Metabolic Biomarker Responses in Acute Cerebral Events

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

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'Ease, is a greater threat to progress than hardship'

D. Washington

ABSTRACT

Aims

The aims of this study were to identify a potential blood biomarker for acute stroke and additionally to identify biomarkers capable of differentiating hyper-acute ischaemic from haemorrhagic stroke using metabonomic techniques.

Methodology

Following ethical approval participants were recruited from the hyper-acute stroke unit at Charing Cross Hospital and acute blood samples taken from patients who were suspected of having an acute stroke. Serum was extracted and frozen prior to MS and NMR analysis. Sub-acute TIA patients were used as a comparative group and patients with known atherosclerotic disease as a non-acute control group. A total of 90 participants were recruited for analysis.

Positive and negative mode reverse phase UPLC-MS and additionally ^{1}H-NMR spectroscopy were used to analyse prepared serum samples. Modelling was performed using OPLS-DA and CA-PLS techniques where appropriate following permutation analysis to detect discriminatory metabolites between participant groups.

Results

Positive mode mass spectrometry detected metabolites that could differentiate between participants who had suffered an acute cerebral ischaemic event (inclusive of stroke and acute TIA) and those participants that were known to have established atherosclerotic disease. The metabolites detected include phosphatidylcholines, sphingomyelins and a ganglioside. Unfortunately, due to a relatively small sample size the CA-PLS false discovery rate analysis found the initial results to lack statistical

significance (Q value = 0.95). It was not possible to metabolically differentiate between acute ischaemic and haemorrhagic strokes.

Conclusion

Despite the relatively small sample size leading to a confirmed false discovery rate analysis the initial findings of elevated phosphatidylcholines, sphingomyelins and the peripheral detection of a ganglioside in relation to acute cerebral ischaemia is promising and follows a trend in the published literature. Further studies with a larger sample size, rigorous follow up and temporal trend analysis using dedicated lipidomic techniques may find a sensitive diagnostic serological biomarker for hyper-acute stroke and transient ischaemic attack.

STATEMENT OF ORIGINALITY

I hereby declare that the work presented in this thesis is original and of the author. Where diagrams are reproduced from the work of others, they have been made available to the public, are freely available and the original source is clearly stated.

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ACKNOWLEDGMENTS

The completion of this thesis is worth so much more than the sum of its parts. It has been borne through the ebb and flow of a complex web of human emotions – not all of them positive! It has spanned significant events in my personal and professional life: the continual flourishing of my daughter, Theadora, who was only 20 months old when my PhD began and not even born when the initial round of funding applications were (unsuccessfully) applied for. During the course of writing up this thesis my son, Raphael, has been born and is showing himself to be as mischievously inquisitive as I always hoped he would be. This thesis has also inspired a career change from surgery to (interventional) radiology – and the pursuit of neurointerventional radiology – with the view of continuing acute stroke research.

Above all else this thesis would not have reached an end-point were it not for the continual support of my wife Yezenash Ayalew, who so often shines a bright light on how best to proceed through difficult times and encourages me to push the limits. At times, this PhD, this thesis, has pressed hard on those limits. Without you, Yezenash, I often wouldn't realise my own potential or attempt to reach it. I owe you the biggest thanks.

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To my parents, for their continual support and for instilling within me the values of hard work, honesty and the importance of family.

ABBREVIATIONS

AHA	American Heart Association
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole
ASA	American Stroke Association
ATP	Adenosine triphosphate
BBB	Blood-brain barrier
CA-PLS	Covariate-Adjustment Projection to Latent Structures
CAM	Cell Adhesion Molecules
CBF	Cerebral Blood Flow
CBV	Cerebral Blood Volume
CSF	Cerebrospinal fluid
CT	Computed Tomography
CW	Continuous wave
DBS	Dried blood spot
DCE-CT	Dynamic contrast enhanced-Computed Tomography
DModX	Distance to Model
ECM	Extra Cellular Matrix
ER	Endoplasmic reticulum
ESI	Electrospray ionisation
ESO	European Stroke Organisation
FID	Free induction decay
FT	Fourier Transform
GFAP	Glial fibrillary acidic protein
HASU	Hyper-acute Stroke Unit
HNE	4-hydroxinonenal
ICH	Intracranial haemorrhage
IMA	Ischaemia modified albumin

ISF	Interstitial fluid
IVH	Intraventricular haemorrhage
LC-MS	Liquid chromatography-mass spectrometry
m/z	Mass-to-charge ratio
MCA	Middle cerebral artery
Mi-RNA	Micro-RNA
MMP	Matrix metallproteinase
MRI	Magnetic Resonance Imaging
MS	Mass spectrometry
MTT	Mean Transit Time
NADH	Nicotinamide adenine dinucleotide + hydrogen
NMDA	N-methyl-D-aspartate
NMR	Nuclear Magnetic Resonance
NO	Nitric Oxide
NSE	Neuron specific enolase
NVU	Neurovascular unit
OPLS-DA	Orthogonal Projections to Latent Structures Discriminant Analysis
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PEMT	Phosphatidylethanolamine N-methyltransferase
PI	Phosphatidylinositol
PUFA	Polyunsaturated Fatty Acids
RF	Radiofrequency
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
SM	Sphingomyelin
TJ	Tight junction
TMS	Tetramethylsilane

TOF	Time of flight
TSP	Trimethylsilylpropanoic
TTP	Time To Peak
U.S.	United States (of America)
UPLC	Ultra-performance liquid chromatography
WHO	World Health Organisation
WSO	World Stroke Organisation

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1 INTRODUCTION

1.1 The Definition of Stroke

"It is impossible to cure a severe attack of apoplexy, and difficult to cure a mild one"

- Hippocratic Aphorism (Clarke, 1963)

Hippocrates could never have imagined the progress made in stroke care since this statement, yet a universal definition of stroke does not exist. The first recorded use of "stroke" as a lay term was in 1599, attributing the sudden onset of symptoms to a "stroke of God's hande" (Pound et al., 1997, Dictionary, 2015). It was not adopted into the medical lexicon of the time and physicians used the term 'apoplexy'; a diagnosis that had been in existence since the Hippocratic writings (Clarke, 1963).

The word 'stroke' is related to the Greek word 'apoplexia' which implies being struck with a deadly blow (Schutta and Howe, 2006), but it would be incorrect to draw direct parallels between our modern concept of stroke and what has been classically referred to as apoplexy.

Apoplexy was an umbrella term, describing a condition in which the patient had a "sudden abolition of all activities of the mind with the preservation…of the pulse and respiration" (Schutta, 2009). Hippocrates describes a patient experiencing sudden pain, losing speech, with a rattle in his throat, urinating without awareness and being unresponsive (Clarke, 1963). These events describe a dramatic pathology and some cases of apoplexy were likely strokes, but the presentation encompassed conditions we now consider 'stroke mimics' such as seizures, migraine and even sudden cardiac death (Leak et al., 2014).

To strengthen the correlation between apoplexy and our modern concept of stroke, the condition has long been associated with paralysis. In the Hippocratic writings the word used to describe such findings was 'paraplexy' and though now obsolete, is understood to be synonymous with what we now term 'paraplegia'(Clarke, 1963). In the 19th century Dr James Copland recognized the intimacy between

apoplexy and 'palsy' (paralysis) stating that they are "so intimately connected... it [is] most difficult, if not altogether impossible, to treat… one apart from the other" (Copland, 1850).

Hippocrates hypothesized that the pathogenesis of 'apoplexy' was linked to humoral theory. He believed that blood (one of the four humors alongside yellow bile, black bile and phlegm) held our spirit or 'vitality'. Consequently, any interference with the flow of vitality to the brain would result in apoplexy.

His hypothesis was supported by his proponent, Galen (born AD 131) (Pound et al., 1997) who also believed that the causes of apoplexy were due to humoral aberrations such as the accumulation of phlegm or black bile in the cerebral ventricles (Schutta, 2009). It was not until the 17th century and the rise of physician-anatomists that this position was challenged.

Among the most important advances in the understanding of stroke were those made by the Swiss physician Johan Jakob Wepfer (1620-1695). Wepfer is credited with being the first to observe that apoplexy was associated with cerebral haemorrhage (Schutta and Howe, 2006). He published the results of four autopsies in his *Historiae Apoplecticorum* (published 1658) (Pearce, 1997) and with a lack of black bile or phlegm in the cerebral ventricles, Galen's hypothesis began to be doubted and the pathogenesis of apoplexy recast in pathological terms.

Over time, the number of autopsies being performed increased and the most significant work published in the 17th century was Theophile Bonet's *Sepulchretum sive Anatomia Practica* (published 1679), of which Section II of Book I is dedicated to apoplexy. At this time, varying causes of apoplexy had begun to be recognised, and though Bonet did not formally categorise aetiologies, the autopsy findings did point to a number of possibilities. It was recognised that apoplexy could be caused by intracranial haemorrhage, tumours and even cerebral abscesses. In Bonet's autopsies, excess fluid, or serum, was also found in the head. Unlike the other causes of apoplexy, the mechanism of this fluid causing disease was more difficult to explain, particularly since it was recognised that hydrocephalics could live for

many years with large amounts of cerebral fluid, without becoming apoplectic (Schutta, 2009). The main hypothesis was that it blocked pores in the brain and hence, the passage of 'spirits' (Schutta and Howe, 2006).

Bonet's *Sepulchretum* was highly regarded, but eventually superseded by the publication of Giovoanni Morgagni's (1682-1771) *De Sedibus et Causis Morborum Per Anatomen Indagatis* in 1761. Morgagni, considered to be the founder of pathology (Schutta, 2009), built on Bonet's work but with fundamental differences: he did not recognise head trauma as a cause of apoplexy (though he acknowledged it could cause an "apoplectic condition") and he divided the causes of apoplexy into 2 principle groups: 'sanguineous' and 'serous'(Schutta, 2009).

The move towards dichotomous causes (sanguineous and serous) was a significant step. The 'sanguineous' form represented intracranial haemorrhage and the excess fluid referred to in the 'serous' form (also found by Bonet) has been postulated as being normal cerebrospinal fluid. The corresponding cerebral infarcts may have been missed, though he did point to abnormalities in the corpus striatum in some cases that may have represented infarcted tissue (Schutta, 2009).

With such observations, apoplexy began to be understood as a predominantly vascular disease, a position strengthened by the discoveries of John Abercrombie (early 19th century) and Rudolf Virchow (early 20th century). The former recognising a link between arterial occlusive disease and areas of cerebral softening (caused by infarctions), with the latter reclassifying the causes of apoplexy as: sanguinea (haemorrhagic) and ischaemica (Virchow's term) (Storey, 2009).

In the 1960s transient ischaemic attacks (TIAs) were considered to be sudden, focal neurological deficits of vascular origin lasting less than 24 hours (an arbitrarily assigned end-point). A stroke was considered to have occurred if a neurological deficit remained for greater than 7 days. Those neurological events that lasted between 24 hours and the 7 day stroke threshold, were classified as a reversible ischaemic neurological deficit – a term now rendered obsolete. Its removal from the clinical

lexicon arose when it was proven that most events lasting 24 hours to 7 days were associated with cerebral infarction (Easton et al., 2009) and thus should carry the diagnosis of stroke. This led to a divergence in the North American and World Health Organisation's view of stroke, one emphasising the evidence of infarction and the other clinical symptoms.

In 1970, the World Health Organisation (WHO) defined stroke as "rapidly developed clinical signs of focal (or global) disturbance of cerebral function, lasting more than 24 hours or leading to death, with no apparent cause other than of vascular origin (Aho et al., 1980)". Although still widely used, the WHO definition relies heavily on clinical symptoms and is now considered outdated by the American Heart Association (AHA) and American Stroke Association (ASA) due to significant advances in the "nature, timing, clinical recognition of stroke and its mimics, and imaging findings that require an updated definition" (Sacco et al., 2013).

There has been a stepwise progression to the endorsement of a new definition of stroke in the United States that began with reclassifying transient ischaemia. TIAs were previously defined as "a sudden, focal neurologic deficit that lasts for less than 24 hours, is presumed to be of vascular origin, and is confined to an area of the brain or eye perfused by a specific artery"(Albers et al., 2002). Due to mounting evidence that the arbitrary 24-hour time period for diagnosing a TIA was inaccurate, with up to 50% showing brain injury on diffusion-weighted magnetic resonance imaging (Easton et al., 2009), in 2002 Albers et al proposed a new definition of TIA (Albers et al., 2002). It stepped away from the assumption that transient neurological deficits could not be accompanied by cerebral infarction.

Subsequently, the Stroke Council of the AHA/ASA removed time as definitional factor and in 2009 endorsed their current definition of TIA: "a transient episode of neurological dysfunction caused by focal brain, spinal cord or retinal ischaemia, without acute infarction" (Easton et al., 2009). The transition to a new definition of TIA that includes radiological exclusion of brain infarction had inherent consequences for the definition of stroke.

In 2013, the AHA/ASA updated their endorsed definition of stroke to one that includes silent infarctions (inclusive of cerebral, spinal and retinal) and silent haemorrhages (Sacco et al., 2013). The 'traditional' clinical definition of stroke is still included by the AHA/ASA, but the inclusion of 'silent' pathology is a significant addition. The rationale behind such a change was to move towards a radiological demonstration (tissue-based definition) of infarction or haemorrhage.

The new American definition is at odds with the definition endorsed by the WHO, European Stroke Organisation (ESO) and the World Stroke Organisation (WSO) none of which consider silent pathology to be equivalent to stroke. The implications of not arriving at a universal definition of stroke are far reaching.

Firstly, the International Classification of Diseases 11th Revision (ICD-11) includes definitions for the first time and continues to be used as a classification system for reporting morbidity and mortality data. The definition of stroke within ICD-11 aligns with the WHO and differs from the definition endorsed by AHA/ASA and therefore survival data, incidence and prevalence rates for stroke in the United States (U.S) may differ dramatically to other countries worldwide. This is especially important in ageing societies as prevalence rates for silent brain infarctions approach 20% in those older than 70 (Fanning et al., 2014).

Secondly, the impact of being diagnosed with a silent stroke on individual health and life insurance premiums will need to be considered in the United States. Silent brain infarctions confer an increased risk of future stroke (Gupta et al., 2016) (WHO definition), but the cumulative risk is perhaps less than the risk of stroke recurrence following the first presentation of stroke (WHO definition)(Mohan et al., 2011), raising the question of whether silent and symptomatic infarctions can be considered the same clinical entity.

Thirdly, surgeon outcome data will also differ across the Atlantic. Silent cerebral infarctions following carotid intervention would be reported as iatrogenic strokes. The synthesis of U.S. trials such as

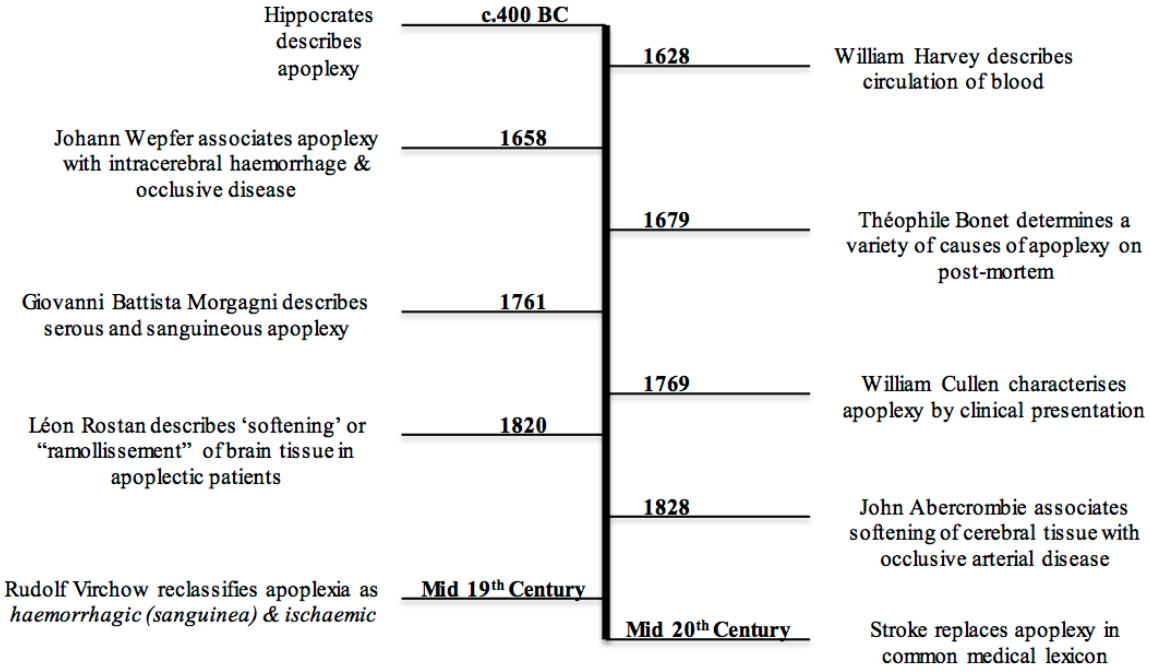
CREST-2 - which compares carotid revascularisation and best medical therapy vs medial therapy alone for asymptomatic carotid atherosclerosis - with European trials will be more difficult as any silent infarction/strokes will have to be removed for parity.

The definition of stroke now endorsed by the AHA/ASA follows in the footsteps of history and places faith in tissue findings reminiscent of the scientific advances made by Wepfer, Bonet and Morgagni. What separates this definition from historical precedent is the inclusion of 'silent' brain, retinal, and spinal infarcts and silent cerebral haemorrhages, thereby removing an association with clearly defined clinical symptoms.

The inclusion of silent pathology within stroke epidemiology has the potential to dramatically alter incidence and prevalence rates in the United States and will likely have an impact on mortality and morbidity data. It will be important in the coming years for international bodies to arrive at a consensus in order to standardise data reporting and research end-points.

For the purpose of this thesis, the WHO definitions of stroke and TIA are used and hence, stroke is considered a clinical syndrome.

Figure 1.1: Historical timeline for the definition of stroke



1.2 Epidemiology

Stroke is the second leading cause of death worldwide after ischaemic heart disease and has remained so for the last 15 years (World Health Organization, 2018). It is the fourth leading cause of death in the United Kingdom using WHO data groupings (Office for National Statistics, 2017). There are more than 100 000 strokes in the United Kingdom (UK) each year, which accounts for one stroke every 5 minutes (Royal College of Physicians, 2016) .

The cost to the UK economy is staggering. Nearly two thirds of all stroke survivors leave hospital with a disability and those that are of working age are up to three times more likely to be unemployed eight years after having a stroke. The societal cost of stroke is approximately £26 billion per annum (Stroke Association, 2018).

The most common cause of stroke is ischaemia (85%) and the remaining 15% are haemorrhagic. Of those people that suffer a haemorrhagic stroke, 1 in 10 will die before reaching hospital (Intercollegiate Stroke Working Party, 2016). Of the ischaemic strokes, approximately 20% are the result of embolism from the carotid artery. The rest have embolic origins in either other large arteries such as the aortic arch or cardiac mural thrombus secondary to atrial fibrillation. A small number of strokes are also secondary to small vessel disease and a smaller number secondary to arterial-venous shunting. For up to a third of patients, the cause of stroke remains unknown despite adequate clinical work up (Nouh et al., 2016).

Moreover, although some anterior circulation strokes (and TIAs) are treated with a carotid endarterectomy - should a causative significant carotid artery stenosis be found – there has been a steady decline in the number of endarterectomies performed with a 25% reduction between 2011 and 2017 (Johal et al., 2019). Despite this, a reduction in stroke prevalence is not observed. The need for further improvements in stroke diagnostics and treatment is as compelling today as it was before the onset of thrombolysis. The personal and societal costs are staggering. Disability burden reduction is paramount.

1.3 Stroke Treatment

1.3.1 Ischaemic stroke

Ischaemic stroke diagnostic and treatment pathways are algorithmic and imperfect. Unlike the diagnosis of myocardial infarction clinical symptoms that correlate with a readily performed and reproducible non-invasive test (ECG) and a rise in a biochemical maker (troponin) does not exist.

Patients are admitted from the community or referred to stroke clinicians from within a secondary care setting with an acute onset of neurological symptoms made famous by the F.A.S.T campaign. That is the onset of facial weakness; arm weakness or speech problems that require the patient or the observer of clinical symptoms to call the emergency services within a short time frame.

Despite the effectiveness of the F.A.S.T campaign the neurological deficits encountered during an acute stroke are not necessarily confined to hemiplegia or profound speech disturbances. More subtle findings such as 'Alien hand' syndrome characterized by involuntary motor disturbances of a hand can also be the sequelae of acute stroke. Complicating matters further are the concepts of 'stroke mimics' and 'stroke chameleons'. Stroke chameleons are acute strokes that do not present with classical stroke symptomatology but the underlying mechanism is cerebral infarction. Stroke mimics are those conditions that present similarly to an acute stroke but have a different underlying pathophysiology. Patients suffering from a condition mimicking a stroke do not require the same acute and time-critical treatment that those with an acute stroke require.

The Gold Standard care is for a patient suspected of having an acute stroke to be admitted to a hyper-acute stroke unit where they can be clinically assessed by a stroke physician with the possibility of being given intravenous thrombolysis if they present within a 4.5 hour time window from symptomatic

onset. And now, in select centres within the United Kingdom, should the patient be found to have a middle cerebral artery occlusion, offered an acute mechanical thrombectomy to remove the occluding clot from the vessel.

Prior to initiating acute ischaemic stroke therapy, it is imperative to exclude the presence of a haemorrhagic stroke. An unenhanced CT scan of the head is performed for this purpose. It may, in addition, display areas of low attenuation or loss of grey-white matter differentiation increasing the positive predictive value of ischaemic stroke but this is not the primary reason for performing a scan. If a patient has a convincing clinical history for acute stroke with no evidence of haemorrhage and they present within the 4.5 hour time window, they are eligible for thrombolysis if they do not have any absolute or relative contraindications.

More recently, convincing evidence for the effectiveness of mechanical thrombectomy for acute stroke has arisen from a number of trials that show a significant reduction in long term disability (McCarthy et al., 2019) with a number needed to treat (NNT) of less than 3 (Evans et al., 2017). The evidence base was so strong in favour of mechanical thrombectomy over conventional treatment (including intravenous thrombolysis) that some of the trials were terminated early.

1.3.2 Haemorrhagic stroke

Haemorrhagic strokes can be classified into either intracerebral haemorrhage (ICH) or subarachnoid haemorrhage (SAH). Intracerebral haemorrhages carry the worst outcomes of all stroke subtypes and immediate therapy is largely supportive with a subgroup of patients eligible for surgical intervention. The chance of survival and/or a favourable clinical outcome depends largely on the size of the haemorrhagic insult; those patients with a smaller haemorrhage with less or no mass effect fair better.

The management options for intracerebral haemorrhage are, for the most part, focused on conservative management and preventing haematoma expansion and resultant increases in intracranial pressure that worsen prognosis.

Any intracerebral haemorrhage is considered a medical emergency and acute therapy is required. More than 20% of patients experience a drop in GCS of >2 and up to 23% will have haematoma expansion with deleterious clinical effects. An ABC approach is taken to ensure adequate oxygenation and lessen the impact of secondary hypoxaemia. Those patients that develop hydrocephalus, mass effect or brainstem herniation should be considered for ventriculostomy and hyperosmolar therapy. Those patients who are hypertensive should receive anti-hypertensive treatment to maintain a systolic blood pressure of approximately 140 mm Hg (Dastur and Yu, 2017). Coagulopathies increase the risk of haematoma expansion and should be corrected, including the rapid cessation of anti-coagulants.

Intraventricular haemorrhage (IVH) can occur in up to 45% of patients and in those with a GCS <8, significant IVH or transtentorial herniation, the placement of an external ventricular drain should be considered plus the consideration of hyperosmolar therapy if intracranial pressure rise above 20 mmHg (Dastur and Yu, 2017).

In some circumstances, there is a role for surgical intervention. However, it has been shown that there is no benefit to early surgical haematoma evacuation in patients with supratentorial haemorrhage. Cerebellar ICH however is a neurosurgical emergency and haematoma evacuation is recommended if the haematoma is >3cm in diameter and/or there is brainstem compression or hydrocephalus (Becker et al., 1999, Dastur and Yu, 2017).

1.3.3 Mimics and chameleons

The diagnosis of acute stroke is not always straight forward. There are various conditions that may mimic a stroke such as Todd's paresis or a hemiplegic migraine. Up to 25% of all potential stroke

admissions can be attributed to these mimicking conditions (Fernandes et al., 2013). For those patients incorrectly diagnosed there is a huge risk to receiving acute stroke thrombolytic treatment as well as the more insidious adverse effects associated with acute investigations such as excess radiation exposure following a CT head. Acute imaging does not hold the diagnostic key either for acute stroke, since a number of unenhanced CT heads may be negative even in those patients who have suffered an acute ischaemic event and access to acute MRI is limited by resources and acquisition time. The absence of a highly sensitive quick and ubiquitously available diagnostic test, leaves the clinician making treatment decisions based on clinical history and examination findings.

Stroke chameleons complicated diagnostic accuracy further where symptomatic onset is perhaps more gradual or not clearly defined by a vascular territory. These are known as stroke chameleons – strokes that present as other diagnoses and are often missed by clinicians in the acute phase and therefore patients are often not offered thrombolysis (Fernandes et al., 2013). Examples of this are the 3% of strokes that may present with dizziness or vertigo (Kerber and Helmchen, 2012) or those patients suffering a non-dominant anterior circulation stroke that present with symptoms such as loss of spatial orientation and disinhibition incorrectly diagnosed as acute delirium (Fernandes et al., 2013).

The common occurrence of stroke mimics and the less common but equally important stroke chameleons offer further strength to the need for a highly sensitive and readily available acute serological diagnostic test for acute stroke. Its advent would help not only tailor acute treatment to those patients that would otherwise have a delayed diagnosis potentially rendering them outwith the acute treatment window; it would also help in reducing the over investigation and potential over treatment of those patients with mimicking conditions.

1.4 The Brain: Structure and Physiological Function

The brain is 2% of the body's total weight but comprises 25% of the human body's total cholesterol (Bjorkhem and Meaney, 2004) and demands 15% of cardiac output to maintain optimal metabolism.

The gross anatomy of the brain is encased within the skull and is divided by the falx cerebri into two cerebral hemispheres. The brain is a complex and incompletely understood organ but distinct anatomical regions control a range of normal physiological functions.

The frontal lobes are the largest and have numerous functions. They control memory and personality traits; Broca's areas is found within the frontal lobes and is integral for speech production. Functions such as executive decision making, emotional traits and reasoning are also functions of the frontal lobe.

The parietal lobe is located posterior to the frontal lobe and anterior to the occipital lobe. The anterior aspect of the parietal lobe contains the primary sensory cortex within the post-central gyrus and is responsible for interpreting touch, position, temperature and pain. The posterior parietal lobe can be split into two areas of physiological function. The superior aspect of the posterior parietal lobe contains the somatosensory association cortex that is involved in higher order functioning such as motor planning. The inferior parietal lobe contains the secondary somatosensory cortex that receives a number of neuronal communications from the thalamus and the contralateral secondary somatosensory cortex that helps integrate visual and auditory inputs (Jawabri KH, 2020).

The temporal lobe contains Heschl's gyrus, the location of the primary auditory cortex which processes and translates sound and tones. Other functional responsibilities of the temporal lobe include semantic memory (the retrieval of common knowledge) and also the processes involved in facial recognition (Jawabri KH, 2020).

