1	4.4	108	82.4	46	4.1	1.05	0.75	2.87	5.47
74	3	68	424	F	0	C	1	1	1	1	27.8	10.8	4.1	70	10	33	6.1	3.15	1.13	3.54	5.4
75	1	54	3	M	SIMVA	C	1	1	0	1	29.5	8.2	6	91	2.5	5	3.1	1.94	1.06	1.16	2.92
76	1	77	2	M	0	A	1	1	0	1	32.1	6.1	5.3	94	1.8	5	3.6	2.41	0.67	1.83	5.37
77	4	57		F	0	0	1	0	0	0	24.9	5.1	4.5	66	0.3	9	6.5	1.08	1.79	4.22	3.63
78	1	65	7	M	ATORVA	A + LMWH	1		0	1	21.7	5.1	10	107	0.9	10	5.7	1.19	1.28	3.88	4.45

No.	Group	Age	Days since event	Sex	Statin	Anti-Platelet therapy	HTN	IHD	DM	Smoke	BMI	WCC	Ur	Cr	CRP	ESR	CHOL	TRIG	HDL	LDL	CHOL:HDL
79	1	65	9	F	ATORVA	A	0	0	0	0	30.1	5.8	3.8	74	1.9		3.8	0.95	1.09	2.28	3.49
80	1	55	1	M	0	A	0	0	0	1	27.9	6	5.4	73	4.1	8	6.5	4.9	1.22		5.33
81	3	76	912	F	SIMVA	C	1	0	0	0	26.2	5.8	6.7	90	0.6	12	4	1.25	1.4	2.03	2.86
82	3	72	273	M	ATORVA	C	1	1	1	1	26.7	10.4	4.5	91	0.2	24	3.1	0.91	1.01	1.68	3.07
83	3	76		M	ATORVA	C	1	1	0	0	23.2	5.3	5.2	73	0.7	5	4.2	0.63	1.4	2.51	3
84	4	71		M	0	A	1	0	0	0	30.9	7.4	3.9	68	2.8	9	6.1	3.28	0.74	3.87	8.24
85	1	80	3	F	0	A	0	0	0	1	18.6	9.9	6.4	103	1.6	28	6.9	1.3	1.42	4.89	4.86
86	1	85	2	F	0	0	1	0	0	1	28.5	9.1	3.8	68	19.2	14	5.3	0.95	1.54	3.33	3.44
87																					
88	1	81	8	M	SIMVA	C	1	0	1	1	19.9	4.9	7.3	91	0.5	7	3.9	0.94	1.51	1.96	2.58
89	3	75		F	ATORVA	A + LMWH	1	1	1	1	32	12.5	5.3	67	16.7	44	4.2	2.45	0.85	2.24	4.94
90	4	67		M	SIMVA	A	0	0	0	1	25.1	7.3	5.4	86	3	5	4.9	2.14	0.97	2.96	5.05
91	3	66	1096	M	ATORVA	A	1	1	1	0	32.1	10.1	7.5	103	0.5	20	3.1	1.3	0.79	1.72	3.92
92	3	72	730	M	SIMVA	C	1	1	1	1	30.9	6.1	6.8	74	0.4	8	3.17	1.48	1.01	1.52	3.17
93	1	42	2	M	ATORVA	C	1	0	0	1											
94	1	66	5	M	SIMVA	A	1	0	0	1	24.2	9.1	5.4	75	25	51	4.1	1.78	0.75	2.54	5.47
95	1	79	115	F	0	C	1	0	0	1	30.1	8	5.2	59	2.7	34	6.4	1.06	1.52	4.4	4.21
96	3	65		M	ROSUVA	A	1	0	1	1	35.3	7.4	4.8	63	0.9	7	3.7	1.28	0.87	2.25	4.25
97	3	81	2191	F	SIMVA	A	1	0	0	1	19.9	6.4	5.7	65		6	4.6	1.76	1.28	2.52	3.59
98	1	71	3	M	SIMVA	A + C	1	0	0	1	30.4	11.4	7.3	110	11.6	14	4.1	1.82	1.15	2.12	3.57
99	3	64	1826	M	ATORVA	W	1	1	0	1	22.5	3.9	5.3	72	0.7	18	3.4	0.53	1.47	1.69	2.31
100	1	78	5	F	1	C	1	0	0	0	33.6	8	4.7	66	18.6		4.3	1.83	1.49	1.98	2.89
101	1	78	6	M	ATORVA	A + C	1	1	0	1	24.6	9.7	9.3	139	18	72	3.7	0.99	0.91	2.34	4.07
102	3	68		F	ATORVA	A	1	0	0	1	29.1	9.5	7.4	82	7.2	38	4.1	2.33	1.34	1.7	3.06
103	3	47		M	PRAVA	A	1	0	0	1	28.3	8.4	17.5	367	5.2						
104	3	68	0	M	ATORVA	A + C	1	0	1	1											
105	3	66		M	ROSUVA	A + C	1	0	1	1	35.3	5.6	3.9	65	1.2						
106	3	73	1034	M	SIMVA	C	1	1	1	1	29.8	6.1	6.8	74	0.4	8	3.2	1.48	1.01	1.52	3.17