The occipital lobe is the smallest of all the cortical lobes and is primarily responsible for visual processing an interpretation. It houses the primary visual cortex that receives visual information from the thalamus. Visual information received and processed within the occipital lobe is transmitted to other regions of the brain such as the parietal lobe for secondary processing (Jawabri KH, 2020).

1.4.1 The Neuron

Neurons function as the communication system for the nervous system. They are unique in structure and function. As a communication system, neurons have a range of qualities that enables optimal functionality. They may be excitatory, inhibitory or modulatory or have motor, sensory or secretory attributes (Raine, 2006). Greater dynamism of function exists between abutting neurons at synapses where a variety of neurotransmitters can be secreted.

The basic structure of a neuron is common to all, regardless of sub-specialised function. Each contains four elements: (1) a cell body, (2) dendrites, (3) axon and (4) axon terminals.

The cell body contains the nucleus and synthesise nearly all proteins and membranes required for neuronal functioning. Proteins and substances required for membrane renewal once synthesised in the cell body are transported along axons towards the axon terminals in vesicles or multiprotein particles by microtubules. Damaged organelles from axonal terminals undergo retrograde transport from the axonal terminals to the cell body where the substances are then disposed of by lysosomes (Raine, 2006).

Neurons have a single axon that transports the action potentials required for neuronal signalling away from the cell body and towards the axon terminal. The action potentials are changes in electrical voltage from a resting state of around -60mV to a up to +50mV and are propagated by a process of depolaristion as sodium ions flow inwards through voltage-gated channels. To facilitate the rapid proliferation of the chemical signal resultant from depolarisation, an action potential can travel along axons at 100 metres per second. Following depolarisation the neuronal membranes return to their resting state, a process known as repolarisation marked by the closing of the voltage-gated sodium channels and the efflux of sodium ions (Raine, 2006).

Dendrites are the long processes that are used by neuronal cell bodies to receive chemical information from adjacent neurons. Within the central nervous system particularly, dendrites are long and numerous

with complex interactional branches forming up to a thousand synapses. It is the function of synapses that facilitate the unidirectional passage of electrical signals throughout the nervous system. At chemical synapses on the arrival of an action potential, the rise of calcium ions leads to fusion of the vesicles with the membrane of the pre-synaptic cell which subsequently leads to the release of the neurotransmitters within the vesicle into the synaptic cleft. The neurotransmitter then fuses with the membrane surface of the post-synaptic cell and induces increased ion permeability. Thereby changing the electrical signal within the cell and inducing an action potential (Raine, 2006).

The whole of the central nervous system is bathed in cerebrospinal fluid (CSF). The CSF has a vital role in CNS function. It is an ultrafiltrate that not only helps to maintain CNS homeostasis but protects the brain from mechanical trauma and helps maintain ventricular pressure (Raine, 2006). The CSF occupies the subarachnoid space between pia and arachnoid mater and is produced by the choroid plexus in the ventricular system. During its circulation is resorbed by arachnoid villi that populate the leptomeninges of the brain (a collective term for both pia and arachnoid mater) (Raine, 2006).

1.4.2 The Neurovascular Unit

On a cellular level, tight homeostatic control of the brain is essential to for it to function effectively. For this reason, the brain's internal homeostatic environment is regulated by functioning barriers that largely protect it from metabolic perturbations in the peripheral blood system.

The brain is separated from its external environment by four barriers (Liddelow, 2011):

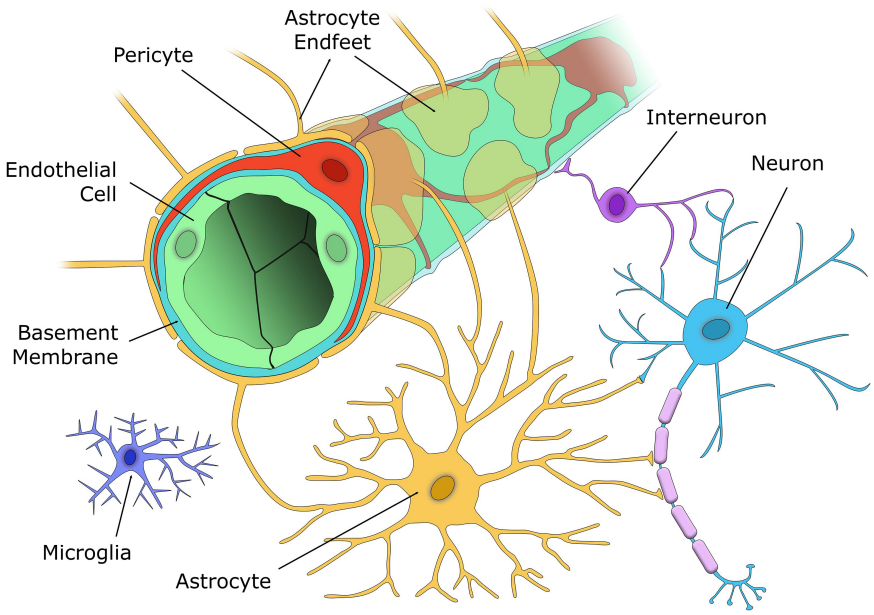
1. Blood-cerebrospinal fluid (CSF) barrier (at the choroid plexus epithelial cells)
2. Blood-brain barrier (at the endothelium of cerebral blood vessels)
3. Arachnoid barrier (between the CSF in the subarachnoid space and the dura mater and overlying tissues)

4. CSF-brain barrier (separation of ventricular system from extracellular fluid of the brain, only present in the embryo)

The first three are relevant to ongoing stroke research, but of primary concern to this thesis is the BBB; its normal functioning and disruption during and following an acute stroke and transient ischaemic attack. Understanding the BBB in context of the neurovascular unit is paramount.

The BBB is one component of the 'neurovascular unit' (see Figure 1.2) a concept developed in 2002 (Harder et al., 2002) that refers to the complex interplay between neurons, microglial cells, pericytes, astrocytes and components of the extracellular matrix (Abbott and Friedman, 2012, Obermeier et al., 2013). The complex relationship begins during embryonic development with juxtaposition of the neuronal progenitor cells derived from the neural tube and vascular progenitor cells derived from the neural crest (Muoio et al., 2014).

Figure 1.2: The Neurovascular Unit



The endothelial cells and their tight junctions comprise the BBB. Note the close relationship of astrocytic end feet and pericytes Reproduced from (Brown et al., 2019).

The over-arching complex functional interplay between the cell types of the neurovascular units is to maintain *hyperaemia* i.e. the microscopic regulation of blood flow to ensure oxygen and nutrient delivery during times of changing metabolic need. The physiological benefits of hyperaemia are accompanied by the process of autoregulation that maintain a near constant blood flow to the brain by altering macroscopic cerebral vascular tone (Muoio et al., 2014).

As neurons experience relative deoxygenation or lack of nutrients, electrical and chemical signals are passed to adjacent interneurons and astrocytes. The astrocyte is a versatile cell that communicates with both neurons and vessels via astrocytic projectional processes or 'end feet'. It is through these end feet that neurovascular communication takes place. Neuronal signals are able to reach the smooth muscle cells of vessels and pericytes and directly influence microvascular function (Muoio et al., 2014).

The function of pericytes is incompletely understood and until recently they were thought to be supportive cells. In more recent literature however, they have been shown to contract in response to rising ATP levels and to some degree control capillary diameter as well as having a secretory role for growth factors and adhesion molecules (Peppiatt et al., 2006, Muoio et al., 2014). Pericytes are separated from astrocytic end-feet and mostly from endothelial cells by a thin basement membrane. Pericytes and endothelial cells are in direct communication at peg-and-socket connections which are free from the otherwise embedding basement membrane. In the brain, pericytes number endothelial cells with a 1:1 ratio; a far higher ratio than is seen in peripheral tissues where pericytes may have a number 10% that of associated endothelial cells (Ferland-McCollough et al., 2017).

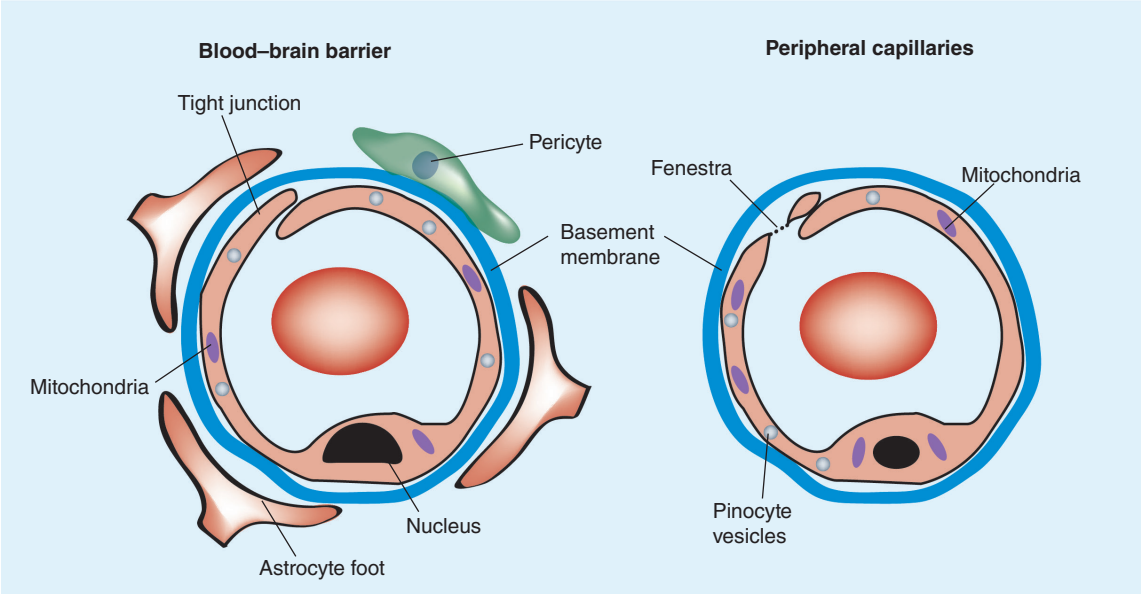
1.4.3 The Blood-Brain Barrier

The BBB was first demonstrated experimentally in 1885 by Paul Ehrlich and its presence confirmed later by his student Edwin Goldmann in 1913 (Hawkins and Davis, 2005). Ehrlich's experiments demonstrated that water-soluble dyes injected into the circulation did not penetrate the brain and spinal cord; Goldmann's contribution demonstrated the reverse phenomenon and he was the first to coin the

phrase *bluthirnschranke* (blood-brain barrier). Interestingly, current scientific experimentation of the BBB is still concerned with the transport of dyes or tracers (including contrast agents) across its (often damaged) interface (Kassner and Merali, 2015).

The function of the normal BBB is to maintain the neuronal environment within a narrow homeostatic range. This is done by a layer of specialised endothelial cells and their basement membrane, as well as tight cell-to-cell junction proteins that limit the flow of substances across endothelial surfaces. The endothelial cells of the BBB are unique in comparison to those in other tissues as they have continuous intercellular tight junctions (TJs) that limit the paracellular and transcellular movement of molecules (Abbott et al., 2006) and prevents the formation of an abnormal plasma ultrafiltrate with in the CNS (Banks, 2009) (See Figure 1.3 below). Additionally, the endothelial cells are surrounded by astrocytic foot processes and pericytes that form an additional layer between blood vessels and brain parenchyma (Serlin et al., 2015). The basal lamina surrounds both endothelial cells and the astrocytic end feet to anchor the BBB and regulate intercellular interaction (Khatri et al., 2012). Regulation is a dynamic process and the blood-brain barrier functions as a metabolic transport barrier as well as a physical barrier (Abbott et al., 2010). The physical barrier properties maintain BBB integrity but the transport control of substances that both influx and efflux the brain across the BBB is regulated on a metabolic level (Yamazaki and Kanekiyo, 2017). Lipid soluble substances pass via diffusion whilst large molecules, such as proteins, have restricted and regulated access (Serlin et al., 2015).

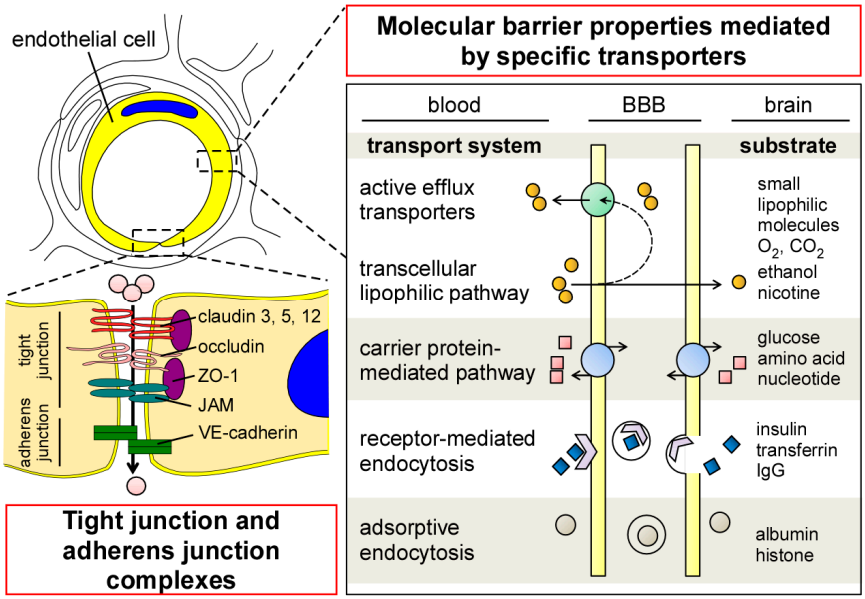
Figure 1.3: Structural comparison between BBB and peripheral capillaries



Notice the presence of TJs within the BBB. Peripheral capillaries contain fenestrations. Reprinted from (Geldenhuys et al., 2015)

Endothelial TJs are a defining feature of the BBB and are composed of transmembrane proteins. The principal protein is claudin. Specifically, the isoforms claudin-5 and claudin-3 as well as claudins -1 and -12 have important roles in the correct functioning of the BBB. The importance of claudin-5 to the integrity of the BBB has been shown in mice. The lack of claudin-5 allowed extravasation of tracers with a molecular mass of <0.8 kDa to pass into the brain parenchyma and spinal cord; such extravasation was not apparent in control mice (Nitta et al., 2003). A secondary physical barrier is formed by vascular endothelial-cadherin at the adherens junction. The cell-to-cell adherens junctions open and close to allow variable degrees of BBB permeability. The role of the adherens junction is particularly important to processes such as leucocyte extravasation (Yamazaki and Kanekiyo, 2017).

Figure 1.4: Molecular properties that control transport across the BBB



Transport across the BBB in molecule dependent. Small lipophilic molecules cross by a transcellular lipophilic pathway. Larger molecules such as amino acids have dedicated carrier protein transport. Transport of substances such as insulin are transported by receptor mediated endocytosis. Albumin can cross by adsorptive endocytosis. Reproduced from (Yamazaki and Kanekiyo, 2017)

Figure 1.4 above depicts the range of mechanisms that control the transport of substances across the BBB. Most molecules do not have free access to the brain parenchyma due to the effective restrictions in place at the BBB. Small, non-polar, lipophilic molecules are the most likely to cross the BBB lipid bilayer by transcellular transport. Saturated fatty acids for example, are more lipophilic as they do not contain any double bonds. Unsaturated compounds with one or more double bonds are less lipophilic. But even these molecules can be prevented by entering the brain by effective efflux ATP-transporters that limit the passive transcellular route (Yamazaki and Kanekiyo, 2017). The concentration gradient of solute across the BBB has a large impact on the degree of initial transcellular movement.

The carrier protein-mediated pathway delivers a greater number of substances to the brain by way of an effective transport system across the lipid bilayer. Substances delivered by this route include glucose (essential for brain metabolic function), hormones, amino acids and nucleotides (Pardridge, 1995).

Receptor-mediated endocytosis allows larger proteins and peptides to be delivered to the brain across the BBB. The process of adsorptive endocytosis, is the mechanism through which molecules such albumin cross the BBB and is reliant on positively charged molecules interacting with the negatively charged membrane (Pulgar, 2018).

1.4.4 Waste disposal

The brain has a high metabolic turnover and a requirement for effective homeostasis that maintains an environment suitable for neuronal function. For this reason, the brain requires an effective efflux system that allows metabolic end-products within the interstitial fluid to either be recycled or removed from the ISF and CSF into the peripheral circulation. Surprisingly, little is known about the brain's waste disposal system and it is only in recent years that some key discoveries have been made in animal models and extrapolated to human biology. Knowledge of potential waste disposal routes is essential to the search for peripherally obtained diagnostic biomarkers for stroke.

It has been known since the 1980s that (in animal models) it is possible for substances within the brain and the CSF to drain into the peripheral lymphatic system (Bradbury et al., 1981). The mechanism for which was unclear. It was not until the discovery of the glymphatic system (see below) that a deeper understanding of waste disposal has been gained.

1.4.5 Perivascular routes

There are two suggested mechanisms for how solutes are transported via the perivascular route. The first is that secretion of fluid by the BBB creates a pressure gradient and hence perivascular flow for solutes to travel from the ISF down preferential routes. The greatest criticism of this route is that it would potentially require larger volumes of fluid secretion than daily CSF production. This is based on a calculation for sucrose requirements by the brain and its known volume distribution. It is suggested

that the clearances would need to be, at least 1.2L per day, which is twice the amount of CSF that is believed to be produced per day (Hladky and Barrand, 2018).

The second is the glymphatic proposal. The glymphatic system has been recently described as a waste disposal system for the brain that has some resemblance to the lymphatic system of the peripheral circulation. It describes the continual, convectional interchange of CSF and brain ISF, as the CSF is carried into the brain parenchyma along arterioles (by arterial pulsation) and interchanges with the ISF before the CSF drains out of the parenchyma following a perivenous route and eventually into the cervical lymph nodes. This system is 'glymphatic' as it resembles the function of the 'lymphatic' system in peripheral circulation, by returning interstitial fluid to circulation and because the glial aquaporin-4 water channels have an integral role to play in the physiological functioning of the glymphatic system by allowing CSF to enter the brain parenchyma (Jessen et al., 2015).

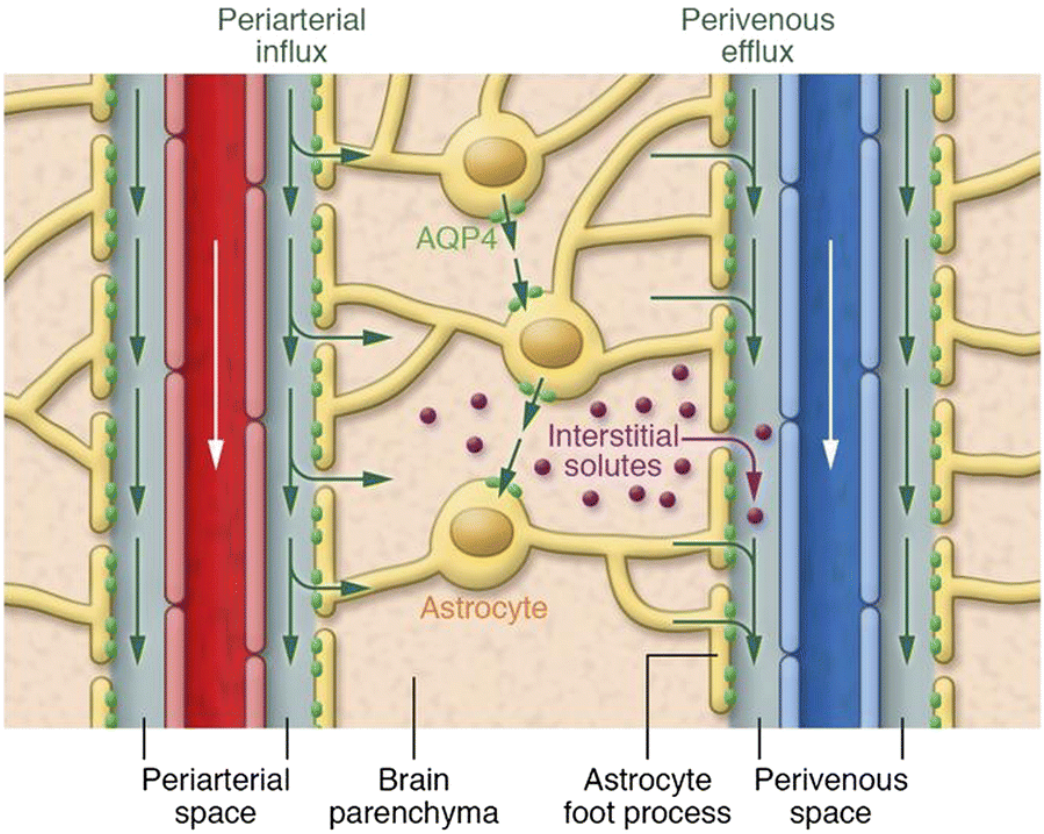
Iliff et al. (Iliff et al., 2012) studied CSF flow in mice and in 2012 described a "brain-wide pathway for fluid transport" whereby CSF flows into the brain parenchyma following a para-arterial route, interchanges with interstitial fluid and drains following a para-venous pathway and eventually into the peripheral circulation. The findings from Iliff et al. reinforced other theories of CSF drainage and also the bulk flow theory of CSF flow – which is facilitated by astroglial water transport enabled by astroglial aquaporin-4 (AQP4) water channel at the perivascular end feet. One of the key features of the bulk flow theory is that the flow of solutes is independent of molecular size, which contrast with what would occur by simple diffusion.

The concept of CSF and ISF fluid interchanging is not new and previous researchers have commented on the peri-vascular route of CSF flow (Abbott, 2004). Until now however, the details of the CSF-ISF interchange had not been documented and a new line of scientific enquiry is beginning to explore the impact the glymphatic system, and its dysfunction, may have on the understanding of cerebral pathophysiology.

The brain wide bulk CSF-ISF interchange has been imaged in rats using contrast-enhanced MRI. Interestingly, this study used to gadolinium chelates as contrast agents with different molecular weights and the distribution of these agents throughout the perivascular spaces and the brain parenchyma differed. The smaller molecular weight diethylenetriaminepentaacetate (Gd-DTPA) was detectable in larger regions of the parenchyma than the larger molecular weight polymetric gadolinium chelate (Gado-Spin) that had a distribution that largely along the perivascular conduits and had sparse parenchymal uptake (Iliff et al., 2013).

The differences in parenchymal infiltration occurred despite similar initial passage along the perivascular conduits. It is postulated by the authors that the presence of overlapping astrocytic endfeet that envelop the cerebral microcirculation restricted the passage of larger molecules (Iliff et al., 2013, Iliff et al., 2012).

Figure 1.5: The glymphatic system.



This image depicts the glymphatic system with mixing of CSF and interstitial fluid that travels down along periarterial spaces with periarterial flow, mixes with solutes with the insterstitium and then drain along perivenous spaces to eventually efflux from the brain parenchyma. Reproduced from (Ray et al., 2019)

1.4.6 BBB efflux

Concerning efflux at the blood brain barrier, this is far more selective than the perivascular route. Most of the research surrounding BBB efflux concerns drug delivery and metabolism but the difficulties faced with CNS drug delivery sheds light on the nature of compounds that may freely efflux across the blood brain barrier and those mechanisms that contribute to the active efflux process.

Passive diffusion (efflux) is possible across the BBB particularly because there is a large surface area for solute transfer and deep penetration of microvessels within the brain parenchyma. Cells are usually no farther than 20micrometres, a distance short enough to allow for efflux by passive diffusion.

There are 2 possible routes for passive efflux: via a paracellular route that is largely impaired by TJs or a transcellular route that involves crossing both the lumen and abluminal cell surface. It has been noted that substances more likely to cross the BBB from the peripheral circulation (and hence the same properties can be extrapolated to efflux) are those compounds that are of low molecular weight, lipophilic, have reduced polarity and less able to donate hydrogen bonds (Geldenhuys et al., 2015).

Of the active BBB efflux transporters, the most highly recognized is the P-glycoprotein transporter and has been described as the 'gate-keeper' for the BBB (Schinkel, 1999). This transmembrane transporter undergoes conformational change upon the binding of ATP that results in effect efflux of substrates. The role of the P-glycoprotein transporter has been studied in relation to CNS disease processes. For example, it is found to be downregulated in patients with Alzheimer's disease which may be implicated in the reduced efflux of β-Amyloid. Interestingly, it has been found to be upregulated following stroke and ischaemic brain injuries, likely due to the increased metabolic need to remove ischaemic metabolites (Erdő and Krajcsi, 2019).

1.5 Pathophysiology of Stroke

1.5.1 Overview

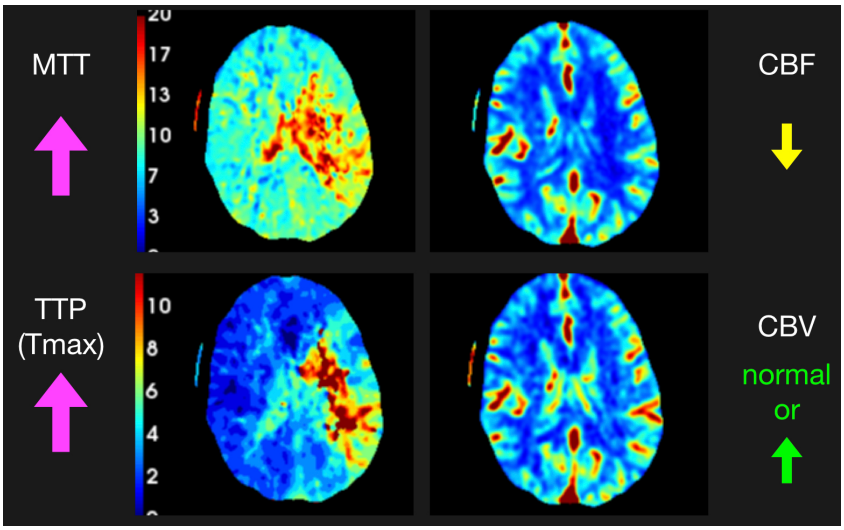
This section explores the metabolic and cellular disturbances encountered following acute stroke. The brain is unique in its energy requirements. It has a high metabolic rate with limited energy stores and depends on aerobic metabolism of glucose. Thus, the brain is highly vulnerable to ischaemic injury and the pathophysiology of stroke is complex.

Fundamental to the understanding of stroke pathophysiology is the distinction between the infarcted tissue core and the ischaemic penumbra. The penumbra that has the potential to either undergo reperfusion and return to normal functioning brain tissue or conversely, be exposed to further ischaemic insult and expand the infarcted core volume.

The penumbra has a reduced blood supply but high-energy metabolism is preserved. Secondly, the penumbra retains electrical activity and ion homeostasis is preserved as well as transmembrane potentials. ATP levels are also preserved but protein synthesis is reduced. In short, though the penumbra tissue does not function normally, the damage is reversible. Restoring blood flow may prevent the onset of irreversible damage (Belayev et al., 2012). Modern imaging techniques such as CT perfusion imaging allow for clear graphical displays of the infarcted core and ischaemic penumbra based on algorithms that calculate the mean transit time (MTT) and time to peak (TTP) of intravenous contrast to the affected area of brain tissue as well as cerebral blood flow (CBF) and cerebral blood volume (CBV) levels. Within the infarcted core there is prolonged MTT and significantly reduced CBF and CBV. This is contrasted with the ischaemic penumbra which like the infarcted core has a prolonged MTT but only moderately reduced CBF and can have almost normal CBV which is retained by autoregulation.

It is helpful when considering the biochemical changes discussed below to bear in mind the graphical display of infarcted core and ischaemic penumbra depicted in Figures 1.6 and 1.7.