No.	Group	Age	Days since event	Sex	Statin	Anti-Platelet therapy	HTN	IHD	DM	Smoke	BMI	WCC	Ur	Cr	CRP	ESR	CHOL	TRIG	HDL	LDL	CHOL:HDL
A1	5	82		F	0	0	0	0	0	1	19.9	6.7	7.1	90	2.9	7	4.3	0.98	1.37	2.48	3.14
A2	5	75		M	SIMVA	0	0	0	0	1	27.1	6.3	11	162	41.1	89	3.6	1.01	1.06	2.08	3.4
A3	5	81		F	0	0	0	0	0	1	23.7	13.7	8.7	89	215		2.6	0.95	0.79	1.38	3.29
A4	5	79		M	SIMVA	A	1	1	1	0	23.6	9.6	15.4	183	4.7						
A5	5	83		F	0	0	1	1	0	1	26.2	8.4	8.9	100	20.1	35	5.3	2.08	0.94	3.41	5.64
A6	5	70		M	SIMVA	A	1	1	0		35.8	7.2	6.7	124	7.7						
A7	5	73		M	1	A	1	1	0	1	27.5	9.5	6.7	102	15.4	27	2.6	1.12	0.64	1.45	4.06
A8	5	65		M	ROSUVA	A + C + W	1	1	1	1	40	7.4	7.7	109	3.1	60	2.3	1.71	0.58	0.94	3.97
A9	5	67		M	SIMVA	A	0	0	1	1	31	6.2	8.1	95	3.6		3.9	1.06	1.3	2.12	3
A10	5	70		M	ATORVA	0	0	0	0	1	24.2	11.8	4.9	84	7	15	4.6	2.15	0.84	2.78	5.48
A11	5	70		M	SIMVA	A	1	0	0	1	28.9	8.9	6.4	74	1	24	4.1	1.84	1.04	2.22	3.94
A12	5	64		M	ATORVA	W	1	0	0		26	9.4	2.7	67	2.1	5	6.2	2.01	1.25	4.04	4.96
A13	5	64		M	1	0	1	0	1	1	43.6	6.4	4.1	69	3.2	5	4	0.8	1.3	2.34	3.08
A14	5	66		M	SIMVA	0	0	0	0	1	27.5	5.8	7.1	89	1.3	8	4	0.84	1.54	2.08	2.6
A15	5	65		M	ATORVA	0	1	0	0	0	33	7.9	4.7	77	0.8	5	5.1	3.05	1.28	2.43	3.98
A16	5	64		M	0	0	0	0	0	1	31.3	7.3	6.1	106	5.2	5	5.7	2	0.93	3.86	6.13
A17	5	65		M	SIMVA	0	1	0	0	1	26.6	6.3	7.1	75	1.4	5	4.3	1.02	1.55	2.29	2.77
A18	5	65		M	ATORVA	A	1	0	1	1	25.6	8.4	8.4	92	9.3	19	2.9	1.18	0.7	1.66	4.14
P1	6	65		M	ROSUVA	A	1	1	0	1	17.5	6.1	4.3	61	2	26	4.8	1.44	1.11	3.04	4.32
P2	6	56		M	ATORVA	A	1	1	0	1	26.7	9.3	3.6	77	1.9	7	5.3	1.6	1.09	3.48	4.86
P3	6	72		F	ATORVA	A	1	0	0	1	26.6	7.5	4	57	0.9	7	4.5	0.93	1.58	2.5	2.85
P4	6	61		M	1	A	1	0	0	1	33.2	10.4	8.8	81	1.3	11	3.9	0.85	1.22	2.29	3.2
P5	6	79		M	ATORVA	A	1	1	0	1	29.7	5.9	8.1	120	1.1	7	5.2	1.21	1.39	3.26	3.74
P6	6	66		M	ATORVA	A	1	0	0	1	28.4	7.6	7	80	1.7	17	4.2	2.92	1.07	1.8	3.93
P7	6	65		M	SIMVA	A	1	0	0	1	30.4	5.7	4.9	72	3.4	10	9.9	1.65	1.64	7.51	6.04
P8	6	71		M	0	A	1	0	0	1	24.5	9.2	6.7	159	2.4	4	4.9	2.17	1.48	2.43	3.31
P9	6	50		F	0	C	1	0	0	1	19.9	7.4	8.3	172	5.3	34	5.1	2.95	0.74	3.02	6.89
P10	6	65		M	ATORVA	A	1	1	1	1	27.1	9.7	4.9	106	2.2	18	3.9	0.95	1.03	2.44	3.79
P11	6			M	SIMVA	A	1	1	1	1	18.2	8.6	4.3	82	2.6	4	4.5	0.75	0.93	3.23	4.84