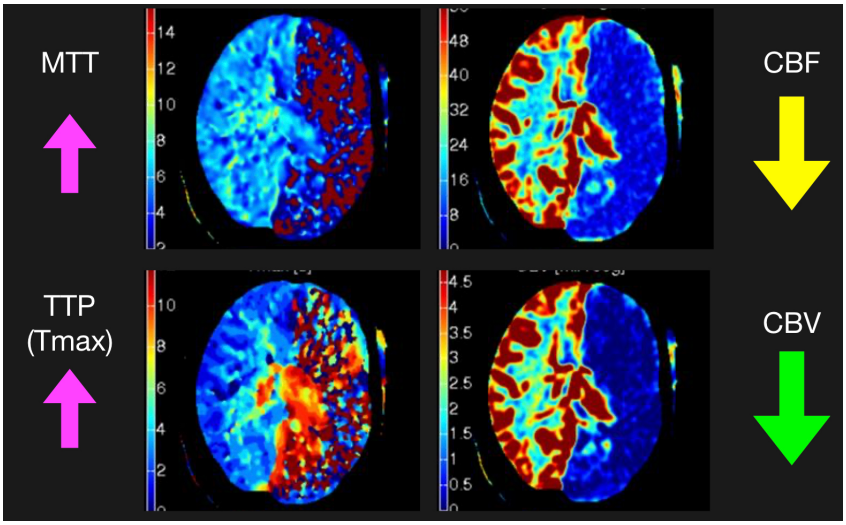
Figure 1.6: Ischaemic penumbra



This CT perfusion image shows a left MCA penumbra core with increased MTT and miniamlly reduced CBF and normal CBV. MTT = mean transit time; TTP = time to peak; CBF = cerebral blod flow; CBV = cerebral blood volume

Case courtesy of Assoc Prof Craig Hacking, Radiopaedia.org, rID: 72596

Figure 1.7: Infarcted Core



This CT perfusion image shows a left MCA infarct core with increased MTT and significantly reduced CBF and CBV. MTT = mean transit time; TTP = time to peak; CBF = cerebral blod flow; CBV = cerebral blood volume

Case courtesy of Assoc Prof Craig Hacking, Radiopaedia.org, rID: 72596

Ischaemia results in cellular energy demands not being met; which leads to a fall in ATP. Loss of ATP leads to an impairment of ion pump function, most importantly, the Na^+/K^+ ATPase pump. The loss of the ion pump that ordinarily maintains high intracellular K^+ concentrations and low intracellular Na^+ concentrations, results in altered transmembrane gradients, depolarisation and the opening of voltage-sensitive ion channels. This cascade, if left unaltered, will eventually lead to cell death (Belayev et al., 2012).

Following ischaemic insult, normal brain activity ceases. K^+ channels open and widespread hyperpolarisation occurs. Cerebral ischaemia results in the continued uptake and use of ATP in the setting of ATP synthesis failure. This leads to a net reduction in ATP and the onset of lactic acidosis and the loss of ionic homeostasis (Xing et al., 2012). The lack of O_2 also leads to the production and build-up of lactic acid as metabolism shifts from relying on aerobic metabolism to a reliance on glycolysis. As a result, cellular pH falls from the normal 7.3 to as low as 6.2. The maintained high extracellular K^+ due to ion channel opening leads to persistent neuronal depolarisation. This is known as the spreading depression (Belayev et al., 2012) and leads to the expansion of the infarct core (Xing et al., 2012).

Other cellular ion gradients are also lost during ischaemia that result in higher concentrations of intracellular Na^+ and Ca^{2+} and a corresponding reduction in intracellular Mg^{2+}. These shifts also compound the additional release and prevented uptake of glutamate leading to sustained elevations of glutamate concentration (Belayev et al., 2012).

Glutamate is the most common excitatory neurotransmitter. Its function is dependent upon concentration. Small amounts promote physiological neurotransmission, but in higher concentrations it is toxic. The increase in glutamate concentration following an ischaemic insult is known as

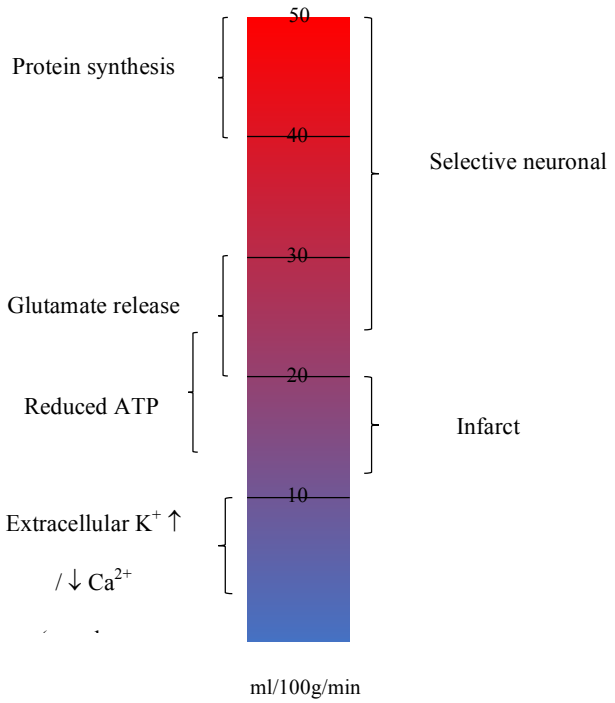
excitotoxicity. As glutamate pools at synapses in response to neuronal ischaemia, N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazole (AMPA) and kainate receptors are over-stimulated. NMDA-receptors are dependent on extracellular Ca^{2+} and thus it is transported intracellularly through receptor-gated ion channels. Ca^{2+} overload leads to lethal cellular reactions which include oxidative stress as well as mitochondrial dysfunction (Xing et al., 2012). The same process results in Na^{+} and H_2O influx and resultant cellular oedema and reduction in the extracellular space. The pathophysiological impact of a significant influx of Ca^{2+} is the activation of phospholipases and proteases that have a role in the degradation of cellular membranes and proteins. Over-stimulation of glutamate receptors leads to energetic failure and neuronal cell death in stroke (Belayev et al., 2012).

Oxygen free radicals are produced intracellularly by the rapid influx of Ca^{2+}, Na^{+} and ADP by mitochondria. These free radicals damage lipids, proteins and nucleic acids. The damage caused is not offset by the slow counter-production of antioxidants such as catalase and glutathione and their concentration levels are not reduced by free radical scavengers such as vitamin C or α-tocopherol (Xing et al., 2012).

When CBF reduces to less than 20% of normal (approximately <10ml/100g/min), it is at this point that the sodium and potassium ATPase pumps begin to fail and neurons begin to lose their ionic gradient (Hansen, 1985). The ischaemic thresholds that lead to impairment of specific functions are illustrated in Figure 1.9 below. These thresholds are based on animal models but appear to correlate with blood flow calculations that result in an ischaemic core and penumbra in human studies that have assessed blood flow using diffusion-weighted MRI. The infarcted core and penumbra thresholds of 13 and 21ml/100g/min have been respectively described (Rohl et al., 2001). Impairments in protein synthesis may begin to manifest at slightly higher thresholds, as detailed from animal models, at <50ml/100g/min (Hossmann, 1994) although inter-species discrepancies may exist. Nevertheless though the specific threshold may vary, the important pathophysiological theme is that there is a gradual loss of cerebral/neuronal function that occurs before necrosis within the infarcted core.

Broadly, though the models used to derive these results vary by anaesthesia and animal model (Hossmann, 1994), the loss of functionality can be described as: firstly, a reduction of protein synthesis by 50% at a blood flow of approximately 55ml/100g/min which is completely suppressed below 35ml/100g/min. The utilisation of glucose diminishes below 25ml/100g/min and at flow rates below 26ml/100g/min tissue acidosis is pronounced which results in loss of ATP production. As CBF continues to decline anoxic depolarisation occurs. Levels of extracellular K^+ begin to increase and extracellular Ca^{2+} declines as due to the opening of Ca^{2+} channels (Hossmann, 1994). The onset of anoxic depolarisation marks the onset of irreversible neuronal damage.

Figure 1.8: Cerebral ischaemic blood flow thresholds for neuronal function



1.5.2 Inflammation

Under physiological conditions secondary to the protection offered by the BBB, the brain is protected from systemic inflammatory response mediators. But following ischaemic stroke, the neurovascular

unit and particularly the BBB undergo changes secondary to inflammatory responses that are haematogenous in nature (Danton and Dietrich, 2003).

Activated platelets interact with the vascular endothelium to turn it from an anti-inflammatory, anti-thrombotic to pro-inflammatory, pro-thrombotic state. This pro-inflammatory process leads to the secretion of endothelin-1 an active vasoconstrictor as well as the upregulation of the one of the cell adhesion molecules, E-selectin. Of the other selectins, L and E- selectins are present on leucocytes and endothelial cells and P-selectins found on platelets and endothelial cells (Danton and Dietrich, 2003). The selectins, along with the release of other call adhesion molecules such as the integrins and the immunoglobulin superfamily IgCAM promote cellular adhesion and interaction between inflammatory mediators. Polymorphonuclear leucocytes are recruited to the site of ischaemia from the peripheral circulation highlighting a complex interplay between the activation of the peripheral immune system and the central pooling of inflammatory mediators. Leucocytes express oligosaccharide antigen sialyl Lewis-X (SLe^x) which binds to P and E-selectin and allows leucocytes to roll along the endothelium and eventually migrate through the vascular wall with the assistance of ICAM-1. The inflammatory process is further propagated by the binding of platelets to leucocytes promotes the inflammatory process (Danton and Dietrich, 2003).

In addition to the activation of the peripheral immune system, the CNS' own immune cells – the microglia – play a role in mediating the inflammatory response. In general, the role of the microglia is reminiscent of the role of macrophages in the peripheral circulation; they have phagocytic properties and can secrete pro-inflammatory cytokines (such as TNF or IL as well as being involved in antigen presentation (Xing et al., 2012). Microglia are known to pool at infarcted tissue and within the penumbra and it is now understood that there are two distinct types. A pro-inflammatory type (M1) and anti-inflammatory type (M2). M1 are responsible for releasing cytokines such as oxygen free radicals, nitric oxide and arachidonic acid derivatives (Danton and Dietrich, 2003) and M2 for releasing anti-inflammatory cytokines such as IGF-1 and VEGF (Xing et al., 2012).

An ongoing microvascular inflammatory response with marked interstitial and endothelial oedema leads to microvascular occlusion and the 'no reflow' phenomenon, even despite recanalisation of proximal feeding vessels (Brouns and De Deyn, 2009).

1.5.3 Lipid peroxidation

The oxidation of lipids, or lipid peroxidation, is a critically important step in cerebral ischaemic damage and is produced by the interaction of reactive oxygen species (ROS) or reactive nitrogen species (RNS) with polyunsaturated fatty acids that are present within fatty acid membranes. When molecules containing carbon and hydrogen are oxidised by oxygen, ROS are produced, two of which are free radicals. A free radical is defined as any chemical species that is capable for independent existence and contains one or more unpaired electrons (Gutteridge, 1995).

Some common ROS of the Fenton/Haber-Weiss pathway are listed below with their chemical equations. Other notable physiological free radicals are the RNS nitric oxide (NO) and nitric dioxide (NO_2). These chemicals are part of the Beckman-Radi-Freeman pathway. Crucially, NO is released via vascular endothelium from the amino acid L-arginine (Gutteridge, 1995).

Below are generic formulae for the production of free radicals:

$O_2 + e + H^+ \rightarrow HO_2$ (hydroperoxyl radical)

$HO_2 \rightarrow H^+ + O_2^-$ (superoxide radical)

$O_2^- + 2H^+ + e \rightarrow H_2O_2$ (hydrogen peroxide)

$H_2O_2 + e \rightarrow OH^- + OH$ (hydroxyl radical)

Lipid peroxidation is a chain reaction with 3 phases: initiation, propagation and termination. It is initiated by a hydrogen atom being abstracted from a methylene ($-CH_2-$) group. This leaves behind –

CH- and the presence of a double bond on the adjacent carbon, which weakens the C-H bonds that promotes H^+ removal. For this reason, the fatty acid side chains of membrane lipids are highly sensitive to peroxidation (Gutteridge, 1995). The carbon containing radical –CH- left behind by the first abstraction of H^+ is chemically rearranged to form a conjugated diene (that is, two double bonds in the molecule are separated by only one single bond) and reacts with oxygen forming a peroxyl radical that can then abstract a hydrogen atom form another fatty acid. Thus, a chain reaction is initiated and continues until substrate use is complete and two free radicals react with each other or, lipid peroxidase is interrupted by an anti-oxidant (Gutteridge, 1995).

The ongoing chain reaction can be chemically summarised as:

RO. + RH → ROH + R

RO2. + RH → ROOH + R.

R. + O2 → RO2.

*R = R group: any group in which a carbon or hydrogen atom is attached to the rest of the group.

The result of lipid peroxidation is the destruction of membrane lipids resulting in loss of integrity and pathologically altered function as well as yielding secondary (metabolic) end-products; in short, barrier function is impaired or lost. The membrane damage results from interruption of fatty acyl chains, lipid-lipid or lipid-protein cross-links and endocyclization reactions (producing organic products with closed rings of atoms) that produce isoprostanes (prostaglandin-like compounds usually produced from the peroxidation of fatty acids, such as arachidonic acid) and neuroprostanes (prostaglandin-like compounds produced from the peroxidation of docosahexaenoic acid). These reactions cause a loss of enzymatic function and toxic effects on cellular function (Rapetto, 2012).

Lipid peroxidation can be driven both non-enzymatically and through enzymatic reactions. Non-enzymatic peroxidation (autoxidation) is driven by the previously described free radical chain. The polyunsaturated fatty acids (PUFA) that are susceptible to lipid peroxidation in cellular membranes include linoleic acid and arachidonic acids. The lipid hydroperoxides produce among other products, short and long chain aldehydes, phospholipids and cholesterol ester aldehydes which are all able to abstract hydrogen from unsaturated fatty acids (Rapetto, 2012). Catalytic metal ions also play an important role in lipid peroxidation by generating free radicals that can continue hydrogen abstraction. An example is detailed below:

$Fe^{2+} + ROOH \rightarrow RO^{\cdot} + OH^{-} + Fe^{3+}$

$Fe^{3+} + ROOH \rightarrow RO2^{\cdot} + H^{+} + Fe^{2+}$

Free radicals produced by NO reactions are important due to NO being released from macrophages and neutrophils recruited during inflammatory reactions. The NO-derived species are also able to dissolve across cell membranes and are important because of the concentration of nitrogen in the hydrophobic core of cell membranes and lipoproteins (Rapetto, 2012).

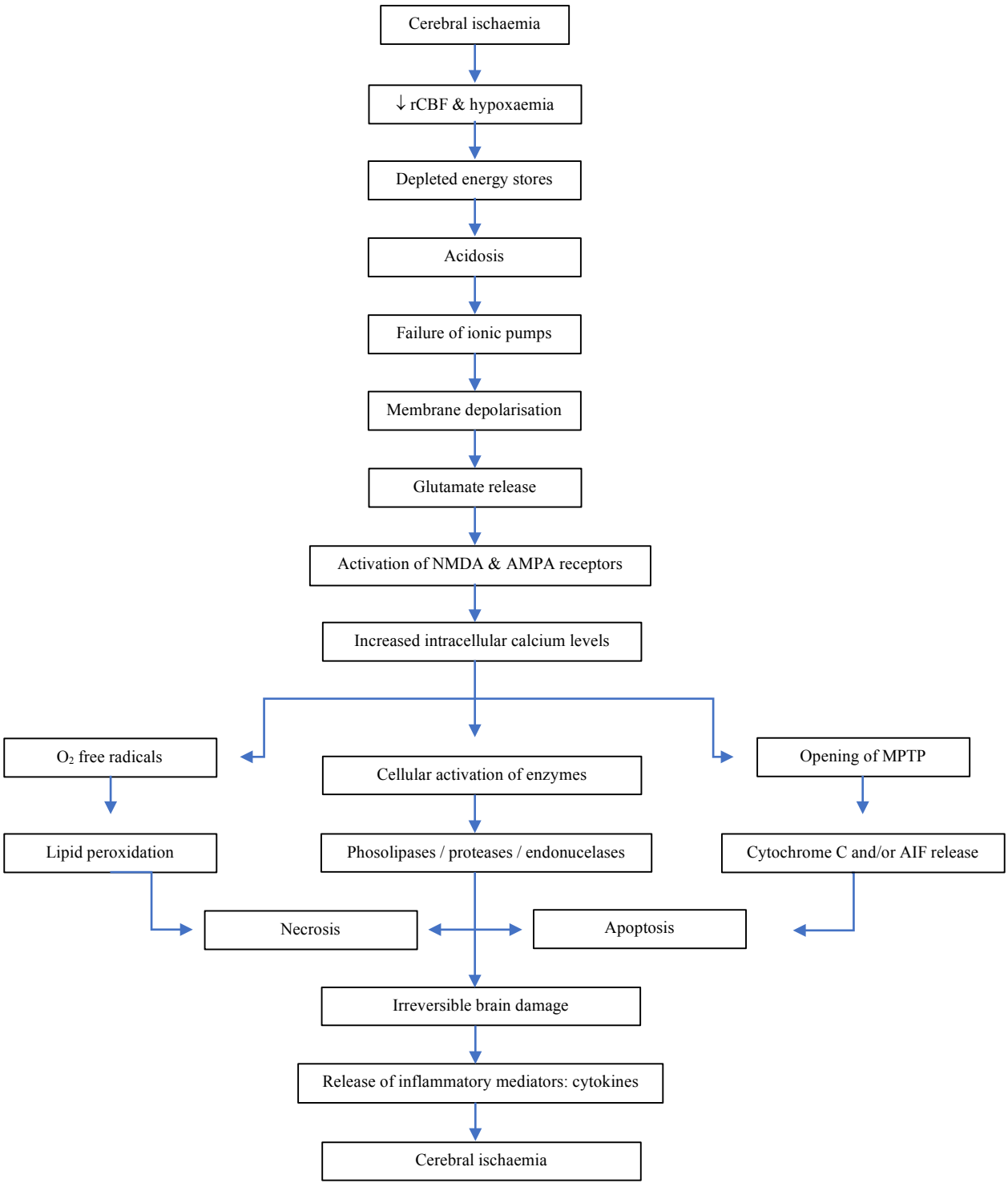
In addition to the production of free radicals, aldehydes are produced aplenty during lipid peroxidation of PUFA. Aldehydes (an organic group contain –CHO group formed from the oxidation of alcohols) are stable structures that can diffuse out of a cell and have biological effects at distant sites. The biologically important aldehyde produced by lipid peroxidation is thought to be 4-hydroxinonenal (HNE). The biological impacts of HNE are wide ranging: from modulation of gene expression to effects on cellular proliferation and apoptosis (Rapetto, 2012). Protein damage also occurs due to additional reactions with lysine amino groups (Rapetto, 2012).

As well as effects on cellular membranes, lipid peroxidation effects subcellular compartments. Microsomal membranes have a high concentration of PUFA and is largely dependent on the biological

effect of reduced iron (Rapetto, 2012). Mitochondrial function is impaired by free radicals with mitochondrial complex I being particularly sensitive to oxygen free radicals. Mitochondria produce most of the energy required for cellular functioning through the process of oxidative phosphorylation – a process whereby electrons are passed along an electron transport chain - that allows effective proton pumping across cell membranes and the production of ATP. The electrons used during oxidative phosphorylation are produced by NADH which in turn is produced by the oxidation of glucose and other nutrients (Saraste, 1999). Mitochondrial complex I is an enzyme that oxidises NADH and transfers electrons to Ubiquinone (Coenzyme Q, CoQ) a lipid soluble electron carrier in the lipid bilayer of the inner mitochondrial membrane (Lenaz et al., 2006). The inhibition of complex I results in oxidative protein damage and functional inactivity (Rapetto, 2012).

Figure 1.9: Schemata of the pathological mechanisms of cerebral ischaemia

Adapted from (Janardhan and Qureshi, 2004)



1.5.4 Blood brain barrier dysfunction

Blood brain barrier dysfunction occurs following the onset of ischaemia to varying degrees, dependent on the size of ischaemic insult. This dysfunction also appears to be biphasic, which is especially the case following reperfusion (Brouns and De Deyn, 2009).

The BBB begins to breakdown following acute insult. It begins with endothelial damage which leads to increased BBB permeability. The ongoing insult and permeability of the BBB can either be improved or worsened by eventual reperfusion which is discussed in more detail in the next section.

The increase in the BBBs permeability allows the passage of the aforementioned inflammatory mediators by paracellular and transcellular transport mechanisms (Cipolla et al., 2004) and eventually, via adhesion molecules, the passage of leucocytes. In particular, MMP-9 may lead to the digestion of the basal lamina that creates a level of sustained permeability, specifically in the delayed phase of BBB dysfunction that occurs between 24-72 hours post initial injury. Furthermore, mast cells present in the perivascular spaces and the meninges contribute to BBB breakdown by release gelatinase (Anrather and Iadecola, 2016).

1.5.5 Reperfusion injury

Tissue reperfusion is essential for its survival. A reperfusion injury following acute stroke is mediated primarily by free radicals that are formed during re-oxygenation rather than during ischaemic periods. Any damage sustained to the BBB during this period increases the risk of haemorrhagic transformation (Khatri et al., 2012).

The degree of reperfusion is dependent on the volume of initial ischaemia and the amount of reperfusion achieved. The process of reperfusion can be split into 3 phases. The first is reactive hyperaemia: an increase in microvascular blood flow during reperfusion is accompanied by increased BBB

permeability. This leads to cellular oedema and eventually microvascular occlusion and subsequent hypoperfusion - the 'no re-flow phenomenon', the hallmark of phase 2. The metabolic result of no re-flow is nutrient deficiency, neutrophil adhesion and inflammation. Phase 3 is marked by increased paracellular permeability at the BBB which follows a biphasic course – the first over the initial 8 hours of reperfusion and the second between 18-96 hours post reperfusion. It is phase 3 that leads to the development of vasogenic oedema, as opposed to the cytotoxic oedema of phase 1 that does not affect the BBB TJs (Khatri et al., 2012).

Damage to the BBB during reperfusion increases the risk of haemorrhagic transformation and there is evidence that having higher levels of circulating MMP-9 increases the risk. Due to the degrading effects MMP can have on the ECM, vessel walls are weakened leaving them prone to rupture (Turner and Sharp, 2016).

1.5.6 Haemorrhagic strokes

The onset of a haemorrhagic stroke leads to a more profound inflammatory response than an ischaemic stroke (Danton and Dietrich, 2003) but many of the subsequent pathological processes are similar. Following the onset of an acute intracranial haemorrhage, there is an immediate and potentially increasing mass effect on the immediate brain tissue from the extravasated blood plasma products.

Haemoglobin (Hb) and heme are potent cytotoxic agents capable of damaging brain parenchyma and eventually cell death. This is done by the production of free radicals again via the Fenton/Haber-Weiss mechanism as described above as well as the oxidation of lipids and proteins (Aronowski and Zhao, 2011). Under physiological conditions free haemoglobin is neutralized by binding to the enzyme haptoglobin (Hp). Under the circumstances of intracranial haemorrhage insufficient Hp can be produced by oligodendrites to neutralize the effect of Hb on the brain parenchyma.

Further physiological balance is produced by the phagocytic processes of microglia that attempt to remove erythrocytes and free Hb. The phagocytic cells express heme oxygenase that converts heme to biliverdin, carbon monoxide and iron. The activation of phagocytic microglia also induces the release of pro-inflammatory cytokines and chemotactic mediators that recruit inflammatory cells to the site of ICH (Aronowski and Zhao, 2011). Evidence of a sustained inflammatory response is seen by the recruitment of neutrophils for up to 4 days post haemorrhagic insult and the longer-term recruitment of haematogenous macrophages (Gong et al., 2000).

1.6 Serological biomarkers: a rationale for the hunt

The cornerstone of stroke diagnostics is currently acute brain imaging, in addition to sound clinical acumen. Great advances have been made in the imaging field but the detection of acute cerebral infarction remains imperfect. Moreover, the need for a hospital admission to gain access to accurate diagnostic tests places a significant strain on acute care services. In an ideal clinical scenario, diagnostics that have a sensitivity beyond that of the clinical signs made popular by the F.A.S.T campaign would be done by ambulance teams before admission to hospital. A point-of-care blood test that is both highly sensitive and specific for acute cerebral ischaemia could offer a diagnosis 30 minutes quicker than current standards (Mattila et al., 2016) which could open an avenue for pre-hospital thrombolysis and/or more accurate triage to a Hyper-Acute Stroke Unit (HASU).

A large amount of research has been focussed on the peripheral detection of tissue-specific proteins such as S100β, neuron specific enolase (NSE) and glial fibrillary acidic protein (GFAP) and although some correlations have been found, the detection of these biomarkers has not been clinically validated. S100β levels have a stronger correlation with infarction volume the longer that is left between the acute event and sample collection, peaking at 48 hours(Ahmad et al., 2012) which is unhelpful in a clinical scenario that requires hyper-acute, accurate diagnostics. Varied research methodologies, such as the length of time from acute event to blood sampling, have also compounded efforts. However, there is very likely a fundamental pathological reason for difficulties encountered in correlating protein levels with acute stroke: the molecular weight of proteins is too high and their transport mechanisms across the BBB too complex and reliant on membrane transporters to allow for rapid, tissue specific detection in a time of pathological NVU dysregulation. The previously explained perivascular efflux of solutes is also likely impaired by acute ischaemia or haemorrhagic stroke (Gaberel et al., 2014) as the required para-arterial flow to drive the convective ‘bulk flow’ theory of solute movement (Cserr et al., 1981) is likely to be disrupted by the onset of vessel occlusive ischaemia and eventually impaired by interstitial oedema.

The attempt to detect solutes that can more readily cross the (damaged) BBB or those that are components of NVU cell membranes is a more compelling research avenue.

1.6.1 Parallels with blood brain barrier imaging

It is a well-established fact that the contrast media used in medical imaging can cross the damaged blood brain barrier. The ability of imaging modalities to detect blood-brain barrier dysfunction is dependent on a number of factors that include, but are not limited to, intra-arterial vs intravenous contrast injection; blood vessel size; flow rate; contrast transit time; the design of the imaging protocol used (Kanal et al., 2014) and the molecular weight of the contrast (Strbian et al., 2008).

It can be argued that of all variables, the molecular weight of the contrast agent (the tracer) is most important. The ability of a substance to cross the BBB is a rate-limiting step in the detection of blood-brain barrier permeability. It is known that tracers with a smaller molecular weight extravasate to a larger cerebral area than larger molecules following insult (Strbian et al., 2008, Nagaraja et al., 2008). This correlates with basic scientific experiments focused on the BBB, whereby smaller molecules penetrate deeper into the interstitium than larger molecules (Iliff et al., 2013). Small molecules therefore, are more sensitive to BBB disruption.

In humans, it is possible to visualise BBB disruption using either dynamic contrast enhanced MRI or CT scan (DCE-MRI and DCE-CT respectively) though there is a greater body of literature for the application of DCE-MRI (Veksler et al., 2014, Heye et al., 2014). The principle behind dynamic contrast enhanced imaging is that as the contrast agent enhances tissue at different time points post injection a temporal-concentration curve is produced that can be used to assess physiological/pathophysiological features of the vasculature (O'Connor et al., 2011). DCE-MRI and DCE-CT use contrast agents that do not readily cross the *normal* BBB, but do so when the BBB *damaged*.

DCE-MRI uses gadolinium chelates and DCE-CT iodinated contrast agents such as Iohexol (Omnipaque). The latter has a molecular weight of 0.821 kDa (Healthcare, 2007) and the molecular weights for a range of gadolinium chelates licenced for use are between 0.558-1.058 kDa (see table in Appendix I).

BBB imaging has also recently questioned the assertion that BBB breakdown is biphasic. A recent animal study utilising micro-computed tomography and intra-arterial contrast infusion post middle cerebral artery (MCA) infarction has quantitatively demonstrated monophasic opening of the BBB (Park et al., 2014). An earlier study has also identified continuous opening of the BBB following transient cerebral ischaemia (Strbian et al., 2008). Raising the question of whether the extent of blood brain barrier dysfunction observed is dependent on the tracer or molecule used for its assessment.

As will become apparent in the next chapter, the low molecular weight of the contrast agents that cross a damaged blood brain barrier along a concentration gradient is of great theoretical importance to the study of metabolites for acute stroke.

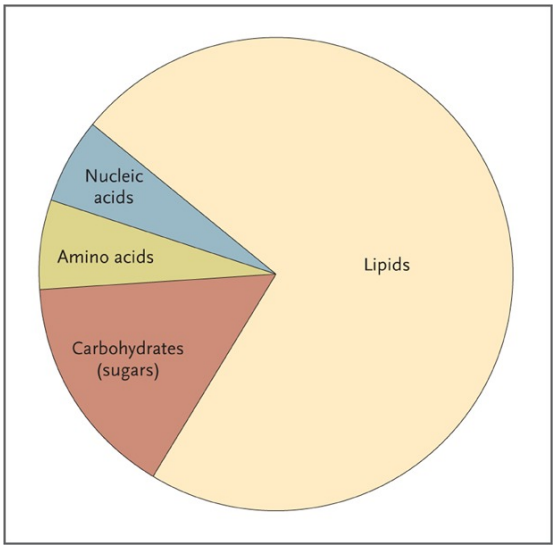
1.6.2 Lipids and the brain

Crucial to the premise that lipids may hold the key to discovering a tissue specific serological biomarker for acute stroke, is the concept that the brain is distinct from peripheral tissues in terms of its lipids content and metabolism.

The lipid content of plasma (the lipidome) has been investigated by the LIPID MAPS consortium (Fahy et al., 2009). What is apparent from this research is that the lipid content of plasma is extremely complex with over 600 molecular species identified within the 6 known mammalian lipid categories. These are: fatty acids, glycerolipids, glycerophospholipids, sphingolipids, sterol lipids and prenol lipids (Fahy et al., 2009, Quehenberger and Dennis, 2011). The relative content of all biological molecules in human

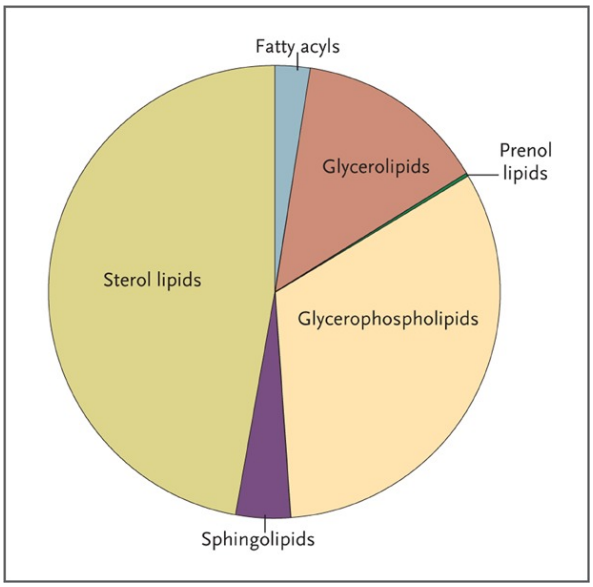
plasma (Figure 1.10) and the relative proportions of lipid species within human plasma (Figure 1.11) are displayed below.

Figure 1.10: The relative proportions of biological molecules present in human plasma



Reproduced with permission from (Quehenberger and Dennis, 2011), Copyright Massachusetts Medical Society.

Figure 1.11: The relative distribution of lipid species within human plasma



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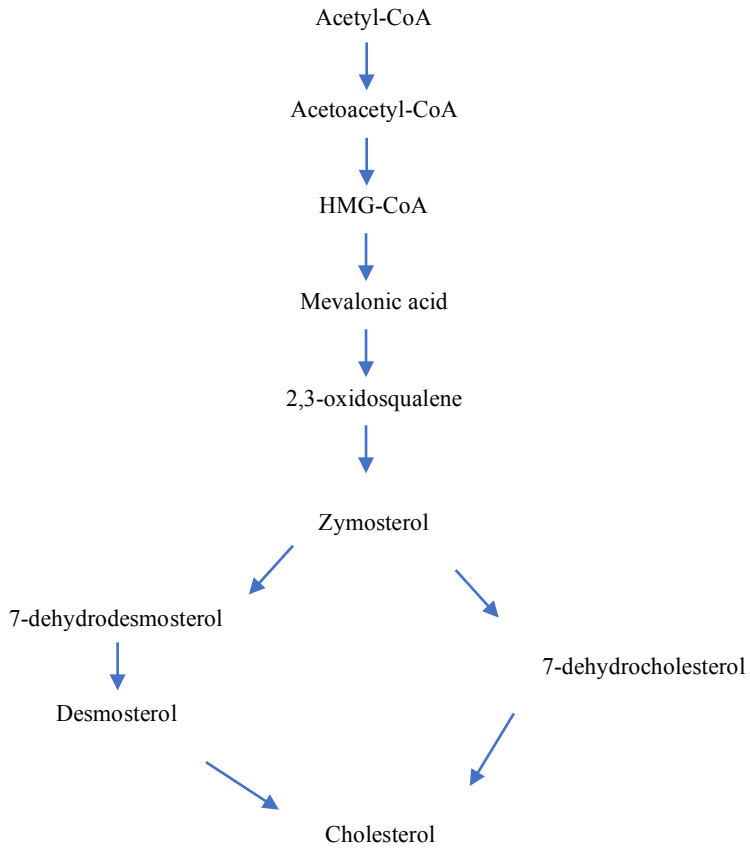
The relative proportions of lipid species within plasma is particularly important as the concentration of lipids within the brain differs. 60% of the brain's dry-weight is composed of lipids, with an additional 20% composed of soluble metabolites involved in a myriad of functions including cell signalling, homeostatic regulation and energy synthesis. Brain lipid content is mainly separated into glycerophospholipids, sphingolipids and cholesterol in roughly equal proportions (Zhang and Liu, 2015), whereas a large proportion of lipids within plasma are of the sterol family.

Cholesterol is synthesised de novo within the brain's endoplasmic reticulum and is distinct from peripheral cholesterol synthesis. In the adult brain, the requirements for cholesterol synthesis are reduced compared to the stages of development that require active myelination (Saher et al., 2005, Zhang and Liu, 2015). The half-life of cholesterol in the adult brain is approximately 5 years (Bjorkhem et al., 1998) which is far longer than the half-life of peripheral cholesterol being only a few days (Zhang and Liu, 2015). Newly synthesised cholesterol is transferred from the endoplasmic reticulum to plasma membranes.

The brain has 2 synthesis pathways for cholesterol. Within neurons most cholesterol is synthesised by the Kandutsch-Russell pathway and within astrocytes synthesis is primarily achieved by the Bloch pathway. Mature neurons rely on astrocytes for cholesterol delivery (Zhang and Liu, 2015). Both pathways begin with a common mevalonate pathway stem and differ in the sequence of double bond reduction steps. This occurs early in the Bloch pathway and as a final step in the Kandutsch-Russell pathway.

Figure 1.12: Kandutsch-Russell and Bloch pathways



As neurons rely on the astrocytic delivery of cholesterol, synthesis can outweigh demand. In these circumstances, cholesterol is stored as intracellular lipid droplets. When cholesterol is required to be excreted from the brain, it is done so with the enzymatic conversion of cholesterol to 24S-hydroxycholesterol. This oxidised cholesterol is far more lipophilic than its non-oxidised precursor and more easily crosses the BBB. In humans, almost all peripherally detected 24S-hydroxycholesterol is derived from the brain (Bjorkhem and Diczfalusy, 2002). Alternatively, cholesterol may be directly exported from the CNS via ABC transporters that are present at plasma membranes (Zhang and Liu, 2015).

1.6.3 Glycerophospholipids

Glyerophospholipids constitute 20-25% of the total dry brain weight (Farooqui et al., 2000) and are constructed of a glyceryl backbone with the C1 and C2 hydroxyls are esterified to fatty acids. The C3 carbon in esterified to a phosphate group (making the glycerophosphate group). The fatty acid backbone is hydrophobic. The structure of a fatty acid back bone and a phosphate group makes up the basic structure of phosphatidic acid (PA). The phosphate group can then be further esterified to a polar head group that determines the glycerophospholipid species such as: phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylinositol (PI) and phosphatidylserine (PS) species.

Glycerophospholipids are essential components of the cell membrane lipid bilayer with the non-polar fatty acid component residing in the aqueous environment and the PUFA in the non-polar tail control the physical properties of the lipid bilayer. Approximately 68% of the brain glycerophospholipids are PC and PE with roughly a 50:50 split. However, different tissue types within the brain have varied relative distributions. Myelin for example, has the highest glycerophospholipid content overall, but grey matter contains a far higher abundance of PE and PC compared to myelin (Farooqui et al., 2000, O'Brien and Sampson, 1965).

1.6.4 Sphingolipids

The sphingolipids are complex lipids with a high concentration within nervous tissue (Quehenberger and Dennis, 2011) and also have hydrophobic and hydrophilic moieties. All sphingolipids contain the spingosine backbone that is a long acyl chain with 2 hydroxyl groups and an amino group. A fatty acid can link to the amino group forming ceramide which forms the precursor for all sphingolipid species. Further polar heads can also attach to the terminal hydroxyl group forming sphingolipid species such as sphingomyelin, glycosphingolipids and gangliosides.

Like glycerophospholipids, sphingolipids play an important role in the function of the neuronal cell membranes. Sphingomyelin in particular, is found in high quantities in the lipid myelin, which contrasts

with the approximate 15% distribution of SM within the human plasma lipidome (see figure 1.14). The relative distribution of sphingolipid species also differs throughout the brain with SM mainly found in the white matter and to a far lesser degree in grey matter (O'Brien and Sampson, 1965). The sphingomyelins are also integral to cellular functioning beyond contributing to the stability of the lipid bilayer. They are integral to cell-cell interaction, the regulation of signal transduction pathways and are particularly important to neuronal-glial signalling (Hussain et al., 2019).

1.6.5 Parallels and divergences from Troponin

The natural comparator for a diagnostic biomarker for acute stroke is the protein Troponin. Discovered in 1965, it has dramatically changed the clinical course of all people diagnosed or suspected of having ischaemic heart disease. Its function is to aid the contraction of actin and myosin. Highly sensitive assays can detect levels of cardiac-specific troponin-I or troponin-T and when cells die it is released into the systemic circulation and detectable from blood taken via peripheral venepuncture approximately 2 hours after the ischaemic insult with a 12-48 peak concentration and remains elevated for up to 10 days. Crucially for troponin, there is a direct correlation between the amount detectable and volume of infarcted/ischaemic cardiac muscle (Giordano et al., 2018).

The real value in the discovery of cardiac troponins has been its addition to a complex decision making tree for determining myocardial infarction especially when ECG changes are not evident and/or when the patient suffers a silent infarction secondary to diabetes mellitus.

Like troponin a diagnostic biomarker for acute stoke would need to be detectable in peripheral blood acutely, but to meet clinical time frames, it would need to be detectable immediately. Ideally, it would need to tissue-specific i.e. released from either neurons, glial cells are other components of the neurovascular units such as the BBB. It would also be preferable if the biomarker were absent or present in far lower concentrations following stroke mimics or when neurological symptoms are secondary to a brain tumours or seizures.

The other crucial difference between the clinical use of troponin and the expected use of a stroke diagnostic test is the clinical setting. Troponin measurement is widely and commonly used in the emergency setting within hospitals for patients who are usually being clinically worked up following a presentation of chest pain, shortness of breath or possibly collapse. A diagnostic blood test for stroke would ideally also be available as a point-of-care test that could be utilized by ambulance crews for either immediate treatment or improved patient triage. There is now increased interest in improving the delivery of pre-hospital stroke care with the formation of the Pre-Hospital Stroke Treatment Organisation (PRESTO) in 2016. A diagnostic blood test for stroke would be highly beneficial to this pursuit.

1.6.6 Special consideration for not sampling urine

It is possible to perform metabonomic analyses on a range of bodily fluid and tissue types. Alongside the analysis of serum, urine is often used as a readily available fluid that is amenable to analysis. It does have some relevance to the pursuit of diagnostic or phenotypc biomarkers for related risk factors for stroke such as hypertension and atherosclerotic disease.

Urine metabolic associations have been found to be significantly increased in people with essential hypertension compared to healthy controls and link with established pathophysiological pathways. High urinary levels of methionine and homocysteine are linked with the effect that homocysteine, in particular, is known to have on the abnormal functioning of endothelial cells (Zhao et al., 2018).

The activation of the oxidative stress pathway is closely linked with the onset of cardiovascular diseases, including essential hypertension. Zhao et al. also found that melatonin was more highly expressed in the urine of people with hypertension compared to healthy controls. This is of interest because melatonin is known to be a potent anti-oxidant capable of mitigating the impact of oxidative (Zhao et al., 2018) stress and the physiological impact of free radicals.

The upregulation of anti-oxidants such as melatonin in urine also reflects the known advantage of urine sampling for representing the influence of chronic metabolic signatures that helps establish the causal links between pathological processes and redox balance (Il'yasova et al., 2012).

The knowledge that metabolomics studies of urine are inherently useful to the assessment of chronic diseases rather than acute metabolic perturbations makes urine an imperfect fluid to analyse in the search for acute diagnostic stroke biomarkers. Especially when blood / serum is readily collected at the point of acute admission to hospital and is more likely to reflect hyper-acute metabolic signatures. Furthermore, the collection of a suitable fluid for analysis must not impede the acute investigation and treatment of a patient within a 4-hour thrombolysis window. A dedicated attempt to collect urine from such acute patients would potentially delay thrombolysis. The consequences of which may be more profound long term disability or possibly death. For the above reasons, urine was not sampled in this study.

1.6.7 Summary

The known disruption of the BBB and its permeability to compounds/solutes that are lipophilic and/or low in molecular weight (preferably <1kDa), the high lipid content of the brain and the difference in relative lipid composition between peripheral blood plasma and the brain, all support the rationale that the search for an acute stroke biomarker should be a lipid or fatty acid.

As is indicated by imaging techniques that rely on BBB permeability, small molecules penetrate further into the parenchyma. Therefore, assuming flow down a concentration gradient is not impeded, the same theory should hold true for the flow of metabolites from brain parenchyma to the blood across a damaged BBB.

Metabonomics which studies small molecules (metabolites) that are generally <1kDa is particularly suited to acute stroke biomarker discovery and holds promise for disease-specific phenotyping. 'Lipidomics' is a subgroup of metabonomics concerned with mapping the lipidome. A lipid is defined as an organic molecule that is soluble in an organic solvent and are highly lipophilic (Vorkas, 2018). To date, only a small number of studies have used metabonomics in the search for diagnostic biomarkers of acute stroke.

1.7 Brain injury: a narrative review

There is a wealth of research into diagnostic or prognostic biomarkers for both acute brain injury (from trauma) and 'brain injury' relating to non-trauma pathology such as transient ischaemic attacks and acute stroke.

This section reviews the current literature for diagnostic serological biomarkers for both acute stroke and traumatic brain injury. Though the pathologies are distinct in their aetiology and the initial mechanism of injury, there are some similarities in the processes that result in neuronal death. A thorough understanding of pathological pathways is essential for stroke biomarker research. The purpose of the narrative review is to review the pathophysiological research streams for biomarkers of brain injury (excluding metabonomic studies which are explored in the next chapter) and any overlap that exists between the research streams of traumatic brain injury and acute stroke.

Medline and Embase databases were search on 3rd March 2018. Search terms are found in the Appendix.

The biomarker studies have been split into categories for analytical ease and to offer insight into the pathways studied. The stroke biomarker literature is discussed first. Traumatic brain injury papers have been included if they explored biomarkers that have not been investigated within the stroke biomarker section.

Those studies that assessed more generic biomarkers such as CRP and ESR and genetic studies are not discussed. In addition, the relationship between brain injury/stroke and protein biomarkers has been extensively reviewed elsewhere (Misra et al., 2017, Hasan et al., 2012) and therefore studies that included proteins were excluded from this narrative review with the intent of narrowing the review to an analysis of other molecules. An exception is made for ischaemia-modified albumin which is thought to be of interest for this thesis and is not found in previous reviews. For completion, the well-studied protein biomarkers are summarised below. None are clinically validated for acute stroke diagnostics.

1.7.1 Proteins

S100β

S100β is a member of the S-100 family of proteins and is a calcium-binding protein with a molecular weight of 10.5 kDa (Kawata et al., 2016). First discovered in 1965 it was initially thought to be tissue-specific to the brain, but in 1981 this theory was disproved by the presence of S100 proteins in other tissues (Cocchia et al., 1981). It is an intra-cellular protein found in the brain with its origin predominantly from astrocytes but is found to a lesser degree in oligodendrites. The primary function of S100β is in modulating second messenger calcium signaling. Consequently, it is involved in a wide range of cellular activities that depend on a cytoplasmic rise in Ca^{2+} including but not limited to, cellular differentiation and migration as well as an inhibitor of differentiation and apoptosis (Donato, 1999).

It has been included in a number of studies in the search for diagnostic biomarker panels for stroke with variable results. Some studies have reported that even with the addition of S100, BNP has been the most sensitive marker of ischaemic stroke (Misra et al., 2017). Others have reported that S100 levels with a cut off of 67pg/mL can differentiate between ischaemic stroke and haemorrhagic stroke within 6 hours but this has not been clinical validated (Zhou et al., 2016, Misra et al., 2017).

Neuron-specific enolase

NSE has been investigated as a surrogate marker for neuronal damage. NSE is a catabolic enzyme that in the glycotic pathway converts 2-phosphoglycerate to phosphoenolpyruvate that is essential for ATP production. It is a cytosolic protein essential for axonal transport. When axons are injured, NSE is upregulated to maintain homeostasis.

NSE is an enzyme that is confined to neurons under normal physiological conditions and only minute amounts are detectable in peripheral blood under normal circumstances. Kim et al explored the relationship between NSE and acute stroke in 83 patients. Although blood samples were not specifically taken in the hyper-acute phase, NSE levels were determined on the day of admission and subsequently, serial bloods were taken each morning for 6 days. The authors classified NSE levels into either continually increasing, continually decreasing or where there were peaks and troughs in NSE levels, as following either a 1 peak or 2 peak pattern (Kim et al., 2014).

Despite NSE being neuron specific, the authors did not find a significant correlation between imaging DWI volume infarction or NIHSS severity scoring and NSE levels (Kim et al., 2014). The authors did note a trend in NSE patterns in those patients that were found to have haemorrhagic transformation of the initial infarct. Although statistically significant levels are not reported, it is noted by the authors that up to 30% of patients who had a haemorrhagic transformation displayed a 2 peak pattern to NSE levels. Providing a tentative link between further, clinically significant, neural damage and the peripheral detection of NSE.

Glial fibrillary acidic protein

GFAP is a structural protein within the CNS and is expressed in astrocytes. There are 10 different isoforms of GFAP and GFAP-α is astrocyte specific (Kawata et al., 2016). GFAP is intermediate

filament protein, responsible for supporting the structure of the astrocytic cytoskeleton. Two independent systematic reviews in 2012 (Hasan et al., 2012) and 2017 (Misra et al., 2017) have found that GFAP is able to differentiate between acute haemorrhagic stroke and ischaemic stroke with the highest efficacy for doing so within 3-4 hours (Hasan et al., 2012) with sensitivity and specificity levels of up to 84.2% and 96.3% respectively (Misra et al., 2017). Differentiation however, is not of diagnostic value for a serological test when an unenhanced CT head of the brain is highly sensitive for acute intracranial haemorrhage and those conditions that may be occult on CT head, such as subarachnoid haemorrhage, can be identified by high bilirubin levels within the CSF following lumbar puncture.

Tau

Tau is a microtubule binding protein and is found in axons. By binding tubulin subunits, tau facilitates axonal signaling and promotes the property of viscoelasticity that allows neurons to stretch and retract against mechanical forces, protecting the brain until the viscoelastic threshold is reached (Kawata et al., 2016). Tau levels in CSF have been studied extensively in association with neurodegenerative conditions such as Alzheimer's disease where the half- life of tau is monitored over weeks. In plasma, the half-life of Tau is only a few hours. Some studies have explored the relationship of plasma Tau levels with acute brain injury and have reported high area under the curve correlation (0.9) with mild traumatic brain injury and with symptoms of concussion. However, to the author's knowledge a Tau blood test is not used routinely in clinical practice (Zetterberg, 2017).

A summary of the commonly studied proteins and their known cerebral and extra-cerebral sources and mechanism of release are tabulated below.

Table 1.1: Protein biomarkers, cerebral and extra-cerebral origins

Adapted from (Kawata et al., 2016)

Biomarker	Mechanism of release	Primary Source	Extra-cerebral source
S100β	Astrogliosis	Astrocyte Oligodendrite	Adipocytes Chondrocytes Cardiomyocytes Alveolar cells
NSE	Neuronal damage	Neuron	Neuroendocrine cells/tumours
GFAP	Glial damage	Astrocyte	
Tau	Axonal injury	Neuronal axon	

1.7.2 Neurotransmitters

The peripheral detection of neurotransmitters and their enzymes is a compelling strategy for acute stroke diagnostic. Castellanos et al. (Castellanos et al., 2008) obtained blood samples from 197 stroke patient on admission and correlated glutamate and Il-6 and L-arginine with DWI volume and worsening neurological function. Interestingly, the strongest correlation came with those patients that were found to have an expanding DWI volume at 72 hours ($r = 0.71$, $p < 0.001$). This was despite weak correlations with initial DWI volume on the admission DWI. Suggesting that serological concentrations of glutamate at admission may correlate with stroke severity beyond what is demonstrated on initial DWI imaging. (Refer to Table 1.2 for further study details).

1.7.3 Amino acids

Jensen et al. monitored the N-terminal fragment of brain natriuretic peptide (BNP) prohormone (NT-proBNP) which although are established prognostic factors for coronary artery disease and heart failure, had not been studied in detail following ischaemic stroke (Jensen et al., 2006). They measured levels at admission and daily to day 5 and then at 6 month follow up. The level of NT-proBNP was highest at day 1, with a significant difference between levels at day 0 (admission) and day 1 ($p = 0.007$). Given that BNP is synthesised by myocardium rather than having a neuronal or glial source, the finding of a peak 24 hours after the clinical presentation is not clinically useful.

1.7.4 Ischaemia-modified albumin

Although levels of ischaemia-modified albumin (IMA) may rise in response to an ischaemic insult to any organ, these papers have been included in the review on the basis that when combined with the clinical syndrome of stroke, a specific test for ischaemia (even if not organ specific) could be useful. This is in contrast to other non-organ specific biomarkers that have been excluded for the purposes of this narrative review that are not specific to organ ischaemia.

IMA is known to be a marker of ischaemic and of oxidative stress since the N-terminal of albumin is altered in response to oxidative stress. A few studies have investigated the relationship of IMA with acute stroke. The first paper was published in 2007 and established a significant difference in acute IMA levels taken within 3 hours of acute neurological insult, between three groups of ischaemic stroke, haemorrhagic stroke patients and those subsequently diagnosed with a TIA or epileptic seizure (Abboud et al., 2007). The ability to differentiate between ischaemic and haemorrhagic strokes was not replicated by a subsequent study that also compared IMA levels to a control group. This study did include SAH in addition to ischaemic stroke and parenchymal haemorrhage and found a significant difference between SAH and acute stroke (Gunduz et al., 2008). Similarly, Herisson et al. did not find acute levels of IMA (within 4.5hr of symptomatic onset) able to differentiate between ischaemic and haemorrhagic stroke. They did note a difference between those patients admitted with acute symptoms and a small group (n=13) of healthy controls but concluded that IMA was not a useful diagnostic biomarker for

acute stroke (Herisson et al., 2010). Ma et al took the study of IMA a step further in 2011 by assessing whether it could potentially be used to augment the diagnosis of cerebral ischaemia compared to both healthy controls and those that already have a diagnosis of type II diabetes. IMA levels were significantly elevated in the acute stroke patients compared to both healthy controls and those that had an established cardiovascular risk factor. However, IMAs ability to differentiate between type II diabetic patients without acute lacunar infarction and those proven to have an acute ischaemic event was marginal, with a p value that just reached statistical significance at $p = 0.044$ (Ma et al., 2011). (Refer to Table 1.3 for further study details).

1.7.5 Micro-RNA

Micro-RNA (Mi-RNA) has been explored as a novel biomarker for stroke in an attempt to identify highly sensitive markers that are earlier in the biological process than protein expression with the additional advantage that they can be monitored on a whole transcriptome level (Swyngedouw and Jickling, 2017). The detection of Mi-RNA is thought to be useful as they are non-coding RNA but are influential in the regulation a number of biological processes including intracellular communication (Wang et al., 2014). The association between circulating cell free Mi-RNAs and ischaemic and haemorrhagic stroke subtypes is discussed.

Chen et al identified from a cohort of 128 acute stroke patients that compared to healthy controls following an initial search of a 17 Mi-RNA panel, that miR-146b was significantly upregulated in the acute stroke group and that miR-21 and miR-221 were significantly downregulated in sampled serum (Chen et al., 2018). Furthermore, miR-146b was moderately positively correlated with both infarct core ($r=0.51$) and NIHSS score (0.64). There was a particularly strong association between miR-146b levels and infarct cores $>3cm^3$ and NIHSS >5 ($p<0.001$) (Chen et al., 2018). The downregulation of miR-221 was also replicated by Jia et al. who also found a significant upregulation of miR-145 which again correlated moderately well with infarct volume and NIHSS scores ($r=0.63$ respectively) (Jia et al., 2015). Following an untargeted transcriptomic approach that identified 3100 differential Mi-RNAs

between acute ischaemic stroke and healthy controls, Li et al. narrowed the most sensitive Mi-RNAs down to 13 (mostly upregulated) molecules (Li et al., 2015). One RNA type miR-106b-5p has also been found to correlate with stroke in an additional study with up to 23 fold increase compared to controls and an AUC of 0.962 (Wang et al., 2014). An additional positive association of mi-RNA34a-5p with acute ischaemic stroke has been documented and interestingly, was found to negatively correlate with NIHSS score and infarct core volume, suggesting a neuroprotective effect, as the authors also claim (Liang and Lou, 2016).

One study followed patients up over a 2-year stroke recovery period, and found that peak upregulation of a 5 biomarker panel was during the acute phase. Unfortunately, this panel does not correlate with other documented studies (Sepramaniam et al., 2014). Thi heterogeneity of results is not uncommon in biomarker literature. Wu et al publishing the strongest correlation with miR-17-5p following multivariate logistic regression demonstrating a 9.9 fold increase (p=0.0002) (Wu et al., 2015). The biological complexity of identifying biomarkers for acute stroke is highlighted by the fact that although mi-RNA let-7e was identified by Sepramaniam in their biomarker analysis, it did not express fold change (p=0.000) compared to controls. Conversely the same molecule is suggested as a putative diagnostic marker by Peng et al (Sepramaniam et al., 2014, Peng et al., 2015) with a specificity of 73% and a sensitivity of 83% in acute stroke patients in the acute phase.