No.	Group	Age	Days since event	Sex	Statin	Anti-Platelet therapy	HTN	IHD	DM	Smoke	BMI	WCC	Ur	Cr	CRP	ESR	CHOL	TRIG	HDL	LDL	CHOL:HDL
P12	6	85		M	0	0	1	0	0	1	24.2	8.3	7	97	4.3	5	4.4	0.54	1.99	2.16	2.21
P13	6	64		F	ATORVA	A	1	1	1	1	36.8	5.6	9	100	0.4	37	5.1	6.26	0.78	>4.5	6.54
P14	6	67		M	0	A	0	0	0	1	26	8.6	4	59	15.4	10	5.2	0.84	1.22	3.6	4.26

Table XI: Participant metadata

Appendix 3: Mass spectrometry checklist for data acquisition

- Check the lock mass spectrum: ensure ion is present and appropriately assigned
- Check disc space is adequate
- Check mobile phase, lock mass, seal wash volumes and top up if needed
- Check every sample and make sure it has been appropriately injected and run
- Check QC samples and assess reproducibility (RT, intensity, mass accuracy)
- Run QC-dilutions and preparation blanks
- Consider running an MSE and DDA
- Make arrangements in order to have the instrument running samples constantly for each of the polarity modes (changing the plates, logging and queuing the samples)
- Check and make sure the waste bottle is not full

Appendix 4: Grants and prizes awarded

2017	New York Venous Symposium Fellowship	**£1500**
2016	UK Stroke Forum Prize for Translational Research	**£100**
2016	First Prize: London Surgical Symposium	**£150**
2015	Imperial College Private Healthcare	**£177,000**
2015	Imperial College Private Healthcare	**£177,000**
2015	Mason Medical Research Trust	**£10,000**
2015	New York Venous Symposium Fellowship	**£1500**
2015	Presentation Prize: MMVD, Nice	**£400**
2015	The Graham-Dixon Prize for Surgery	**£500**
2015	The Graham-Dixon Trust Research Grant	**£5,000**
2015	First Prize: 3MT Competition, Imperial College	**£100**
2015	SARS Bursary	**£100**
2014	Royal College of Surgeons/Dunhill Fellowship	**£110,767**
2014	Anglo Arabian Healthcare Educational Award	**£1,500**
2014	The Rosetrees Trust	**£10,000**
2014	The Graham-Dixon Trust Research Grant	**£3,026**
2013	Circulation Foundation Surgeon Scientist Award	**£55,000**
2013	The Graham-Dixon Trust Research Grant	**£7,500**

Appendix 5: Publications achieved during PhD

The application of metabolic profiling to aneurysm research (Systematic Review)

Qureshi MI, Grecco M, Vorkas P, Holmes E, Davies AH

Journal of Proteome Research. 2017 Mar 13 [epub ahead of print]

Post thrombotic syndrome in children

Vosicka K, **Qureshi MI**, Shapiro S, Lim CS, Davies AH

Phlebology. 2017 Jan 1:268355516686597 [epub ahead of print]

History of the definition of stroke

Coupland A, Thapar A, **Qureshi MI**, Jenkins IH, Davies AH

Journal of the Royal Society of Medicine. 2017;110(1):9-12

Lessons from metabonomics on the neurobiology of stroke

Qureshi MI, Vorkas P, Coupland A, Jenkins H, Holmes E, Davies AH

Neuroscientist. 2016 Oct 1:1073858416673327 [epub ahead of print]

Ambulance smartphone tool for field triage of ruptured aortic aneurysms (FILTR): study protocol for a prospective observational validation of diagnostic accuracy

Lewis TL, Fothergill RT, **Aneurysm-FILTR Study Group**, Karthikesalingam A

BMJ Open. 2016 Oct 24;6(10):e011308

Carotid intervention following TIA: What are we waiting for?

Qureshi MI, Davies AH

Vascular. 2016;24(5):556-8

Temporal trends in the safety of carotid endarterectomy in asymptomatic patients (WriteClick Correspondence).

Munster AB, Franchini AJ, **Qureshi MI**, Thapar A, Davies AH

Neurology. 2016 Jan;86(3):312-3

Thromboprophylaxis for superficial venous intervention.

Qureshi MI, Davies AH

Phlebology. 2016; 31(2):77-80

Temporal trends in the safety of asymptomatic carotid endarterectomy

Munster A, Franchini A, **Qureshi MI**, Thapar A, Davies AH

Neurology. 2015 Jul 28;85(4):365-72

The role of cost-effectiveness for vascular surgery service provision in the UK

Mandavia R, Dharmarajah B, **Qureshi MI,** Davies AH

J Vasc Surg. 2015 May;61(5):1331-1339

The safety of carotid intervention post-thrombolysis

Mandavia R, **Qureshi MI**, Dharmarajah B, Davies AH

Eur J Vasc Endovasc Surg. 2014 Nov;48(5):505-12

A study to evaluate patterns of superficial venous reflux in primary chronic venous disease

Qureshi MI, Gohel M, Wing L, Macdonald A, Lim CS, Ellis M, Franklin IJ, Davies AH

Phlebology. 2015 Aug;30(7):455-61

Patterns of short saphenous vein incompetence

Qureshi MI, Lane TRA, Moore HM, Franklin IJ, Davies AH

Phlebology. 2013 Mar;28 Suppl 1:47-50

Appendix 6: National and international presentations

ORAL

Biomarker Research in Thromboembolic Stroke

Qureshi MI, Vorkas P, Kaluarachchi M, Holmes E, Davies AH

European Stroke Conference. Berlin; May 2017

Biomarker Research in Thromboembolic Stroke

Qureshi MI, Vorkas P, Kaluarachchi M, Holmes E, Davies AH

Charing Cross Symposium. London; Apr 2017

Temporal Trends in Perioperative Safety of Carotid Endovascular Treatment in Average Risk Symptomatic Patients

Masood M, Thapar A, Qureshi MI, Davies AH. ASGBI

Bournmouth; May 2017

Temporal Trends in Perioperative Safety of Carotid Endovascular Treatment in Average Risk Symptomatic Patients