Two studies assessed the detection of Mi-RNA following haemorrhagic stroke. Leung et al. explored the relative concentrations of miR-124-3p and mIR-16 within 24hrs of both haemorrhagic (HS) or ischaemic stroke (IS). They found that in the acute setting (<6hrs from symptomatic onset), miR-124-3p levels were significantly higher in the HS group, compared to both IS and controls but that this difference did not persist in comparison to IS after 6 hrs. Conversely, it was found that mIR-16 levels were significantly higher in IS patients >6hrs but the groups could not be separated at time frames closer to the acute neurological event (Leung et al., 2014). Mi-RNAs have been used to differentiate SAH from healthy controls and miR-502-5p, miR-1297 and miR-4330 were significantly higher in SAH

patients. MiR-502-5p and miR-1297 were significantly higher in those patients with higher mRS scores and poorer clinical outcomes (Lai et al., 2017). (Refer to Table 1.4 for further study details).

1.7.6 Brain injury biomarkers

This section reviews the current literature for serological biomarkers of traumatic brain injury. The purpose is to provide insight into the pathological pathways researchers and research groups are pursuing and compare with the current stroke literature.

Only one study explored a molecule other than proteins in relation to the diagnosis of traumatic brain injury (TBI). Many studies assessed small molecules over the time course of a patient's admission, and although some are pathophysiologically interesting, particularly the assessment of markers of lipid peroxidation or levels of brain specific cholesterol (Bjugstad et al., 2016, Weiner et al., 2008, Scholpp et al., 2004), if initial blood samples were not taken with 24 hours of admission, the studies are not reviewed as their relevance to acute stroke diagnostics is less compelling.

Jeter et al. explored the relationship between branched-chain amino acids and TBI due to the knowledge that they are major source of nitrogen for glutamine production in the brain and are also integral to protein synthesis . They found a significant decrease in amino acids in patient groups with both mild and severe brain injuries compared to healthy controls, but a similar decrease in amino acids was also observed in patients who had undergone orthopaedic surgery (Jeter et al., 2013). (Refer to Table 1.5 for further study details).

1.7.7 Discussion

The search for a diagnostic biomarker for acute stroke as progressed beyond the search for a protein and the path is now leading towards tissue specific biomarkers such as neurotransmitters and amino acids. This is a logical step and follows an understanding of pathophysiological processes and utilises the progress made in analytical chemistry. Smaller molecules are also more compelling as they are more likely to cross or arise from the damaged blood brain barrier. To date, none of the reviewed molecules are clinically validated for acute stroke diagnostics and the results are too heterogeneous to make meta-analytical comparisons. This is particularly relevant to mi-RNA whereby the same molecules is indicated as a biomarker for stroke in one study but not found to be upregulated in another (Sepramaniam et al., 2014, Peng et al., 2015). There is further criticism of the mi-RNA approach as the tissue origin remains unclear (Li et al., 2015). It is possible that some of the mi-RNAs detected are produced by neurons or glial cells, but at present, this is not certain. However, hope for a brain specific mi-RNA is a possibility as they are known to be small enough to cross the BBB (Zeng et al., 2011) and as detailed above, some proteins are specific, or more highly prevalent, within the CNS. It follows therefore, that some RNA molecules may be specific, and this may hold for mi-RNA molecules that although not transcribed into proteins, are central to cell messaging.

Individual studies reporting on associations between acute stroke and IMA are reviewed in this section as a special consideration as it does not appear in the other protein biomarker reviews and is thought to hold interest to acute stroke research due to its ischaemic modulation. Like the other biomarkers reviewed in this section the results are heterogeneous and the inability for IMA to reliably differentiate between ischaemic and haemorrhagic stokes and further between acute stroke and those patients with established cardiovascular risk factors (such as type II diabetes) (Abboud et al., 2007, Gunduz et al., 2008) is a set-back in the pursuit of a IMA as diagnostic biomarker. It is essential for any acute stroke diagnostic marker to be able to differentiate between acute pathology and background atherosclerotic and/or other cardiovascular risk factors disease which is often the causative mechanism that leads to ischemic stroke.

Table 1.2: Summary of studies assessing neurotransmitters

Author	**Year**	**n**	**Study group**	**Controls**	**Sampling time from event**	**Upregulated**	**Downregulated**	**No statistically significant difference**	**Important features**
Castellanos et al.	2008	197	Ischaemic stroke	N/A	Admission	Glutamate, IL-6	N/A	N/A	Increased glutamate associated with ↑ DWI lesion volume

Table 1.3: Summary of studies assessing ischaemia-modified albumin

Author	**Year**	**n**	**Study group**	**Controls**	**Sampling time from event**	**Upregulated**	**Downregulated**	**Statistically significant difference**	**Important features**
Abboud	2007	118	Ischaemic stroke (n =84) ICH (n=18) TIA / seizure (n = 16)	N/A	Within 3 hrs	IMA	N/A	Between all 3 groups (p=0.009) Higher levels of IMA in stroke patients compared to TIA/seizure	Temporal trend analysis over 24 hrs – no significant difference
Gunduz	2008	149	Ischaemic stroke Parenchymal haemorrhage	Non-stroke ED patients	Admission	IMA	N/A	Between acute event (all cause) and control group (p<0.001)	No difference between ischaemic stroke and parenchymal haemorrhage

			Subarachnoid haemorrhage					Between SAH and ischaemic stroke (p<0.001)	
Herisson	2010	56	Ischaemic stroke Parenchymal haemorrhage	Healthy	Within 4.5hr	IMA	N/A	Between acute events and controls	No differen between ischaem and haemorrhag strokes
Ma	2011	97	Symptomatic lacunar infarct	Healthy controls Type II diabetic	Within 6hrs	IMA	N/A	Between stroke and both control groups (p = 0.044 and <0.001 respectively)	More sensitive th homocysteine

Table 1.4: Summary of studies assessing Mi-RNA

Author	**Year**	**n**	**Study group**	**Controls**	**Sampling time from event**	**Upregulated**	**Downregulated**	**Important features**
Chen	2018	230	Acute ischaemic stroke	Healthy controls	Within 24hrs	miR-146b	miR-21 mIR-221	Moderate correlation with infarct core and NIHSS
Jia	2015	60	Acute ischaemic stroke	Healthy controls	Within 24hrs	miR-145	miR-221 mIR-23a	Moderate correlation with infarct core and NIHSS
Li	2015	199	Acute ischaemic stroke	Healthy controls	Within 24hrs	miR-32-3p miR- 106b-5p miR-423-5p miR-451a miR-1246 miR-1299 miR-3149	miR-224-3p miR-377-5p miR-518b miR-532-5p miR-1913.	Initially investigated 3100 mi-RNAs Criteria for further investigation: • High fold change • Well documented • High expression & signal intensity

						miR-4739		
Liang	2016	199	Acute ischaemic stroke	Healthy controls	Within 72hrs	miR-34a-5p	miR-30a, miR-126, and let-7b	Negative correlation of mil 34a-5p with NIHSS score a infarct volume
Sepramaniam	2014	193	Acute ischaemic stroke	Healthy controls	Acute	miR-125b-2 miR -27a miR -422a, miR -488 miR -627	Panel of 58 mi-RNA	Over 2 year stroke recovery period, peak upregulation d acute phase.
Peng	2015	113	Acute ischaemic stroke	Healthy controls		miR-let7e		Highest expression in the ac phase

Wang	2014	136	Acute ischaemic stroke	Healthy controls	Within 24 hrs	miR-106b-5P miR-4306	miR-320e miR-320d	
Wu	2015	226	Acute ischaemic stroke	Healthy controls	Acutely (time window not specified)	miR-15a miR-16 miR-17-5p	N/A	miR-17-5p most significant
Leung	2014	93	Acute ischaemic stroke Haemorrhagic stroke	Healthy controls	Within 24 hrs	miR-124-3p miR-16	N/A	miR-124-3p highest in hyperacute haemorrhagic miR-16 highest in ischaemi stroke >6 hrs
Lai	2017	76	Subarachnoid haemorrhage	Healthy controls	Day 3	miR-502-5p, miR-1297 miR-4330	N/A	Blood samples taken at day post neurological insult

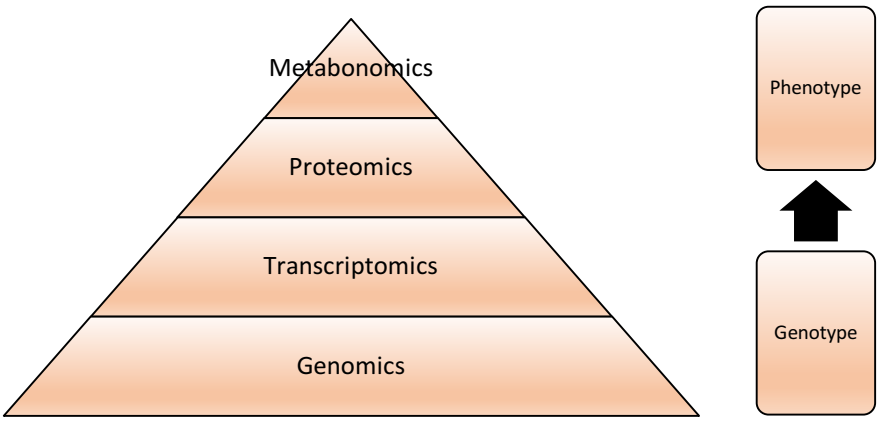
Table 1.5: Summary of the study assessing biomarkers of brain injury

Author	**Year**	**n**	**Study group**	**Controls**	**Sampling time from event**	**Upregulated**	**Downregulated**	**Important features**
Jeter	2013	73	Mild TBI (GCS >12) Severe TBI GCS (<8)	Healthy controls Orthopaedic admission	Within 24 hrs	Methylglutarylcarnitine	Valine Isoleucine Leucine	Methylglutarylcarnitine elevated in severe TBI patients compared to all other groups.

1.8 A summary of metabonomics and its techniques

Metabonomics is defined as 'the quantitative measurement of the dynamic multiparametric metabolic response of living systems to pathophysiological stimuli or genetic modification'(Nicholson et al., 1999). It is the study of small molecules (metabolites) typically <1 kDa and represents the analysis of the molecules present at the end of metabolic processes. The strength of metabonomics lies in its ability to analyse the end-products of metabolism that most accurately represent disease a phenotype.

Figure 1.13: A pyramidal representation of the relationship between metabonomics and other 'omic' disciplines



Metabonomics fits into the wider discipline of systems biology which attempts to understand biological process as a 'whole' from the species down to individual cells. It logically follows the study of genomics, transcriptomics and proteomics. Additional advantages are that there are approximately 7 000 metabolites, 25 000 genes, 100 000 transcripts and 1 000 000 proteins. Making the study of metabonomics attractive to the biochemist. It is naturally limited by concentrations that are often small and fluctuating. There are a few techniques employed for metabonomic analysis such as liquid/gas

chromatography coupled with mass spectrometry (MS), nuclear magnetic resonance spectroscopy (NMR) as well as matrix –assisted laser desorption ionization MALDI for tissue analysis.

Metabonomic analysis can take a non-targeted or targeted in approach. A targeted approach enables the profiling of specific metabolites that have been identified a priori. A non-targeted approach analyses as many metabolites as possible within a given biological specimen and biochemical separation methodology. A non-targeted approach is generally used to comment on peak metabolite differences between 2 biological or clinical states (Shah et al., 2012) and when there is benefit in keeping the search for potential disease-specific metabolites broad.

The following sections deal specifically with the analytical techniques used within this thesis and does not represent a comprehensive list of mass spectrometry or NMR spectroscopy techniques.

1.8.1 Mass spectrometry

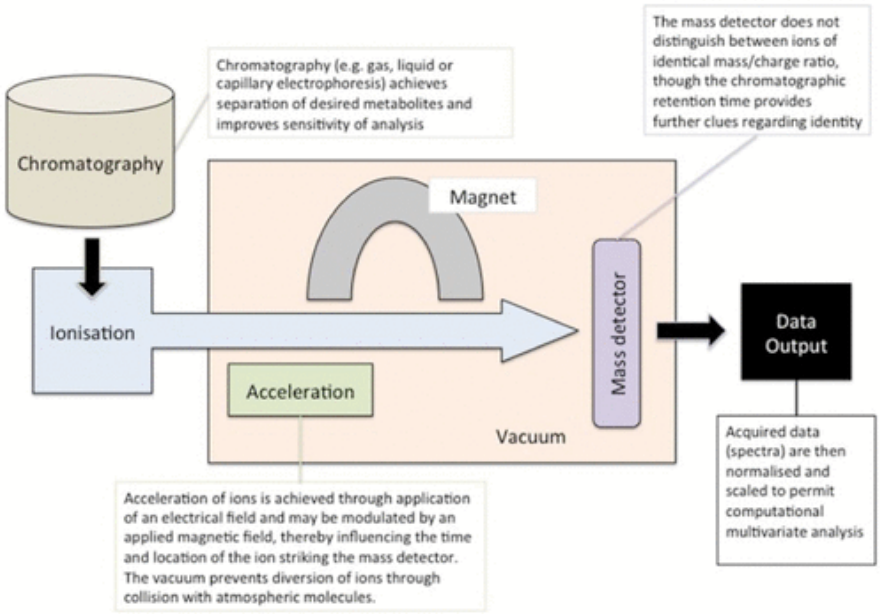
Mass spectrometry is a technique used to analyse compounds by their mass-to-charge ratio (m/z) and the abundance of such compounds. The essential components of a mass spectrometer are a sample inlet, an ionisation source, an accelerator, an electromagnet, ion detector and a data output software.

The liquid compounds are ionised in an ionisation chamber by electrospray ionisation (ESI) – the applications of an electric field - which results in compounds becoming charged. An aerosol is generated by the application of Nitrogen gas. A heated gas is then applied which leads to solvent evaporation. The droplets become smaller as the charges repel each other until the analyte enters the gas phase.

The ions created in the ionisations chamber move at speed within a vacuum and are deflected by the electromagnet according to their mass. The lighter the compound and/or the greater the charge, the

more the ion is deflected. The ions travel within a vacuum to avoid their course being interrupted by air molecules. The m/z of each compound is determined by TOF analysis. Ions are accelerated by an electromagnetic field of known strength and kinetic energy for each ion is the same. Velocity and therefore detection is dependent on the mass-to-charge ratio. Heavier ions reach lower speeds. The time it takes for an ion to reach a detector at a known distance is measured. Time depends on velocity and therefore upon m/z.

Figure 1.14: The process of mass spectrometry



To increase the likelihood of identifying compounds, mass spectrometry is often combined with a separation method or chromatography which is undertaken prior to MS analysis. For this thesis, the methodology employed was ultra-performance liquid chromatography (UPLC). The purpose of chromatography is to reduce the complexity of the samples and reduce the chance of ion suppression i.e. inadequately ionising compounds within a sample that inhibits detection by MS.

UPLC consists of a mobile phase and a stationary phase. The mobile phase consists of two solvents that continually run through the chromatography system and pushes the sample through the column. Two

solvents are used (A and B) so that varying mixture proportions can be used of the 'strong' and 'weak' solvent during chromataograhpy – this is known as gradient elution – that reduces the retention time of those compounds that would otherwise elute slowly.

The samples are stored in vials within an autosampler that selects the sample to be analysed which is then passed through the column. The column represents the stationary phase that separates out the sample. A non-polar stationary phase is used for reversed-phase (RP) liquid chromatography, which is the methodology employed for this thesis. A non-polar stationary phase interacts more strongly with non-polar more hydrophobic compounds within the mobile phase allowing more hydrophilic compounds to elute faster. RP chromatography has become an established separation method for lipidomics as it allows for greater subclass separation (Vorkas, 2018). The exact UPLC-MS protocols are discussed in the methodology section and detailed in the Appendix.

1.8.2 Nuclear magnetic resonance spectroscopy

NMR is concerned with measuring energy released and absorbed from nuclei that are subjected to a magnetic field. Energy shifts are created because the nuclei of most organic isotopes have property known as nuclear *spin* as they spin on their own axis. A spinning charge (inherent to nuclei) creates a magnet. When an external magnetic field is applied (B_0) the nuclei either align (alpha) or are opposed (beta) to the magnetic field. The relative proportions of protons and neutrons within a nucleus affects its overall spin.

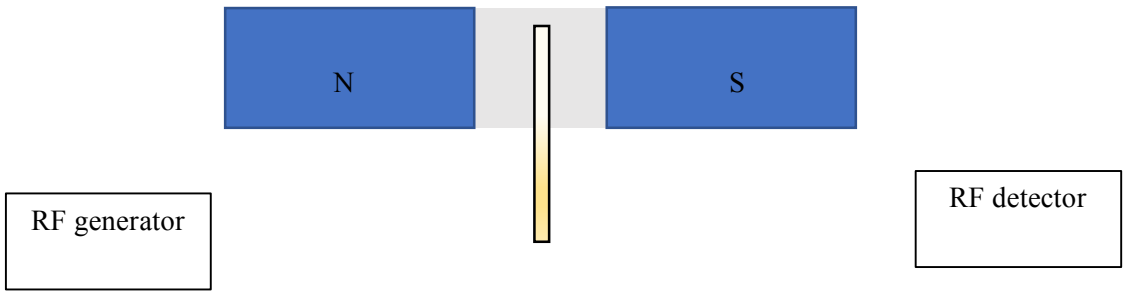
When a radiofrequency pulse is applied to the spinning atoms, nuclei in the low-energy alpha state will switch to the high-energy beta state. The energy difference between alpha and beta is known as ΔE. When the nuclei align with B_0 they are said to resonate with B_0. An NMR spectra captures ΔE and resonance signals are displayed as peaks on the NMR spectra. From these peaks, the composition and molecular structure of compounds can be determined. Protons that are surrounded by electrons due to

the chemical compound in which they exist, are shielded from the magnetic field by these electrons. Shielded protons resonate at lower frequencies at any given magnetic field strength. Hence, spectral differences are apparent on NMR spectra for different chemical compounds.

For the purposes of NMR spectroscopy, we are concerned the relative spin of hydrogen ^{1}H atoms. When an external magnetic field is applied to nuclei and they align in either alpha or beta states, they are said to be polarised. The ΔE detected by the NMR machine is dependent on the strength of the external magnetic field (a perpendicular radiofrequency pulse) and the composition of the target nucleus (its mass and charge). The difference between nuclear spin (angular momentum) and its related, generated magnetic field (magnetic moment) is called the *gyromagnetic ratio*, which is constant for each nucleus. The greater the magnetic field and/or the gyromagnetic ratio, the greater the energy difference. The energy difference is detected by the NMR spectrometer.

The NMR spectroscope is composed of a number of simple elements to facilitate the generation of an NMR spectra. They consist of a magnetic to generate a magnetic field, a radiofrequency generator and a detector. Samples are placed in a cylindrical tube within the magnetic field.

Figure 1.15: Basic components of the NMR Spectroscope



The spinning sample is placed within a magnetic field. Radiofrequency (RF) pulses are delivered to the sample. ΔE is detected by a detector coil and then by the RF detector. The resonance signals are then displayed as peaks on the NMR spectra.

The radiofrequency pulse can be applied to the magnetised sample in two different ways which splits the NMR spectrometer into two types. There are continuous wave (CW) and Fourier transform (FT) instruments. These instruments differ in the way that they alter the RF wave applied to the sample. The strength of the magnetic field needs to be altered to allow all protons within a sample to come into resonance as they will naturally alter their axis at different rates. In an FT spectrometer, energy is applied in short bursts that excites all protons within a sample at the same time. Conversely, with a CW spectrometer, the strength of the magnetic field is altered that allows atoms to be excited at different time depending on the degree of atomic shielding.

Most modern NMR spectrometers employ the FT methodology. This allows sample a period of relaxation between RF pulses whereby atoms return to their resting state. As the nucleus relaxes it emits electromagnetic radiation that is specific to a particular atom. As the atoms relax at different rates within a biological sample, a range of electromagnetic signals are emitted simultaneously. This is termed the free-induction decay signal (FID). The FID signal contains a combination of all the frequencies generated within a sample. Individual frequencies for each nuclei are extracted by performing a Fourier Transform analysis.

As previously mentioned, some nuclei will resonate within a biological sample at different frequencies based on electron shielding. As the differences in resonance are minute, to detect them a known reference compound must be used to provide a relative comparison point of known resonance. These compounds are usually trimethylsilylpropanoic acid (TSP) and tetramethylsilane (TMS). The resonances of the compounds can then be reported based on how far they shift from the reference compound. This shift can then be divided by the frequency of the spectrometer giving the resonant frequency of any given nucleus. The distance from the reference compound is known as *chemical shift*. Consequently, the interpretation of an NMR spectra gives information regarding the structure of a molecule as well as its immediate chemical environment.

1.9 Hypothesis and Aims

1.9.1 Hypothesis

1. Patients presenting to hospital with a hyper-acute stroke have a distinct metabolic profile from patients presenting with conditions that are considered ‘stroke-mimics’ and those patients who have not suffered an acute neurological deficit but have background atherosclerotic disease.

2. The metabolic profiles can be distinguished in serum.

1.9.2 Aims

1. To identify a potential diagnostic serological biomarker for stroke
2. To identify biomarkers differentiating hyper-acute ischaemic and haemorrhagic stroke.

2 LITERATURE REVIEW

2.1 The neurobiology of stroke

2.1.1 Introduction

This section updates a previously published systematic review by the (thesis) author on the known metabonomic associations with stroke (Qureshi et al., 2017).

As has been described in the Introduction chapter above, the pressing need for identifying a sensitive, specific and readily available biomarker for acute stroke is evident. Metabonomics is a methodology that has the ability to detect disturbances in metabolites of low molecular weight (<1kDa). As this low molecular weight favours movement of molecules across the blood brain barrier, metabonomics holds promise for the discovery of a diagnostic biomarker for acute stroke.

The purpose of this chapter is to review the current literature on the metabolic profiling of stroke.

2.1.2 Methodology

A systematic review was performed using PRISMA guidelines. Medline and Embase were initially searched on 29th August 2015 and an updated search was performed on 20th October 2020 using the search terms:

- [(metabonomics OR metabolomics) AND ("cerebrovascular attack" or CVA or stroke)].

The inclusion criteria were:

All full-length English language articles utilising MS or NMR spectroscopy 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

The PRISMA diagram for study selection is presented in Figure 2.1 below. Original studies were selected by 2 reviewers independently. The follow up search was performed by the thesis author.

All outcomes reported in each of the studies were analysed. Particular attention paid to statistically significant metabolites able to separate groups. Heterogeneity of results precluded meta-analysis. Pertinent results from each included paper are tabulated.

The quality of the diagnostic studies was assessed using recognized quality assessment tool for systems biology diagnostic studies QUADOMICS (Lumbreras et al., 2008a) which is an adaptation from a recognised bias assessment tool for diagnostic studies (QUADAS). Details are listed in Table 2.2. Ten studies are included in the quality assessment. All are phase I or phase II studies (Lumbreras et al., 2008b). Two of the newly identified studies are not included as they aim to differentiate between ischaemic stroke and haemorrhagic stroke (Zhang et al., 2017) or with cardioembolic stroke recurrence (Seo et al., 2018). No quality assessment tool exists for prognostic omics studies. Table 2.1 details the included studies and summarises their metabolic results.

2.1.3 Results

Since the initial search in 2015, 9 further studies (Hu et al., 2016, Sun et al., 2017, Wang et al., 2017, Liu et al., 2017, Seo et al., 2018, Sun et al., 2019, Tiedt et al., 2020, Sidorov et al., 2020, Zhang et al., 2017) have been published and a total of 15 are now included in the review.

The most significant update since the initial version of this review is the expansion of studies that aim to identify diagnostic metabolic biomarkers for acute stroke. In the initial review, only three studies had this aim (Kimberly et al., 2013, Jung et al., 2011, Jiang et al., 2011) but all additional studies with the exception of the Seo (Seo et al., 2018) and Zhang (Zhang et al., 2017) are focused on diagnostics. There has been a further progression with regards to the type of biofluid analysed by the studies. In the original review, all studies examined used serum/plasma and one study the addition of urine. However, since the original search in 2015, there are now three studies that have preferentially used whole blood dried blood spot sampling (DBS) (Sun et al., 2019, Zhang et al., 2017, Hu et al., 2016). All additional studies also approach the question of diagnostic biomarkers for acute stroke utilising MS rather than a combination of MS and/or NMR (Jung et al., 2011). A further difference between the previously identified studies and those added, is the number that report only upregulated metabolites. Previously, the studies reported both upregulated and downregulated metabolites. In the 5 year gap between the searches, there is now a greater emphasis on reporting upregulated biomarkers (Hu et al., 2016, Sun et al., 2017, Wang et al., 2017, Liu et al., 2017, Seo et al., 2018, Sun et al., 2019, Tiedt et al., 2020, Sidorov et al., 2020, Zhang et al., 2017).

Sidorov et al. took blood samples from acute stroke patients and followed those patients up for up to 5 months using a targeted LC-MS approach to identify amino acids. Whilst they did not identify significantly different levels of isoleucine or leucine they do report that the metabolic profile of acute stroke could be distinguished on the basis of upregulated metabolites: asparagine (p 0.45), tyrosine (p 0.015) and xylose (p 0.003) which returned to non-significant levels during 5 month follow up (Sidorov et al., 2020).

Wang found that glutamate was increased in the serum of acute stroke patients compared to controls but that a range of metabolites including tyrosine and tryptophan were significantly downregulated. In fact, when tyrosine, tryptophan and lactate were combined a 91.7% precision rate for acute stroke is reported (Wang et al., 2017).

One of the most clinically relevant studies published earlier this year is by Tiedt et al. (Tiedt et al., 2020) which attempts to metabolically separate acute stroke patients from those presenting with a stroke mimic using reverse phase liquid chromatography. Following an untargeted approach to metabolite discovery, four highly discriminatory metabolites were identified that are reported as being able to differentiate acute stroke from stroke mimics with an AUC of 0.9 with sensitivity of 82.5% and specificity 84.9%. The metabolites included in the study are adenosine (which was found to be acutely down regulated) and the upregulated metabolites (symmetric and asymmetric) dimethylarginine and pregnenolone sulfate (Tiedt et al., 2020).

The previously reported metabolic disturbances related to the homocysteine pathway are again found in one study (Zhang et al., 2017). It was previously reported that homocysteine levels were found to be elevated in the Jiang study (Jiang et al., 2011) and along with a panel of other metabolites, the model in this study was well fitted and predictive to differentiate acute stroke patients from controls (R^2Y = 0.998, Q^2 = 0.947). The downregulation of methionine is in keeping with the previous finding, albeit found when differentiating between ischaemic and haemorrhagic strokes rather than from a control group (Zhang et al., 2017).

2.1.4 Discussion

The new studies have continued the search for acute biomarkers of stroke and found – in the untargeted studies – markers that parallel the metabolic pathways initially reported. For example, the perturbations

in branched chain amino acids such as leucine, isoleucine (Kimberly et al., 2013) reported in the first review are again seen in the more current studies however, their associations are heterogeneous. Kimberley (Kimberly et al., 2013) reported a downregulation of leucine and isoleucine which corresponded with infarct size, but both leucine and isoleucine have been found as upregulated compared to healthy volunteers in both acute ischaemic and haemorrhagic stroke patients (Hu et al., 2016, Liu et al., 2017). Furthermore, there is greater evidence for metabolic alterations relating to amino acids regulation in the research published by Sidorov and Wang (Sidorov et al., 2020, Wang et al., 2017).

Sidorov found that after 5 months follow up, the initial metabolic disturbances returned to 'baseline' although the metabolic profile found during follow up was assumed to be each patient's pre-event state. Although the assumption of a return to baseline is premature without population data to support the hypothesis, the alteration of amino acids in response to acute stroke does seem to follow a trend in the literature that consists mostly of untargeted approaches to metabonomics.

The combined test of tyrosine, tryptophan and lactate published by Wang et al. (Wang et al., 2017) has compelling accuracy but the obvious criticism of this study is that the metabolites in question are unlikely to be tissue-specific and, though interesting, a highly accurate biomarker panel for downregulated metabolites is less compelling than an upregulated panel. To be clinically efficacious such an approach would require a baseline knowledge of normal levels and likely a plethora of causes for an altered metabolic state (including iatrogenic, environmental and genetic factors).

The Tiedt study is compelling as it attempts to metabolically separate acute stroke patients from those presenting with a stroke mimic using reverse phase liquid chromatography. The reported results are accurate and the authors make the valid point that the reported sensitivity and specificity are higher than for an unenhanced CT of the brain (Tiedt et al., 2020). Whilst this is true, the criticism of the 4-metabolite panel is that none of the metabolites are neuron / glial cell specific. Asymmetric and symmetric dimethylarginine are endogenous inhibitors of nitric oxide synthase which is implicated in

the acute pathophysiology of stroke, but could be elevated in other disease processes, particularly in other vascular diseases (Zobel et al., 2017).

Homocysteine is derived from S-adenosyl homocysteine which in itself is synthesized from methionine (Ansari et al., 2014) and it is hypothesised that elevated homocysteine levels are associated with oxidative injury to vascular endothelial cells (Jung et al., 2011, Qureshi et al., 2017). The knowledge of homocysteine metabolism, its association with oxidative stress, vascular disease and acute stroke explains the statistically significant down regulation of methionine in the Zhang study (Zhang et al., 2017). These findings help in the quest for pathophysiological consistency and, perhaps in the future, with further attempts at biochemical pathway development but they are less relevant to the initial discovery/development of a diagnostic blood test for acute stroke.

The increased use of DBS (Sun et al., 2019, Zhang et al., 2017, Hu et al., 2016) is interesting and an important methodological step to continue considering. The rationale for this approach is that relying on serum sampling and the complex sample preparation required for MS or NMR analysis may be too slow in hyper-acute stroke diagnostics. Any attempts to speed up the detection of acute metabolites would be a welcome addition to acute stroke diagnostics and treatment pathways.

A further interesting trend in the literature is the increase in reporting of metabolites that are more specific to cellular / membrane function than simply detailing generalized responses to ischaemia. In 2011, the upregulated biomarkers reported by Jung and colleagues (Jung et al., 2011) were generic such as lactate and pyruvate (serum) and nitric oxide (urine), albeit discovered by NMR. With the increasing trend of using MS techniques metabolites that could arise from damaged cell membranes are being discovered. Both Liu and Sun report significantly upregulated concentration of Lyso PE and PC both of which are highly abundant (Sun et al., 2017, Liu et al., 2017, Wurtman, 2009). The elevation of both PE and PC in stroke patients is likely not only related to the more sensitive MS technique compared to NMR, but also due to the time and circumstances of serum sampling. Blood samples were taken during an acute time frame in the Liu study during which time evidence of acute cellular membrane

dysfunction is more likely to be detected. Furthermore, in the Sun study, samples were taken from fasted patients 12 hours after their acute admission. With the knowledge that PC are not only derived endogenously but also acquired through diet (Wurtman, 2009), there is biochemical value in patients being fasted when the analysis is untargeted, even if fasted state blood sampling cannot be directly translated to clinical practice.

2.1.5 The evidence gap

Although slow progress is being made towards the potential identification of a serological or whole blood based biomarker for acute stroke, there are further questions that need to be asked and gaps in the evidence to fill.

It is becoming apparent that MS sample preparation and analytical techniques are being modified and advanced appropriately to explore metabolic insults following acute stroke. Lipid extraction methodology is thought to be adequate for current analytical techniques. To advance the study of acute stroke metabonomics further, it is necessary to address the question of appropriate control populations with which to compare acute stroke patients. With the exception of a small number of studies (Jove et al., 2015, Kimberly et al., 2013, Tiedt et al., 2020), that have compared stroke populations with patients who have suffered a TIA or stroke mimic condition, the remaining studies have stated their control population as ‘healthy controls’. This is a non-specific term and does not fully account for the control groups’ baseline metabolic profile. For example, comparing acute stroke patients with those who have varicose veins (even if age and sex matched) would be an unhelpful clinical comparison. A better comparison would be those patients who are known to have significant background atherosclerotic disease, but whom have not suffered an acute stroke or TIA. Such patients may be those with ischaemic heart disease, peripheral arterial disease or abdominal aortic aneurysms. Including such patients as a control group would advance metabolic enquiry. It would ensure that any positive results could reliably

separate acute metabolic disturbances from significant background atherosclerotic disease. A vital step for any clinically relevant diagnostic biomarker for acute stroke.

In addition to the separation of background atherosclerotic disease from acute stroke metabolic disturbances, there is a need to monitor each acute stroke patient's metabolic profile as it returns to baseline in the acute setting. This would require sampling serum at fixed time points from the index stroke event (including hyperacute blood tests) and, in effect, using each stroke patient as their own control. Such a comparison of temporal metabolic profiles with the profile of the index stroke event, coupled with a control group that has proven atherosclerotic disease would be a powerful exploratory study. It would also increase the likelihood of capturing biphasic blood brain barrier disturbance (Brouns and De Deyn, 2009) and the effects increased blood brain barrier permeability may have on a peripheral serological biomarker profile. Furthermore, more accurately observing acute elevations and subsequent reductions in relevant metabolites is more likely to lead to neuronal or glial specific metabolites involved in, or produced secondary to, ischaemia and oxidative stress.

Once the above methodology has been achieved and putative diagnostic biomarkers identified it would then be prudent to explore direct blood sampling further. This approach has fewer sample preparatory steps and is more likely to be successful using targeted metabonomics to identify elevations in specific metabolites identified in prior untargeted serological analyses.

2.1.6 Limitations

The limitations of this review are common to those of metabonomic studies. They include heterogeneity of sample collection both in terms of time and methodology employed. Results are limited to phase I and II studies and inconsistent matching of patient groups for vital baseline characteristics such as statin use and cardiovascular risk factors, which limits progression to real-world diagnostic test development. The small sample sizes also increase the risk of type I error. Furthermore, as stated in the original review

(Qureshi et al., 2017) the limitations of ‘scaling up’ any clinically validated biomarkers from metabonomic research includes high maintenance and infrastructure costs.

Figure 2.1: PRISMA diagram

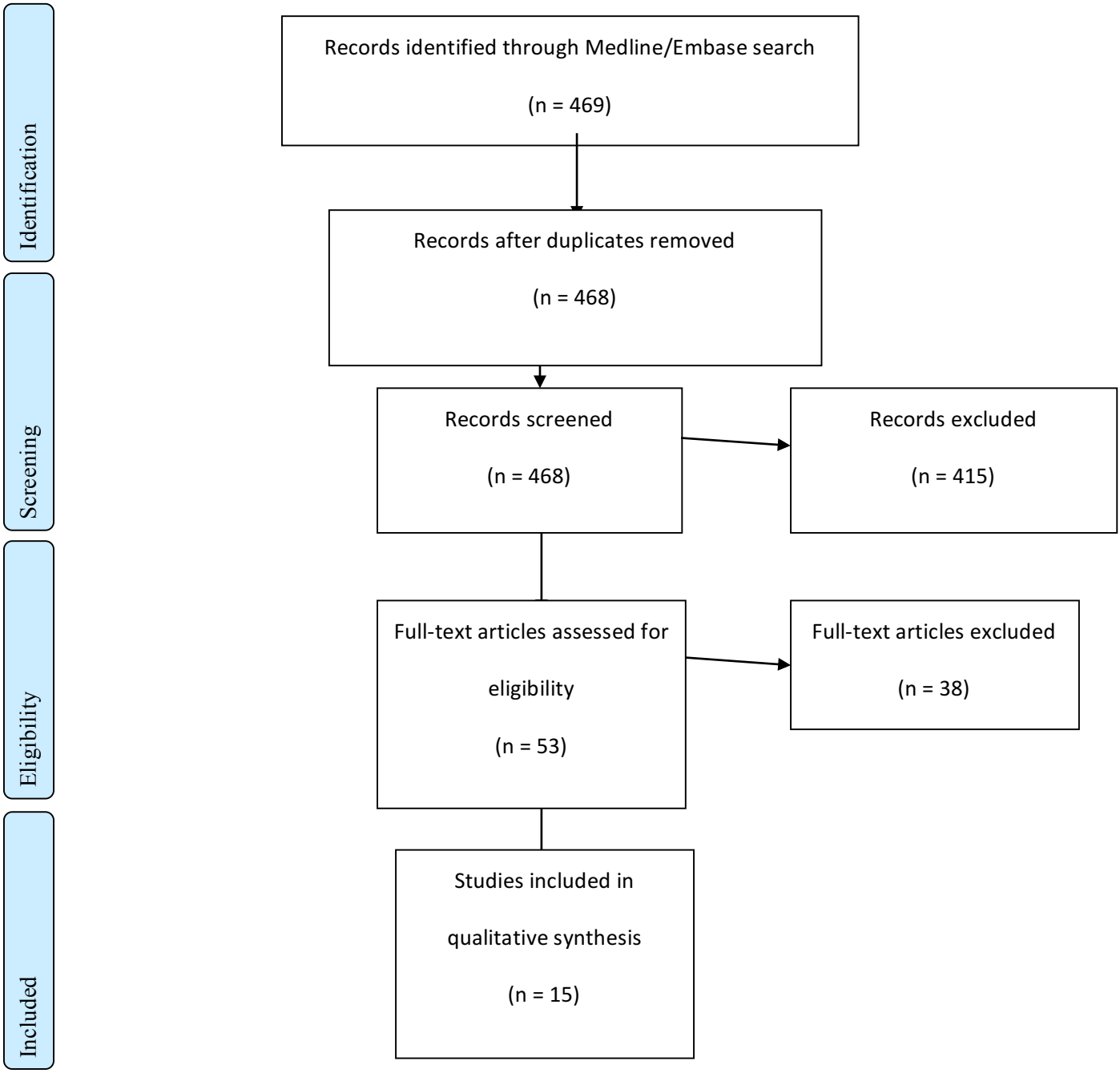


Table 2.1: Studies included for qualitative analysis

Author	Year	Origin	Subjects						Bio-Fluid	Spectroscopic technique	Targeted/ Untargeted	Upregulated Biomarkers	Downregulated Biomarkers
			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
									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
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 ([LysoPC[16:0])
Liu	2015	China	Stroke or TIA	Not stated	60	Healthy volunteers	20	Prestroke cognitive impairment Haemorrhagic stroke	Serum	Ultra–high-pressure liquid chromatography and time of flight MS		Carnitine Creatine Glutamate Proline N-acetyl-neuramininc acid	Citric acid Valine Isoleucine Tryptophan LysoPCs

								Reduced consciousness Aphasia or dysarthria Acute medical illness Cancer Infection				Hypoxanthine Uric acid Tyrosine Kynurenine Phenylalanine Sphingosine-phosphate Palmitoylcarnitine	
Ding	2016	China	Stroke	>14 days	55	Healthy volunteers	32	Antidepressant Mntal health disorder Alcohol excess Metabolims disorder Diabetes Neurological illness	Plasma	Gas chromatography MS	Untargeted	Aspartic acid	Palmitic acid Stearic acid Oleic acid Linoleic acid Phenylalanine Pyroglutamate Serine Proline Isoleucine Valine
Liu	2017	China	Stroke	Acute (timeframe not specified)	66	Healthy volunteers	63	None stated	Serum	Gas chromatography MS Liquid chromatography mass spectrometry	Untargeted	Serine Isoleucine Betaine PC (5:0/5:0) LysoPE (18:2)	Phosphatidic acid Phosphatidyllinositol Phosphatidylcholine Lyso-Phosphatidylcholine
Hu	2016	China	Ischaemic stroke Haemorrhagic stroke	<12 hours	129 73	Healthy volunteers	98	None stated	Dried blood spot	Direct injection MS	Targeted	Tyrosine Carnitine Aspragine Proline Valine Arginine/ornithine Leucine Phenylalanine Methionine Leucine Phenylalanine	Triglylcarnitine Acetylcarnitine Proprionylcarnitine Palmitoylcarnitine Petroselinic carnitine Methionine
Seo	2018	Korea	Stroke	Within 7 days	190	Healthy volunterrs	30	TIA On lipid lowering medication Atrial fibrillation Omega 3 supplement	Serum	Ultra-performance liquid chromatography-MS	Untargeted	Decanoylcarnitine Octanoylcarnitine Glutamine Aspartame acid Phenylalanine	NA
Sidorov	2020	USA	Acute stroke	Witihn 72 hrs	20	NA	NA	None stated	Serum	Liquid chromatography-mass spectrometry	Targeted	Xylose Asparagine Tyrosine	NA

			Chronic Stroke	5 month follow up					Urine			Glycine Acetylcarnitine	
Sun	2017	USA	Stroke	12 hours Fasted	30	Healthy volunteers	30	Autoimmune disease Pregnancy Serious brain disorders Immunosuppressed Regular analgesics Malignancy Liver/kidney failure	Serum	Ultra-performance liquid chromatography time of flight MS	Untargeted	Uric acid LysoPE (0:0/18:3) LysoPC (18:2) Bilrubin Sphinganine Linoelaidyl carnitine LysoPC(16:0) Adrenonylethanolamide	NA
Sun	2019	China	Stroke	Day 1	38	Vertigo	46	Malignancy Infection Autoimmune conditions Cardiovascular disease	Dried blood spot	Direct infusion mass spectrometry	Targeted	Arginine Citrulline Ornithine Carnitine	NA
Tiedt	2020	Germany	Stroke	Within 24rhs	508	Stroke mimics	349	Haemorrhagic stroke Malignancy Inflammatroy/infectious disease Surgery witihn 3 months	Serum	Ultra-performance liquid chromatography	Untargeted	Dimethylarginine Pregnenolone sulfate	Adenosine Proline Adeosine Guanosine Inosine
Wang	2017	China	Stroke	Unclear Fasted	40	Healthy controls	29	Renal disease Liver disease Malignancy Autoimmune disease Hypothyroidism	Serum	Gas chromatography-mass spectrometry	Untargeted	Lactate Carbonate Glutamate	Alanine Citrate Glycine Isoleucine Leucine Serine Tyrosine Methionine Tryptophan Erthronic acid Urea Purine Hypoxanthine Proline
Zhang	2017	China	Ischaemic stroke Haemorrhagic stroke	Within 12 hrs	129 128	Healthy controls	65	Infection Tumour Serious vascular disease	Dried blood spot	Direct injectiom MS	Untargeted	Ornithine Citrulline 3-hydroxyisovalerylcarnitine Arginine Glutarylcarnitine Phenylalanine Tyrosine	Decanoylcarnitine Acetylcarnitine Glutarylcarnitine Palimitoylcarnitine Valine/Phenylalanine Hydroxystearoylcarnitine Citrulline

												3-hydroxyisovalerylcarnitine Propionylcarnitine	Methionine

Table 2.2: Quality assessment of included metabonomic studies using QUADOMICS

QUADOMIC QUALITY ASSESSMENT CRITERIA	**Jung 2011**	**Jiang 2011**	**Kimberly 2013**	**Hu 2016**	**Liu 2017**
1. Were selection criteria clearly described?	YES	YES	YES	YES	YES
2. Was the spectrum of patients representative of patients who will receive the test in practice?*	N/A	N/A	N/A	N/A	N/A
3. Was the type of sample fully described?	NO	NO	YES	YES	YES
4. Were the procedures and timing of biological sample collection with respect to clinical factors described with enough detail?	YES	YES	YES	YES	YES
4.1. Clinical and physiological factors	NO	NO	NO	NO	NO
4.2. Diagnostic and treatment procedures.	NO	NO	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?	NO	NO	YES	YES	-
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?	NO	NO	YES	N/A	N/A
7. Is the reference standard likely to correctly classify the target condition?	-	YES	YES	YES	YES
8. Did the whole sample or a random selection of the sample receive verification using a reference standard of diagnosis?	-	YES	YES	-	YES
9. Did patients receive the same reference standard regardless of the result of the index test?	-	YES	YES	YES	YES
10. Was the execution of the index test described in sufficient detail to permit replication of the test?	YES	YES	NO	YES	YES
11. Was the execution of the reference standard described in sufficient detail to permit its replication?	NO	YES	YES	NO	YES
12. Were the index test results interpreted without knowledge of the results of the reference standard?	NO	NO	NO	NO	NO
13. Were the reference standard results interpreted without knowledge of the results of the index test?	YES	YES	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?*	N/A	N/A	N/A	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?	YES	YES	-	-	-

* Criterion applies to Phase 4 diagnostic study only

N/A = Not applicable

\- = Unclear

Table 2.2 continued (QUADOMICS)

QUADOMIC QUALITY ASSESSMENT CRITERIA	Sun 2017	Wang 2017	Sun 2019	Sidorov 2020	Tiedt 2020
1. Were selection criteria clearly described?	YES	YES	YES	YES	YES
2. Was the spectrum of patients representative of patients who will receive the test in practice?*	N/A	N/A	N/A	N/A	N/A
3. Was the type of sample fully described?	YES	YES	YES	YES	YES
4. Were the procedures and timing of biological sample collection with respect to clinical factors described with enough detail?	YES	YES	YES	YES	YES
4.1. Clinical and physiological factors	NO	NO	NO	NO	NO
4.2. Diagnostic and treatment procedures.	NO	NO	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	YES	YES	YES	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?	N/A	N/A	N/A	N/A	N/A
7. Is the reference standard likely to correctly classify the target condition?	YES	-	-	NO	YES
8. Did the whole sample or a random selection of the sample receive verification using a reference standard of diagnosis?	YES	-	YES	-	YES
9. Did patients receive the same reference standard regardless of the result of the index test?	YES	YES	YES	YES	YES
10. Was the execution of the index test described in sufficient detail to permit replication of the test?	YES	YES	NO	YES	YES
11. Was the execution of the reference standard described in sufficient detail to permit its replication?	YES	NO	NO	NO	YES
12. Were the index test results interpreted without knowledge of the results of the reference standard?	NO	NO	NO	NO	NO
13. Were the reference standard results interpreted without knowledge of the results of the index test?	YES	YES	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?*	N/A	N/A	N/A	N/A	N/A
15. Were uninterpretable/intermediate test results reported?	-	-	-	-	-

16. Is it likely that the presence of overfitting was avoided?	YES	-	-	-	-

3 METHODOLOGY

This section details the study methodology. It details the recruitment of acute stroke and stroke mimic patients via an acute recruitment pathway in the hyperacute stroke unit (HASU). It details problems encountered during recruitment of the acute participants and subsequent substantial amendments to the ethical approval.

In response to low recruitment numbers and an inability to collect serial blood for temporal trend analysis, sub-acute TIA patients as well as study control patients were recruited separately to the acute stroke recruitment pathway. This was to provide suitable groups for meaningful disease group analysis.

3.1 Study design

This is a prospective case-control study that occurred between July 2016 and August 2017. The study time was 13 months. Acute participants were recruited via a HASU. Following routine acute clinical investigation and subsequent clinical diagnosis patients were allocated to the following groups: acute ischaemic stroke or acute haemorrhagic stroke, acute TIA or non-stroke (stroke mimic).

3.2 Clinical Setting

The HASU at Charing Cross Hospital serves as a regional referral unit for Imperial College Healthcare NHS Trust within central London. All acute participants were recruited via the HASU. Control participants were recruited from a vascular outpatient clinic at Charing Cross Hospital. Sub-acute TIA patients were recruited from a dedicated TIA clinic at Charing Cross Hospital.

3.3 Sample size calculation

At the time of the study no recognised methods exist for calculating sample size for untargeted metabonomic studies. No diagnostic metabolites exist for acute stroke. Knowledge of metabolic

disturbances following acute stroke in humans is limited and potential metabolites are numerous. Thus, the study was guided by experts within the Department of Computational and Systems Medicine namely Professor Elaine Holmes.

3.4 Ethical Approval

Ethical approval for the study was granted by London-Camberwell St Giles Research Ethics Committee on 16 May 2016. REC Reference: 16/LO/0528. The ethical approval included satisfying the requirements of the Mental Health Act 2005 for recruiting participants who lack the capacity to consent. Where a participant lacked the capacity to consent, enrolment into the study was sought via either a personal or nominated consultee.

In accordance with Section 32 of the Mental Health Act 2005, personal consultees are individuals who know the person who lacks capacity in a personal capacity and are able to advise researchers about the wishes and feelings of the person who lacks capacity in relation to the research project.

The view of a nominated consultee can be sought should a personal consultee not be identified. A nominated consultee is a person independent of the project who is appointed to the role in accordance with the Department of Health's guidance on nominating a consultee for research involving adults who lack the capacity to consent. For the purpose of this study, A&E consultants who are not involved in the care of hyper-acute stroke patients were enrolled as nominated consultees.

Examples of participant consent and withdrawal forms as well as consultee declaration forms can be found in the Appendix.

3.5 Participant recruitment

Subject to informed consent or consultee declaration, patients were recruited into the following groups:

1. Patients presenting within 6 hours of onset of ischaemic stroke
2. Patients presenting within 6 hours of a haemorrhagic stroke
3. Thrombolysis-call patients whose final diagnosis is non-stroke

Pre-registration evaluations:

1. Be admitted to Charing Cross Hospital with a suspected stroke
2. Not have received acute treatment/medication prior to arrival in hospital
3. Be clinically assessed by a junior member of the stroke team as part of routine NHS care

Inclusion criteria include:

1. Adult patients presenting to hospital within 6 hours of onset of neurological symptoms
2. Age over 45 years

Exclusion criteria include:

1. Active malignancy
2. Preceding stroke within six months
3. Administration of thrombolysis within six months
4. Seizure
5. Sepsis
6. Pre-menopausal women
7. Taking novel medication for a clinical trial

3.5.1 Rationale and definitions for inclusion / exclusion

An age restriction to recruitment was established to attempt to reduce variations in metabolic profile that are influenced by age. Participants were recruited within 6 hours of neurological symptoms because this covered the 4-hour thrombolysis window and aligned with admission criteria at Charing Cross Hospital.

Pre-menopausal women were excluded from the study to reduce the likelihood of metabolic differences.

Acute neurological symptoms were defined as a loss of normal neurological function and were reliant on ambulance crews identifying patients with a suspected stroke. Those symptoms as described by the F.A.S.T campaign are an example. Confirmation of neurological deficit was performed by expert stroke clinicians and Charing Cross Hospital.

Active malignancy was defined as one for which the patient was either actively under surveillance or receiving treatment.

Sepsis was defined as ‘ a life threatening organ dysfunction due to a dysregulated host response to infection’ (NICE, 2016) and was diagnosed by expert and appropriately trained stroke clinicians.

3.5.2 Serum Extraction and Storage

Blood was collected from each patient prior to the administration of any medication, and in particular, thrombolytic therapy. Blood is routinely collected within minutes of the thrombolysis-call patient’s arrival to the emergency department and an additional 12mls of blood was taken during the same needle stick using the same vacutainer set. This minimised the time diverted for research during time-critical acute clinical investigations for stroke. The blood samples were taken by the admitting clinical team.

To streamline research blood sample taking and the patient's timeline for acute investigation, the additional research blood samples were taken prior to the patient's consent. Written consent for inclusion in the study was gained as soon as possible and within 24 hours.

Those patients that did not have or regain the capacity to consent were enrolled into the study following personal or nominated consultee approval. If the patient refused consent to be enrolled in the study or a consultee felt that the patient would not wish to be involved all research serum samples taken at the point of admission were destroyed.

In the event that a participant whom initially lacked the capacity to consent and was enrolled into the study via consultee approval and subsequently regained capacity during their acute admission, informed consent was gained from the participant. If they wished to be withdrawn from the study, a withdrawal declaration was signed and their samples destroyed.

3.5.3 Temporal trend metabolic signatures

To determine the time-dependence of statistically significant biomarkers it was planned that a sub-group of participants would have additional blood samples taken at structured intervals from the time of onset of symptoms. These planned intervals were: 6, 12, 24 and 48 hours. This section of the project was not completed and explanations are given below.

3.5.4 Serum extraction

Following venepuncture and blood collection in serum silica blood vials, the blood samples were permitted to clot for 20-30 minutes prior to being centrifuged to separate the serum from the heavier cellular pellet. The samples were centrifuged using a Hettich EBA 20 centrifuge at 5800rpm for 10

minutes. Serum was pipetted into 1ml aliquots into 2ml eppendorfs and immediately frozen at -80^{o}C. Samples were kept frozen until required for metabonomic analysis.

3.5.5 Data Collection

Baseline data and meta-data relating to the acute symptomatic episode were recorded on pre-designed pro forma. Including the results of flood blood count, biochemical profile and lipid profile. The results of acute CT /MRI imaging were recorded. Past medical history, including smoking status and medication history was accurately documented. Smoking status was defined as 'current' for current smokers, 'ex-smokers' for those who had successfully stopped smoking and 'non-smoker' for those who had never smoked.

Those recruited participants whose final diagnosis was stroke had their baseline National Institutes for Health Stroke Scale (NIHSS) recorded. This is an established stroke severity scale that ranges from 0 – 42. The larger the recorded number on the scale, the greater the initial neurological deficit. In terms of severity, strokes can be classified as the following:

- Very severe >25
- Severe 15-24
- Mild to moderately severe 5-14
- Mild 1-5

The blood baseline parameters recorded were:

- Haemoglobin
- White cell count
- Lymphocytes

- Neutrophils
- Urea
- Creatinine
- CRP
- ESR
- Total cholesterol
- High density lipoprotein
- Low density lipoprotein
- Triglycerides
- Total cholesterol: high density lipoprotein ratio

Diagnoses recorded in the patient's past medical history were taken to be accurate if clearly recorded. It is assumed that diagnoses within each participant's past medical history were accurate for the purpose of the study. Long term follow up and serial testing was not performed. For example, it is assumed that NICE guidelines for the diagnosis of hypertension were followed, prior to reaching a documented diagnosis of hypertension.

3.5.6 Problems and Solutions

It was evident from early recruitment that high numbers of patients presenting to the Hyper-Acute Stroke Unit with a suspected acute stroke had awoken with symptoms having gone to bed the previous evening asymptomatic. Clinically, such patients are classified as having 'wake-up strokes'. These patients could not be recruited under the initial research protocol approved by the ethics committee, as the time of stroke onset could not be verified.

A second group of patients who were initially excluded were those with prevalent prostate cancer. However, once recruitment had commenced, it was evident that excluding these patients may bias the results as fewer men would be eligible for recruitment.

To overcome these two problems, an amendment was successfully submitted to the ethics committee that enabled the recruitment of patients diagnosed with a wake-up stroke and men with prevalent prostate cancer. Ethical approval for the substantial amendment was granted on 3 February 2017.

Another limiting factor for the research, was a Trust policy to limit phlebotomy from those patients who had received thrombolysis. As the ethical approval did not deal specifically with the increased risk of bleeding in this group of patients, serial blood tests were not attempted and hence the planned temporal trend metabolic signature analysis could not be performed. An additional recruitment limitation would also have been not having access to appropriate laboratory equipment out of hours. For the aforementioned reasons, the originally planned serial blood test analysis could not be performed. An attempt to mitigate against this recruitment obstacle was made by recruiting TIA patients from the TIA clinic.