Masood M, Thapar A, Qureshi MI, Davies AH

Association of Surgeons in Training. Bournmouth; Mar 2017

Biomarker Research in Thromboembolic Stroke

Qureshi MI, Vorkas P, Kaluarachchi M, Holmes E, Davies AH

Society of Academic & Research Surgeons. Dublin; Jan 2017

Biomarker Research in Thromboembolic Stroke

Qureshi MI, Vorkas P, Holmes E, Davies AH

Vascular Society of Great Britain and Ireland ASM. Manchester; Nov 2016

Invited presentation: Prevention is key for any public health system

Qureshi MI

National Congress of the Portuguese Society of Cardiothoracic and Vascular Surgery. Algarve, Portugal; Nov 2016

Invited presentation: Limits for EVAR in the elderly

Qureshi MI. National Congress of the Portuguese Society of Cardiothoracic and Vascular Surgery. Algarve, Portugal; Nov 2016

Metabolic profiling of aneurysmal disease

Greco M, **Qureshi MI**, Vorkas P, Holmes E, Davies AH

Italian Congress of Vascular and Endovascular Surgery. Rome; Oct 2016

Biomarker Research in Thromboembolic Stroke

Qureshi MI, Vorkas P, Holmes E, Davies AH

The Surgical Symposium. London; Sept 2016

Invited presentation: Carotid atherosclerosis

Qureshi MI. Royal Society of Medicine. London; Feb 2016

Invited presentation: Delay in carotid endarterectomy following TIA

Qureshi MI. National Congress of the Portuguese Society of Cardiothoracic and Vascular Surgery. Algarve, Portugal; Nov 2015

Invited presentation: Circulation Foundation: The researcher's viewpoint

Qureshi MI. VSGBI Annual Conference. Bournemouth; Nov 2015

Core Training for Surgery

Qureshi MI. Royal College of Surgeons Career Day. London; Oct 2015

Cost Effectiveness of Vascular Surgery

Qureshi MI, Mandavia R, Dharmarajah B, Davies AH. International Healthcare Conference. Oxford; June 2015

Thromboprophylaxis for superficial venous intervention.

Qureshi MI, Davies AH. MMVD. Nice; June 2015

Invited Presentation: 3 Minute BRUITS

Qureshi MI. Imperial College Science Festival (free admission to public). London; May 2015

Invited Lecture: Carotid Atherosclerosis

Qureshi MI. The Royal Society of Medicine. London; February 2015

Biomarker Research in Thromboembolic Stroke

Qureshi MI, Vorkas P, Shalhoub J, Anwar M, Davies AH. Society of Academic & Research Surgeons (SARS). Durham; January 2015

Temporal Trends in the Safety of Asymptomatic Carotid Endarterectomy

Munster A, Franchini A, **Qureshi MI**, Thapar A, Davies AH. Society of Academic & Research Surgeons (SARS). Durham; January 2015

Post Thrombotic Syndrome in Children

Qureshi MI, Vosicka K, Lim CS, Davies AH. Society of Academic & Research Surgeons (SARS). Durham; January 2015

Invited Lecture: So You Want To Be A Surgeon

Qureshi MI. Association of Surgeons in Training Foundation Skills in Surgery. Imperial College London. London; November 2014

Biomarker Research in Thromboembolic Stroke

Qureshi MI, Vorkas P, Shalhoub J, Anwar M, Davies AH. Royal Society of Medicine. London; Oct 2014 . *Shortlisted for the MIA Prize.*

Post Thrombotic Syndrome in Children

Qureshi MI, Vosicka K, Lim CS, Davies AH. Royal Society of Medicine. London; Oct 2014. *Shortlisted for the Norman Tanner Prize.*

Headline Lecture: Evidence Based Management of Carotid Atherosclerosis

Qureshi MI. Anglo Arabia Healthcare. Dubai; Apr 2014

Headline Lecture: Biomarkers & Microbiomes in Atherosclerosis

Qureshi MI. Anglo Arabia Healthcare. Ajman; Apr 2014

Headline Lecture: The Origin of Venous Reflux

Qureshi MI. Anglo Arabia Healthcare. Abu Dhabi; Apr 2014

Temporal Trends in the Peri-Operative Safety of Carotid Endarterectomy for Asymptomatic Stenosis.

Munster A, Thapar A, Rouhani M, **Qureshi MI**, Davies AH.

European Society for Cardiovascular and Endovascular Surgery. Nice, France; April 2014

Safety of Carotid Surgery and Intervention Following Thrombolysis in Acute Ischaemic Stroke

Mandavia R, **Qureshi MI,** Davies AH.

Norman Tanner Prize Meeting, Royal Society of Medicine. London; Oct 2013

Carotid Intervention Post Thrombolysis

Mandavia R, **Qureshi MI,** Davies AH.

World Online Conference; Nov 2013

The Anatomy of Venous Reflux

Qureshi MI, Moore HM, Ellis M, Franklin IJ, Davies AH.

Americal College of Phlebology & International Union of Phlebology. Boston; Sept 2013

POSTER

Biomarker Research in Thromboembolic Stroke

Qureshi MI, Vorkas P, Kaluarachchi M, Holmes E, Davies AH

UK Stroke Forum, Liverpool; Nov 2016

(Winner of the UK Stroke Forum Prize for Translational Research)

Metabolic Profiling of Ischaemic Stroke

Qureshi MI, Greco M, Vorkas P, Holmes E, Davies AH

UK Stroke Forum. Liverpool; Nov 2016

Metabolic Profiling of Aneurysmal Disease

Greco M, **Qureshi MI**, Vorkas P, Holmes E, Davies AH

IMAAD. Brussels, Belgium; Sept 2016

Paediatric Post-Thrombotic Syndrome

Qureshi MI, Vosicka K, Shapiro S, Lim CS, Davies AH

6th Annual Venous Symposium. New York, NY, USA; April 2015

The Additional Benefit of Graduated Compression Stockings to Pharmacological Thromboprophylaxis in the Prevention of VTE in

Surgical Patients

Qureshi MI, Mandavia R, Shalhoub J, Davies AH

Controversies & Updates in Vascular Surgery. Paris, France; January 2015

The Role of Cost-Effectiveness for Service Provision within Vascular Surgery

Qureshi MI, Mandavia R, Dharmarajah B, Davies AH

Controversies & Updates in Vascular Surgery. Paris, France; January 2015

Safety of Carotid Intervention Following Thrombolysis in Acute Ischaemic Stroke

Qureshi MI, Mandavia R, Dharmarajah B, Head K, Davies AH

Controversies & Updates in Vascular Surgery. Paris, France; January 2015

Post Thrombotic Syndrome in Children

Qureshi MI, Vosicka K, Busuttil A, Shapiro S, Lim CS, Davies AH

The American Venous Forum 2015. Palm Springs, USA; February 2015.

Post Thrombotic Syndrome in Children

Qureshi MI, Vosicka K, Busuttil A, Sharipo S, Lim CS, Davies AH

SARS 2015 (Poster of Distinction). Durham; February 2015.

Temporal trends in perioperative safety of carotid endarterectomy for asymptomatic stenosis

Munster A, Franchini A, **Qureshi MI**, Thapar A, Davies AH

British Society of Endovascular Therapy 2014. Warwickshire; June 2014.

The Safety of Carotid Intervention Post Thrombolysis.

Mandavia R, **Qureshi MI**, Dharmarajah B, Davies AH.

Society of Academic & Research Surgery. Cambridge, UK; Jan 2014

Temporal Trends in Peri-Operative Safety of Carotid Endarterectomy for Asymptomatic Stenosis.

Munster A, Thapar A, Rouhani M, **Qureshi MI**, Davies AH.

United Kingdom Stroke Forum. Harrogate, UK; Dec 2013

Dynamic Microbubble Contrast-Enhanced Ultrasound to Assess Human Carotid Plaque Biology

Qureshi MI, Shalhoub J, Thapar A, Davies AH.

Rosetrees Trust 25 Years. UCL, London; Oct 2012

I shall be telling this with a sigh
Somewhere ages and ages hence
Two roads diverged in a wood, and I
I took the one less travelled by
And that has made all of the difference

The Road Less Travelled, Robert Frost