3.5.7 Final acute recruitment inclusion and exclusion criteria:

Inclusion criteria include:

3. Adult patients presenting to hospital within 6 hours of onset of neurological symptoms or wake up stroke
4. Age over 45 years

Exclusion criteria include:

8. Active malignancy (other than prostate cancer)

9. Preceding stroke within six months
10. Administration of thrombolysis within six months
11. Seizure
12. Sepsis
13. Pre-menopausal women
14. Taking novel medication for a clinical trial

3.5.8 Inclusion of TIA patients

Due to recruitment pressures in the acute stroke cohort and the pathological relevance of comparing the metabolic profile of patients with sub-acute transient ischaemic attacks (TIA) (occurring more than 24 hours before clinical review) with those who had an acute-TIA and hyper-acute stroke, approval was sought from the neurology team at Charing Cross Hospital to recruit patients from the TIA clinic.

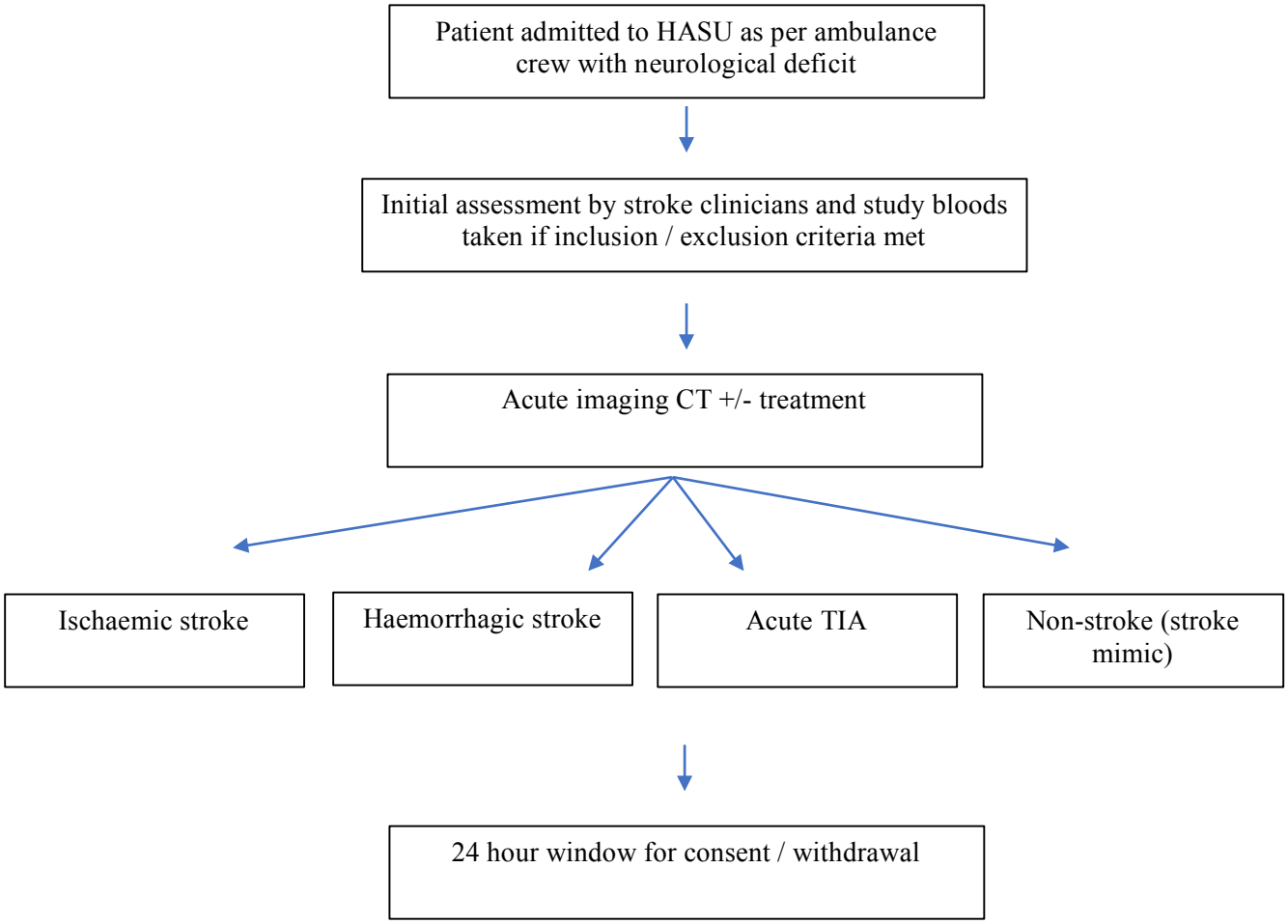
These patients were recruited as a separate cohort to the acute cohort and therefore revisions to the inclusion and exclusion criteria listed above were not necessary. Participant recruitment was performed under existing ethical approval for Investigating Genome-Phenotype Correlations for Human Vascular Diseases (REC reference 13/EM/001). Additional blood tests were taken from patients who attended the TIA clinic and had proven carotid atherosclerosis on carotid duplex sonography. Patients who had increased intima-media thickness without evidence an atherosclerotic plaque were not recruited. This was a requirement stated in the ethical approval for the Genome-Phenotype project.

Blood samples were taken on a participant's arrival to the TIA clinic and prior to final diagnosis. TIA diagnosis was based on the clinical criteria of a transient neurological deficit due to focal brain, spinal cord or retinal ischaemia. Those patients who were diagnosed by expert stroke clinicians as having a pathology other than TIA were grouped as non-TIA. Blood samples were retained for future analyses. Baseline characteristics were collected for each participant as described above.

3.5.9 Justification for control group

The American College of Cardiology Foundation/American Heart Association Task Force definitions of peripheral atherosclerotic vascular disease include aneurysmal disease of the aorta within the definition of PAD (Writing Committee to Develop Clinical Data Standards for Peripheral Atherosclerotic Vascular et al., 2012). This grouping is used within this theses for the recruitment of non-acute control participants who are known to have peripheral arterial disease or an abdominal aortic aneurysm. It was felt of scientific importance to use patients with proven atherosclerotic disease as a control group to establish that any statistically significant metabolites could differentiate acute pathology from background atherosclerotic disease. This is an effort to advance the metabonomic enquiry of acute stroke beyond the known published data as explained in the 'evidence gap' section in Chapter 2.

Figure 3.1: Flow diagram of acute participant recruitment and groups



3.6 UPLC-MS Lipidomics

3.6.1 Lipid extraction

Following serum sample thawing an established lipid extraction process was undertaken before reconstituting the extract lipids into a solution suitable for mass spectrometry analysis. A detailed point-by-point summary is detailed in the Appendix II.

Serum samples were aliquoted into 100µl in Eppendorf tubes. In addition, a pooled sample was made by mixing 100µl of each sample into the same glass vial. The pooled sample was then aliquoted into 10 x 100µl Eppendorf tubes. Samples were prepared in batches of 30 that included 2 blank samples (no biological sample) and 1-2 pooled samples. 700 µl of pre-chilled (-20C) organic solvent mixture (Methyl tert-butyl ether/methanol (3:1)) is added to the eppendorf tubes. The solutions were vortexed for 5 minutes using a multimixer before being centrifuged for 30min (4^oC) at 20,800G. 600 µl of the supernatant for each sample was decanted into a clean Eppendorf, with careful pipetting to ensure that the bottom aqueous layer was avoided. All new Eppendorf tubes were then loaded into an open SpeedVac to evaporate the organic solvent. This process was continued until the samples were dry without temperature adjustment (for 2-3 hours). Extracts were stored at -40^oC until reconstitution.

3.6.2 Sample reconstitution

Following lipid extraction, samples were reconstituted to allow mass spectrometry analysis. 150µl of cooled (4^oC) organic solvent mixture (isopropyl alcohol/acetonitrile/water (3:1:1)) was added in the eppendorf tubes of the lipid extracts. The samples were submitted to intense vortexing for 5 minutes using a multimixer. The samples were centrifuged for 30min (4oC), 20,800G. Following this, 120µl of the reconstituted sample were decanted into a new clean LCMS vial, with careful technique to

avoid decanting the bottom pellet). A pooled sample was prepared by decanting 20 µl of each reconstituted sample.

Samples were randomised (using MS excel random number generator) and placed on racks for mass spectrometry analysis.

3.7 NMR

Following transfer from Charing Cross Hospital, the frozen serum samples were randomised. To be suitable for NMR analysis the samples required pre-processing. Samples were centrifuged at 13 000 rpm for 10 minutes. 300 µl of serum was then transferred to an Eppendorf tube. A 300 µl volume of a pre-prepared buffer solution (detailed in the Appendix III) was added to the mixture before the serum and buffer were mixed by vortexing. Following vortexing, the samples were centrifuged for 10 seconds to ensure appropriate separation. Following this , 580 µl of the supernatant was pipetted into a glass NMR tube and sealed, ensuring that the liquid rested at the bottom of the tube. In addition to the above, at the same time as serum extraction, 50 µl of each sample was added to a pooled quality control sample, that can be run periodically during experimentation that enables adequate monitoring of machine functioning.

3.7.1 NMR sample analysis

A ^{1}H NMR 600 MHz Bruker spectrometer was used to process the samples for NMR analysis. A 1D pulse sequence was used with water suppression. To ensure appropriate temperature monitoring, water suppression testing and continual spectrometer performance, Topspin 3.1 software was used. Spectral files were downloaded to an external hard drive and analysis undertaken on MATLAB.

Once data was uploaded into the MATLAB software, pre-processing of the data prior to statistical analysis was undertaken. It is necessary to remove water from the NMR spectra as it produces a large wide peak that could supress smaller metabolites that would otherwise be obscured from analysis. This process is known as cutting. Additionally, it was necessary to scale (normalise) the signal intensities and to align any superimposition of spectral peaks that may have shifted during experimentation.

Following pre-processing, the data were analysed using SIMCA (Umetrics) software – a well recognised statistical programme for metabonomic analyses.

3.8 Statistical and Analytical Techniques

The inherent difficulty with the results obtained from metabonomic techniques, is that there are many more variables (metabolites) than observations (samples/patients). To find patterns in the data, the basis of multivariate data analysis is Principal Component Analysis (PCA).

PCA involves new axes being drawn through the dimensionality of data to derive meaning from the results whilst maintaining variation. The analysis takes place in K- dimensional space (a higher dimensional space). The first principal component (PC1) is the line drawn through the data in K-space, that is a line of 'best-fit' through the data that spreads the data along an axis that results in maximal variation. The second principal component (PC2) is drawn orthogonal to PC1. These new axes become new X and Y axes for the data set. PCA is an unsupervised statistical method, meaning that class identification of observations is not applied to the analysis.

The second commonly used statistical technique used following PCA is orthogonal partial least squares projections to latent structures-discriminant analysis (OPLS-DA). Briefly, OPLS-DA is the regression extension on PCA; it attempts to connect the information between *X* and *Y*. In metabonomics datasets, *X* is usually spectral data and *Y* is the response. OPLS-DA is performed with the assumption that there

is an inherent connection between *X* and Y and is an extension of partial least squares projections to latent structures (PLS) whereby new variables are created by projecting latent variables from data points onto new lines that well approximate the point swarm and provides correlation with *Y*.

In PCA R^2X and Q^2 relate to the performance of the model in relation to the X-data (the predictors) and in OPLS-DA the values represent performance of the model in relation to the Y-data.

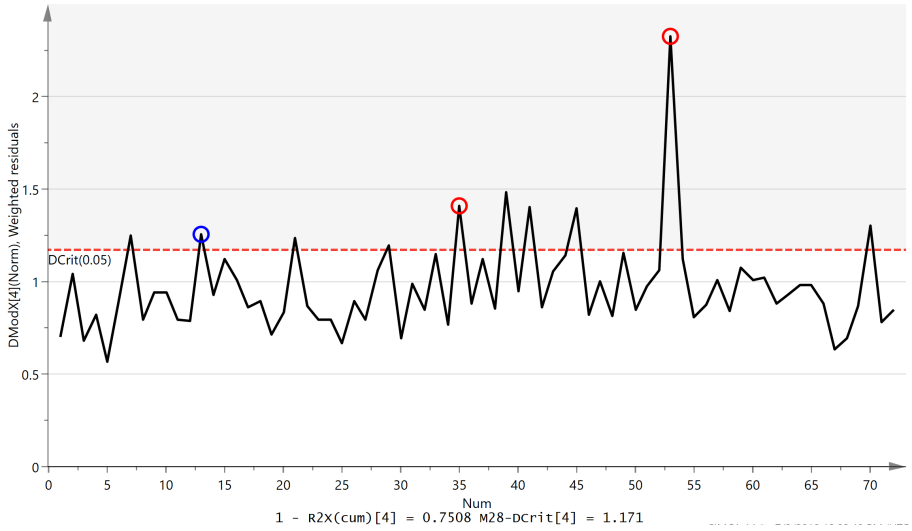
3.8.1 Identifying outliers

Once PCA is complete, there are inevitably outliers present within the dataset. Outliers may result from pathological mechanisms that render certain metabolic profiles divergent from the rest of the data. Or, they may result from methodological problems that occurs during sample preparation or the running of MS and H^1-NMR.

Potential outliers are observed by existing beyond the 95% confidence interval marked by the Hotelling's ellipse. Some or all of these outliers may skew results and effect the predictability of the model.

Decisions regarding which outliers to remove from the PCA as *true* outliers were made by examining the *distance to model* (DModX). If both criteria are fulfilled (outlying beyond the 95% confidence interval and being significantly distant from the model) the samples in question were removed from the analysis.

Figure 3.2: Example of DModX



Those metabolites highlighted that are found to be above the DModX line and additionally beyond the 95% confidence interval on PCA. Such metabolites were removed from the analysis.

Data points that are shown to be above the red line (*DCrit*) indicating the maximal tolerable distance from the model were removed from the PCA dataset for each model. They were not removed from the complete dataset as the same sample may not be an outlier when a different question is asked of the data in subsequent models.

3.8.2 Scores and loading plots

The scores plot displays the relationship of all variables (metabolites) to one another at the same time. Variables that contribute to similar data are grouped together; those that are inversely proportional are diagonal to each other. The further away the variable lies from the plot origin, the greater its impact of the model. Loading plots are used to interpret the scores plot. They unravel the magnitude (large/small correlation) and the manner (positive/negative correlation) in which the variables contribute to the scores (principal components).

The scores plot details which participant serum samples are related to one another based on their metabolic profile. The loadings plot provides further information on which metabolites may be responsible for the separation.

3.8.3 Scaling

Scaling data is an important step prior to modelling to remove the effect of those metabolites with larger values causing the data to be skewed. The method used is Pareto scaling (by dividing each variable by the square root of its standard deviation). Logarithmic transformation is additionally used to reduce the impact of noise and high variance of the variables.

4 RESULTS: POSITIVE MODE MASS SPECTROMETRY

This chapter details the results of positive mode mass spectrometry analysis. The recruitment numbers and the baseline characteristics for the study participants are detailed below and should be used as a reference point for the two further results chapters.

Table 4.1: Recruitment numbers

Patient type	**Number**
Participants attending HASU	39
Ischaemic stroke	*12*
Acute TIA	*9*
Haemorrhagic stroke	*8*
Other pathology	*10*
Participants attending TIA clinic	17
Confirmed TIA	*9*
Non-TIA	*8*
Study controls	34
Total	**90**

Table 4.2: Baseline characteristics

	Any stroke (n=20)	**Any TIA (n=18)**	**Any Control (n=52)**	**p value**
Male - No (%)	12 (60)	12 (67)	34 (65)	0.89
Age -yrs (SD)	69 (±12.46)	71 (±9.53)	72 (±9.78)	0.51
BMI	25 (±4.85)	27 (±4.95)	26 (±4.69)	0.45
Statin use - N^o (%)	9 (45)	8 (44)	33 (63)	0.21
Antiplatelet use - N^o (%)	7 (35)	9 (50)	22 (58)	0.22
Smoking history - N^o (%)	9 (45)	7 (39)	38 (73)	0.011*
Total cholesterol – Mean (SD)	4.6 (±0.33)	5.4 (±0.33)	N/A	0.11
HDL (SEM)	1.2 (±0.09)	1.4 (±0.11)	N/A	0.23
LDL (SEM)	2.8 (±0.27)	3.1 (±0.30)	N/A	0.35
Total cholesterol:HDL ratio (SEM)	3.98 (±0.37)	4.15 (±0.3)	N/A	0.75
Triglycerides (SEM)	1.46 (±0.32)	1.86 (±0.39)	N/A	0.43
Diabetes – N^o (%)	1 (5)	4 (22)	12 (23)	0.19
IHD - N^o (%)	4 (20)	1 (5)	12(23)	0.26
AF - N^o (%)	3 (15)	2 (11)	7 (13)	0.94
Hypertension - N^o (%)	8 (40)	9 (50)	37 (71)	0.034*
ETOH units/week – median (IQR)	0 (22)	10 (22)	2 (14.25)	0.52
Strokes with positive imaging – N^o (%)	9 (45%)	N/A	N/A	N/A
Mean NIHSS (SD)	10 (±8.2)	N/A	N/A	N/A

3 groups compared: Categorical variables using Chi Square; Continuous variables using ANOVA (parametric data) or Kruskal-Wallis (non-parametric data). Normal distribution tested using Shapiro-Wilk test normality test. 2 groups compared: Unpaired T-test.

4.1 Demographic data

Of those patients that were recruited via the HASU 39 participants were successfully recruited into pre-specified groups. During the course of the recruitment period, 78 patients were excluded from the study.

Overall, only 33% of all patients admitted to HASU were successfully recruited. The reasons for exclusion are detailed in Appendix XV.

12 (31%) of the HASU recruited participants had an ischaemic stroke and a further 9 participants (23%) had an acute TIA. Overall, 54% of patients suffered an acute ischaemic cerebral event. None of the TIA patients had evidence of acute ischaemia on unenhanced CT scans. Only 1 of the acute TIA patients had evidence of subacute infarctions on follow-up MRI. As a direct comparator, none of the sub-acute TIA patients recruited from the TIA clinic had positive imaging findings.

Of all stroke participants recruited, 9 (45%) had positive imaging findings on unenhanced CT head. The remaining participants who had negative findings had suffered an ischaemic stroke (by definition a haemorrhagic stroke requires positive image findings) and the diagnosis of ischaemic stroke was made on clinic grounds by experienced stroke clinicians.

Of those patients that suffered an acute stroke (ischaemic or haemorrhagic) the mean NIHSS score was 10. Therefore, by definition, the stokes suffered by participants in this study were of moderate severity.

Regarding those participants recruited via the TIA clinic, 9 (53%) were clinically diagnosed as having a TIA. Those participants that did not have a TIA were diagnosed with either pre-syncope or non-TIA visual disturbances.

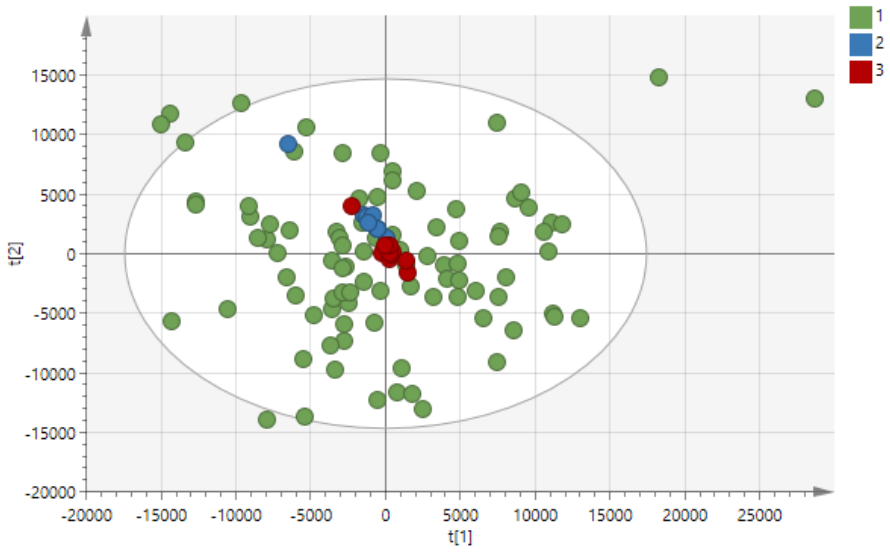
Across the groups presented in the baseline characteristics in Table 4.2 above, only smoking status and hypertension are found to be significantly different confounders. For an acute stroke cohort these are significant and have a direct impact on cardiovascular risk and likely on metabolic profile. These confounding factors are studied further on in this chapter and the subsequent results chapters with other statistically significant confounding variables that became evident as the data were formed into groups for PCA and OPLS-DA analytical models.

The remainder of this chapter is dedicated to positive mode mass spectrometry results.

4.2 Positive mode MS results

Initial modelling ensured that the quality control samples (QC) were well group and centred. Logarithmic transformation improved the analysis by reducing the impact of variations for statistical analysis.

Figure 4.1: PCA of all samples following Pareto Scaling



1 = Samples; 2 = Pooled samples (formed prior to lipid extraction; 3 = QC samples (from reconstituted samples following lipid extraction)

It can be seen above that the QC samples are reasonably well grouped. Following Pareto scaling the metabolites can be represented on an S-plot. The samples at the extreme ends of the S-plot have the greatest impact on separating out the two groups in each model. Those metabolites towards the centre

of the plot contribute the least. Individual metabolites can be selected from the S-plot and their upward or downward regulation determined.

Figure 4.2: S-plot of Acute Admissions Vs Controls

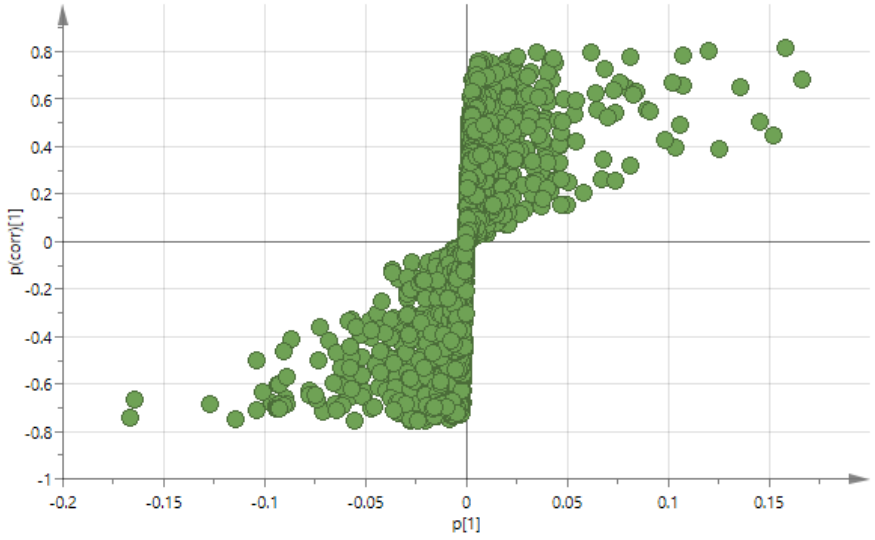
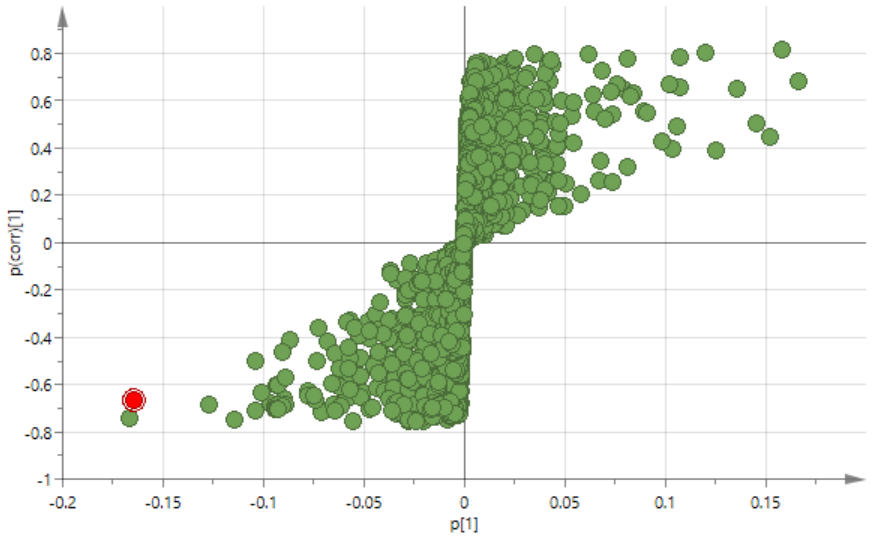


Figure 4.3: S-plot with a phosphatidylcholine highlighted



The models discussed in this chapter attempt to address important clinical questions and find metabolic consistencies with related underlying pathological processes i.e. some models group ischaemic events (stroke, acute TIA and subacute TIA) together to investigate the presence of unifying ischaemic metabolites.

Each model is presented in detail below and in each of the following results chapters, the models are replicated. A summary of each of the OPLS-DA model characteristics is found in Table 4.3. The statistically significant models were explored further.

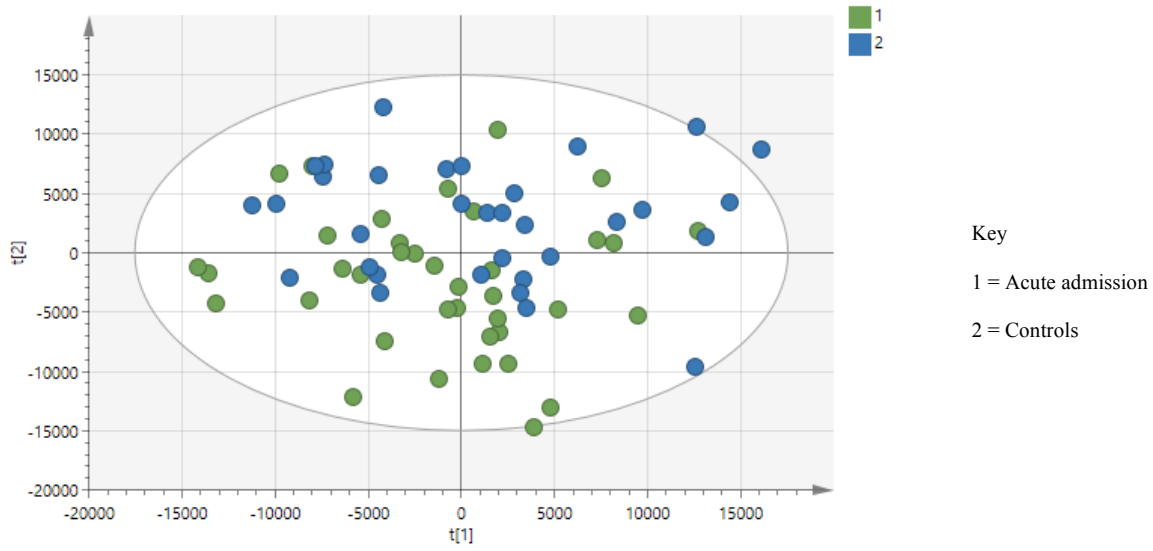
Table 4.3: Summary of positive mode mass spectrometry OPLS-DA models

Model		**n**	**R^2Y**	**Q^2**	**p**
Disease Group	**Control Group**				
Acute admission	Study Controls	73	0.338	0.137	**0.00614589**
All strokes	Study Controls	52	0.67	-0.288	1
Stroke & TIA*	Study Controls	72	0.281	0.0893	**0.0396**
All ischaemic events**	Study Controls	64	0.304	0.086	0.0644
Acute ischaemic events	Study Controls	55	0.387	0.164	**0.00940139**
Acute Stroke/TIA	Mimics	39	0.75	0.345	**0.0075178**
Acute TIA	Subacute TIA	18	0.41	-0.118	1
Ischaemic stroke	Haemorrhagic stroke	20	0.534	0.294	0.0519687

*Disease group includes haemorrhagic and ischaemic strokes and acute/subacute TIA

**Acute and subacute ischaemic events (ischaemic stroke, acute & subacute TIA)

Figure 4.4: Principal Component Analysis Scores Plot of Acute Admissions Vs. Controls



The unsupervised PCA model does not show a clear graphical separation between cohorts which is not unusual for human studies due to significant sample heterogeneity.

It is not uncommon in human studies for cohorts not to separate clearly on PCA. This is because the metabolic profiles of participants are not tightly regulated (as they are in plant models for example), increasing the likelihood of factors beyond acute pathology affecting a large number of metabolites. Such factors include diet and medication, among others.

The first important question to ask of the data is whether the study controls could be differentiated from the acute group independent of whether the diagnosis within the acute group was a stroke or a stroke mimic. As can be seen in Table 4.3 above, this model has moderately good R^2Y and Q^2 values at 0.338 and 0.137 respectively and the model is statistically significant (p=0.006).

The metabolites responsible for the OPLS-DA model were identified with the expectation that some of the metabolites identified in this model would also be identified in subsequent statistically significant models that ask a relevant clinical question. And, that any metabolites identified here that are not

subsequently found to be responsible for disease group separation in further models would point towards the influence of background disease or medication use that would be useful for future work beyond the scope of this thesis.

Table 4.4: Downregulated metabolites for acute admission vs controls

Metabolite	m/z	RT	Regulation
TG(14:0/18:2/18:1)	846.758	913.61	↓
TG(16:1/16:0/18:2)	846.758	913.61	↓
TG(16:0/18:1/20:4)	898.789	924.943	↓
TG(16:1/16:0/18:1)	848.777	929.892	↓
TG(14:0/18:1/18:1)	848.777	929.892	↓
TG(16:0/18:1/18:1)	876.8	947.018	↓
TG(16:0/16:0/18:1)	850.789	947.398	↓
TG(16:0/17:0/18:1)	864.797	954.285	↓
TG(18:/18:1/18:0)	904.83	963.192	↓
TG(16:0/18:1/18:0)	878.817	963.43	↓

Table 4.5: Upregulated metabolites for acute admission vs controls

Metabolite	m/z	RT	Regulation
PC(18:2/18:3)	780.557	260.52	↑
PC(18:2/18:2)	782.57	311.781	↑
PC(15:0/18:2)	744.551	327.167	↑
PC(18:1/18:2)	784.586	384.708	↑
PC(O-16:0/20:4)	742.574	422.385	↑
PC(17:0/18:2)	772.581	430.478	↑
PC(17:1/18:1)	772.581	430.478	↑
PC(18:2/18:0) - dimer	1572.19	495.091	↑
SM(d18:2/16:0)	701.557	265.691	↑
SM(d18:1/20:0)	759.617	593.884	↑
SM(d16:1/22:0)	759.617	593.884	↑
SM(d18:2/22:0)	785.651	617.937	↑
SM(d18:2/23:0)	799.669	710.376	↑
SM(d18:1/22:0)	789.679	768.888	↑
SM(d18:1/23:0)	801.69	787.875	↑
SM(d18:1/24:0)	815.706	804.927	↑

Although the upregulated metabolites were initially thought to be of hopeful pathological significance as a series of phosphatidylcholines and sphingomyelins may point to cell membrane dysfunction, it was also noted that the control group exhibited lower triglyceride levels than the acute group. As the control group contains a cohort of participants known to have either peripheral vascular disease or abdominal aortic aneurysmal disease it was thought likely that they may have been commenced on best medical therapy, inclusive of HMG-Co-A (statin) therapy which would lower triglyceride levels.

It was postulated that although there is not a statistically significant difference in statin use between the cohorts in the presented baseline characteristics that there may be a difference in statin use between the participants that are modelled in the Acute Admission vs Control analysis that would explain the downregulation of triglyceride levels. Therefore, the baseline characteristics for each model were examined. The updated baseline characteristics that included only those groups assessed in the model are presented below in Table 4.6 for the Acute Admission vs Control model.

Table 4.6: Characteristics for the Acute Admission Vs Control Model.

	Acute Admission (n=39)	**Control (n=34)**	**p-value**
Age (mean)	69	71	0.35
Sex (m)	22	26	0.08
BMI (mean)	26 (±0.8)	25.8 (±0.9)	0.91
Smoking status	6	16	**0.004***
Statin use	16	25	**0.009***
Anti-platelet use	25	25	0.45

* Statistically significant difference between the groups for statin use and smoking status.

The model baseline characteristics demonstrated a statistically significant difference between the groups for statin use and smoking status. Until recently, it was not possible to perform confounder adjustment on metabonomic data and thus the risk of a type I error (falsely identifying predictive metabolites) was high. A recent PhD thesis examined at Imperial College London however, developed a method for covariate adjustment that was subsequently validated in large datasets and published subject to peer review (Posma et al., 2018). This statistical method is termed Covariate-Adjustment Projection to Latent Structures (CA-PLS). It was decided that future metabolic identification would occur following CA-PLS analysis using *Matlab* software.

The models that had previously shown to be statistically significant were run again using CA-PLS and the statistically significant confounders adjusted for within each model. The CA-PLS analysis generates new R^2Y and Q^2 values but does not give an overall measure of the model p-value and therefore model

significant cannot be determined. It does however provide data on which of the metabolites are found to significantly affect the model both pre- and post-false detection rate (FDR) analysis.

The baseline characteristics for models with statistically significantly different confounders are presented in the tables below. The remaining models with matched characteristics are presented in Appendix XV.

Table 4.7: Stroke & TIA

	Stroke & TIA (n=38)	Control (n=34)	p-value
Age (mean)	68	71	0.25
Sex (m)	24	26	0.31
BMI (mean)	26.2	25.8	0.70
Smoking status	5	16	**0.001**
Statin use	17	25	**0.017**
Anti-platelet use	16	25	**0.009**

Table 4.8: All Ischaemic events Vs Controls

	Ischaemic events (n= 30)	Control (n=34)	p-value
Age (mean)	71	72	0.8
Sex (m)	18	26	0.18
BMI (mean)	27	26	0.4
Smoking status	3	16	**0.0009**
Statin use	13	25	**0.02**
Anti-platelet use	13	25	**0.02**

Table 4.9: Acute Ischaemic Events Vs Controls

	Ischaemic events (n= 21)	Control (n= 34)	p-value
Age (mean)	71	72	0.89
Sex (m)	12	26	0.15
BMI (mean)	27	26	0.4
Smoking status	2	16	**0.006**
Statin use	9	25	**0.04**
Anti-platelet use	8	25	**0.01**

Table 4.10: Updated model parameters following CA-PLS analysis (far right columns)

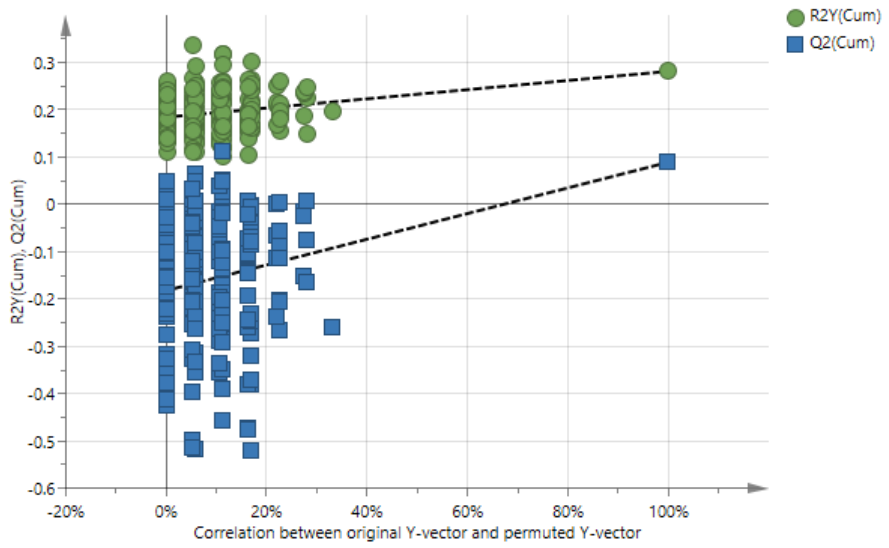
Model		**Parameters**				**CA-PLS**	
Disease Group	**Control Group**	**n**	**R^2Y**	**Q^2**	**p**	**R^2Y**	**Q^2**
Acute admission	Study Controls	73	0.338	0.137	**0.00614589**	0.31	0.12
Stroke & TIA*	Study Controls	72	0.281	0.0893	**0.0396**	0.23	0.106
All ischaemic events**	Study Controls	64	0.304	0.086	0.0644	0.28	0.1
Acute ischaemic events	Study Controls	55	0.387	0.164	**0.00940139**	0.31	0.147
Acute Stroke/TIA	Mimics	39	0.75	0.345	**0.0075178**	0.58	0.0467

*Disease group includes haemorrhagic and ischaemic strokes and acute/subacute TIA

**Acute and subacute ischaemic events (ischaemic stroke, acute & subacute TIA)

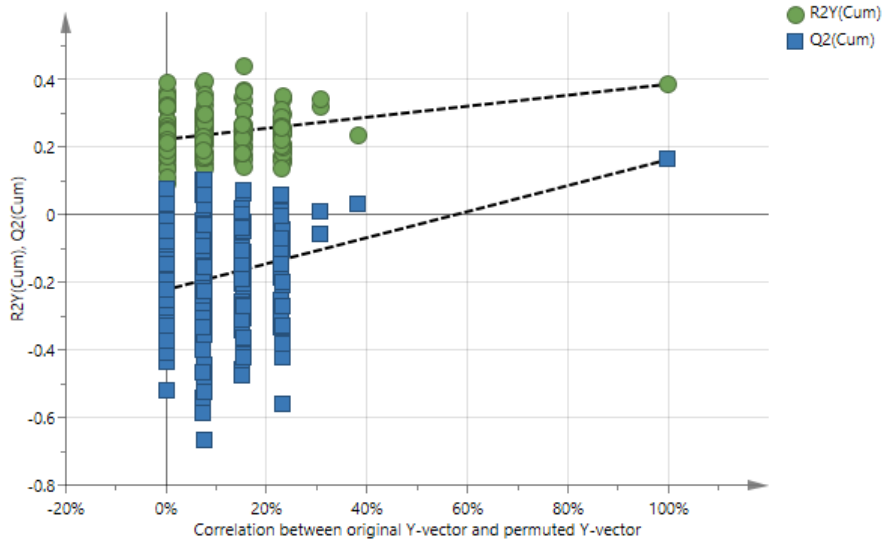
The models that display the most optimal balance between goodness of fit (R^2Y) and predictability (Q^2) are those that compare the control group with either all acute admissions; all stroke and TIAs or with acute ischaemic events. The best model assesses those participants with acute ischaemic events (acute stroke and acute TIA) compared to the study control group. The all stroke and TIA model was found to be over-fitted as shown in the permutation analysis is Figure 4.5.

Figure 4.5: Permutation analysis for the model Stroke & TIA Vs Controls



The model is over-fitted. Not suitable for further analysis. Demonstrated by the permutation analysis being able to demonstrate a higher Q^2 value (most superior blue square) than the model Q^2 value (far right blue square).

Figure 4.6: Permutation analysis for the model Acute ischaemic events Vs Controls



The model is not over-fitted and suitable for further analysis. The permutation analysis does not demonstrate a higher Q^2 value than the one produced by the model.

In the first instance, those metabolites that were identified from the non-adjusted Acute Admission Vs Control model were compared with the metabolites found to be statistically significant from the CA-PLS model for Acute Ischaemic Event vs Controls.

The rationale for this was two-fold: firstly, to identify if the downregulated triglycerides were found to be non-significant following adjustment for statistically significant confounders – one of which being statin use. The other, was the hope that some of the metabolites identified in the broader disease group would remain significant within a narrower, more clinically relevant disease group.

As predicted, many of the triglycerides were identified in the CA-PLS model and all were found non-significant. Two of the previously identified phosphatidylcholines (PC) were found to be significant. Table 4.11 below shows which metabolites were subsequently non-significant and those that remained significant.

Table 4.11: Significance of OPLS-DA upregulated metabolites following CA-PLS analysis

Metabolite	Regulation	Significance (p / NS)
PC(18:2/18:2)	↑	**0.020**
PC(15:0/18:2)	↑	**0.023**
PC(18:2/18:3)	↑	NS
PC(18:1/18:2)	↑	NS
PC(O-16:0/20:4)	↑	NS
PC(17:0/18:2)	↑	NS
PC(17:1/18:1)	↑	NS
PC(18:2/18:0) - dimer	↑	NS
SM(d18:2/16:0)	↑	NS
SM(d18:1/20:0)	↑	NS
SM(d16:1/22:0)	↑	NS
SM(d18:2/22:0)	↑	NS
SM(d18:2/23:0)	↑	NS
SM(d18:1/22:0)	↑	NS
SM(d18:1/23:0)	↑	NS
SM(d18:1/24:0)	↑	NS

*= co-luting; NS = non-significant

Table 4.12: Significance of OPLS-DA downregulated metabolites following CA-PLS analysis

Metabolite	Regulation	Significance (p / NS)
TG(14:0/18:2/18:1)*	↓	NS
TG(16:1/16:0/18:2)*	↓	NS
TG(16:0/18:1/20:4)	↓	NS
TG(16:1/16:0/18:1)*	↓	NS
TG(14:0/18:1/18:1)*	↓	NS
TG(16:0/18:1/18:1)	↓	NS
TG(16:0/16:0/18:1)	↓	NS
TG(16:0/17:0/18:1)	↓	NS
TG(18:/18:1/18:0)	↓	NS
TG(16:0/18:1/18:0)	↓	NS
TG(14:0/18:2/18:1)*	↓	NS
TG(16:1/16:0/18:2)*	↓	NS
TG(16:0/18:1/20:4)	↓	NS
TG(16:1/16:0/18:1)*	↓	NS
TG(14:0/18:1/18:1)*	↓	NS
TG(16:0/18:1/18:1)	↓	NS

Figure 4.7: MS Spectra for PC (18:2/18:2)

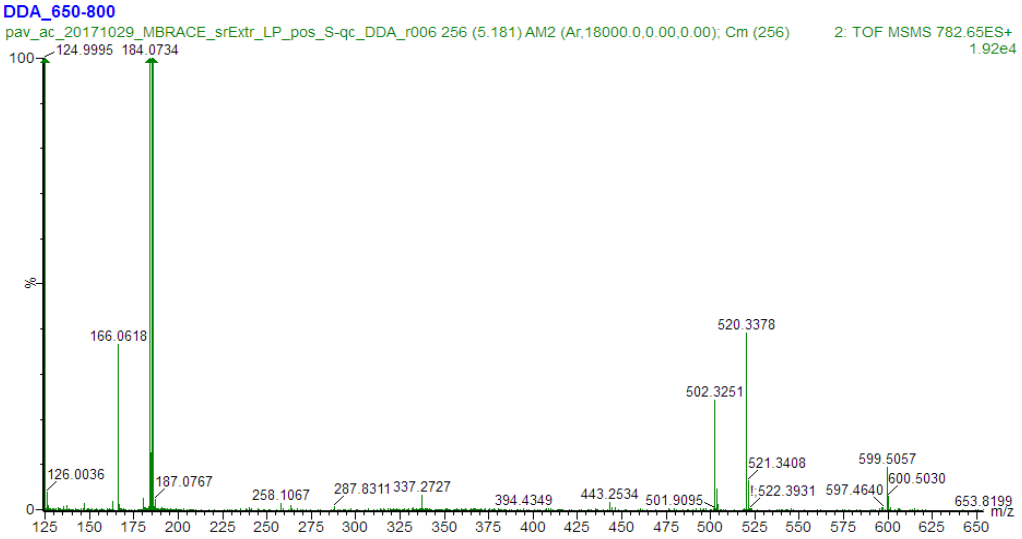
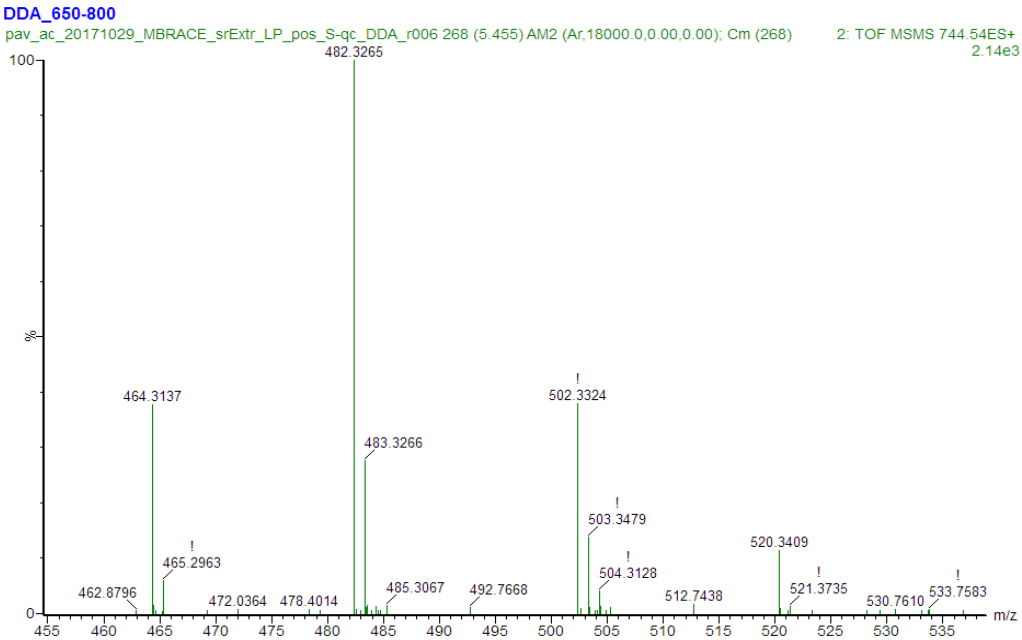


Figure 4.8: MS Spectra for PC (15:0/18:2)



Further metabolites were found to be significantly different in the Acute Ischaemic Event Vs Control model. The identifiable metabolites are listed below:

Table 4.13: Significant metabolites following CAPLS analysis for Acute Ischaemic Events Vs Controls

Metabolite	Lipid Class	m/z	RT	Regulation
SM (d18:2/16:0)	Sphingolipid	701.5570679	265.690988	↑
PI (16:0_18:2)	Glycerophospholipid	835.5333764	272.5154963	↑
Trihexosylceramide (d18:1/16:0)	Sphingolipid	1025.681489	320.0800042	↑
PI (18:0_18:2)	Glycerophospholipid	605.5390015	352.1475016	↑
PC (16:0_18:2)	Glycerophospholipid	1516.118467	374.1790011	↑
PC (16:0/18:1)	Glycerophospholipid	1542.154497	382.3520049	↑
PC (17:0/18:2)	Glycerophospholipid	772.8171207	430.5050022	↑
Ganglioside (d18:1/22:0)	Sphingolipid	1238.81289	530.7279984	↑
SM (d18:1/22:0)	Sphingolipid	1574.336307	768.5559866	↑
HexCer (d18:1/22:0)	Sphingolipid	784.6708341	783.3134837	↑
HexCer (d18:1/24:0)	Sphingolipid	812.7030145	815.7060182	↑
Cer (d18:1/24:0)	Sphingolipid	672.6278687	844.2609816	↑
TG (58:8)	Glycerolipids	948.9279965	918.97498	↓
TG (16:0/18:1/18:0)	Glycerolipids	880.2403177	963.2765366	↓

PC = phosphatidylcholine, PI = phosphatidylinositol, SM = sphingomyelin, HexCer = hexosylceramide, Cer = ceramide, TG = triglyceride

The identification of ganglioside (d18:1/22:0) as a differentiating metabolite between participants diagnosed with an acute ischaemic event compared with controls, all of whom have atherosclerotic disease, is interesting. First discovered in the 1940s the group of lipids assigned as gangliosides were found in brain tissue and ganglion cells (Masson et al., 2015) and although present in all human tissue they are particularly abundant within the brain and central nervous system (up to 200 times greater than peripheral tissues) and can account for up to 6% of lipid content within the brain. Furthermore, they are far more prevalent in grey matter than white matter. They are present predominantly on the outer plasma membrane and have central role pertaining to cell-cell recognition and transmembrane signaling (Masson et al., 2015).

Gangliosides are a glycerosphingolipid and can be considered a sphingolipid with an attached carbohydrate and their biosynthetic pathway is known to begin with ceramide. It is not possible to determine if the identified ganglioside is a known or new member of the lipid species as the current experiment precludes the hydrolysation and subsequent identification of the attached sugar. Nevertheless, its presence in peripheral blood samples adds weight the previous efforts to identify a disease-specific biomarker for acute stroke.

The remaining identified lipids in the metabolite panel are also of interest and most form part of recognized pathways. Ceramide is the backbone of sphingolipid metabolism and can either be produced *de novo* within endoplasmic reticulum or from the enzymatic anabolic hydrolysis of sphingomyelin by sphingomyelinase (Iqbal et al., 2017). Ceramide has been shown to have a central function in the cell membrane lipid raft microenvironment that alters cell signaling pathways and modulates intracellular ion channels. It has also been shown to be a central component for the production of reaction oxygen species and can be synthesized into other sphingolipid species (Li et al., 2010). The presence of a

sphingomyelin is of pathological interest as although it is widely found in human tissues it is found in particularly high concentration in the myelin of nerve cell axons.

The remaining metabolites identified in the panel are glycerophospholipids (PC and PI) both of which are found is cell membranes and are likely related to cell membrane dysfunction. These are not specific to neuronal/glial tissue and therefore it is not possible to postulate on their origin, but there increased expression following an acute pathological insult is not surprising.

4.3 False Discovery Rate Correction

Following False Discovery Rate (FDR) correction, unfortunately none of the identified metabolites in the CAPLS analysis were found to be significant features, based on the Q value cut of <0.05.

The Q value is related to the p-value but instead of focussing on the number of null features in the study that will be erroneously called significant, the Q-value focusses on the number of identified significant features that are truly null.

Table 4.14: Q-values for each identified metabolite following FDR correction

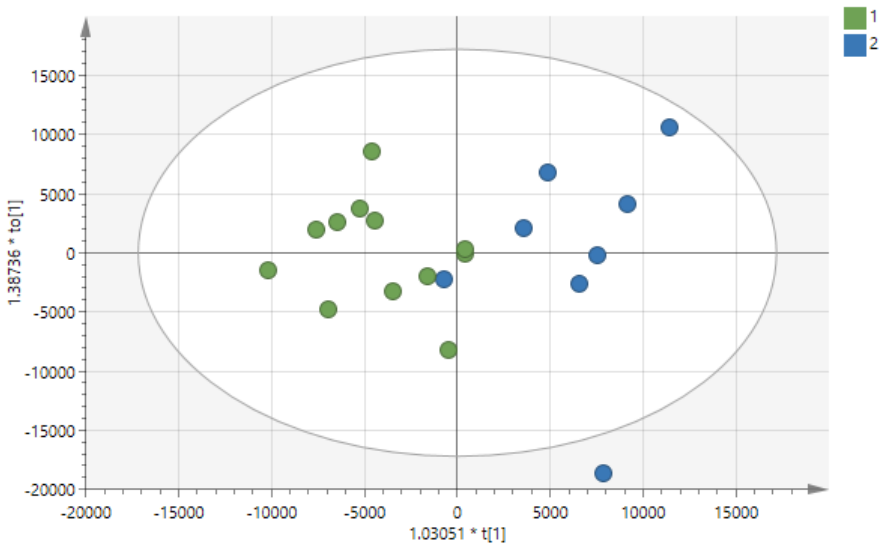
Metabolite	Q-value
SM (d18:2/16:0)	0.95
PI (16:0_18:2)	0.95
Trihexosylceramide (d18:1/16:0)	0.95
PI (18:0_18:2)	0.95
PC (16:0_18:2)	0.95
PC (16:0_18:1)	0.95
PC (17:0_18:2)	0.95
Ganglioside (d18:1/22:0)	0.95
SM (d18:1/22:0)	0.95
HexCer (d18:1/22:0)	0.95
HexCer (d18:1/24:0)	0.95
Cer (d18:1/24:0)	0.95
TG (58:8)	0.95
TG (16:0_18:1_18:0)	0.95

This casts doubt over the results. However, not reaching statistical significance in the current study may simply be due to low sample sizes and heterogeneity of underlying conditions despite a common ischaemic pathway. It still holds true that lipids have been identified, some of which are far more likely to be expressed in the CNS that in peripheral tissue, via peripheral blood sampling in patients who have had an acute cerebral ischaemic insult.

4.4 Ischaemic and haemorrhagic strokes

The model that compares ischaemic and haemorrhagic strokes is a well-matched model (see appendix for model baseline characteristics) and has a reasonable goodness of fit and predictive values for a study of *a priori* uncontrolled human populations: $R^2Y = 0.534$ and Q^2 0.294. Unfortunately, the model does not reach statistical significance p = 0.052. It can be postulated that this model did not reach statistical significance based on only comprising 20 participants.

Figure 4.9: OPLS-DA of the model exploring the differences between acute ischaemic and haemorrhagic strokes

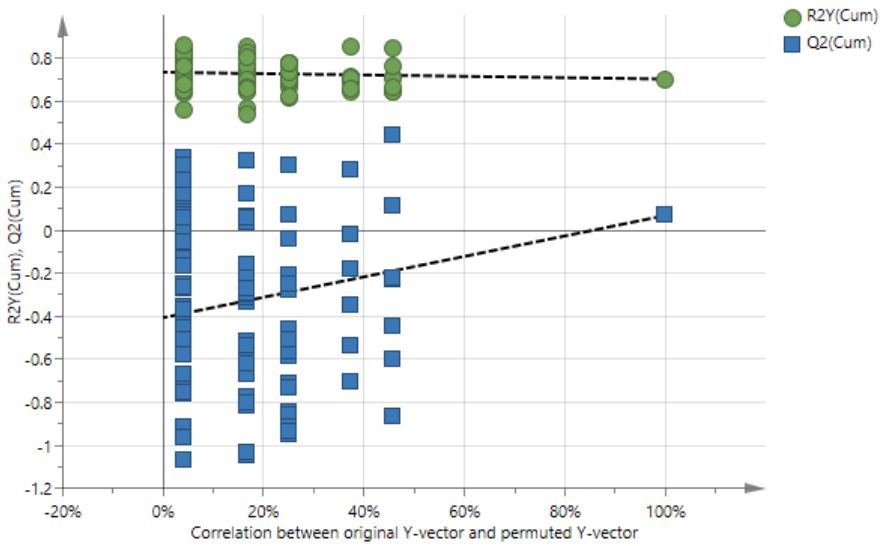


$R^2Y = 0.534$

$Q^2 = 0.294$

1 = ischaemic stroke ; 2 = haemorrhagic stroke

Figure 4.10: Permutation testing for ischaemic and haemorrhagic stroke model



The permutation test clearly demonstrates that the model is over-fitted.

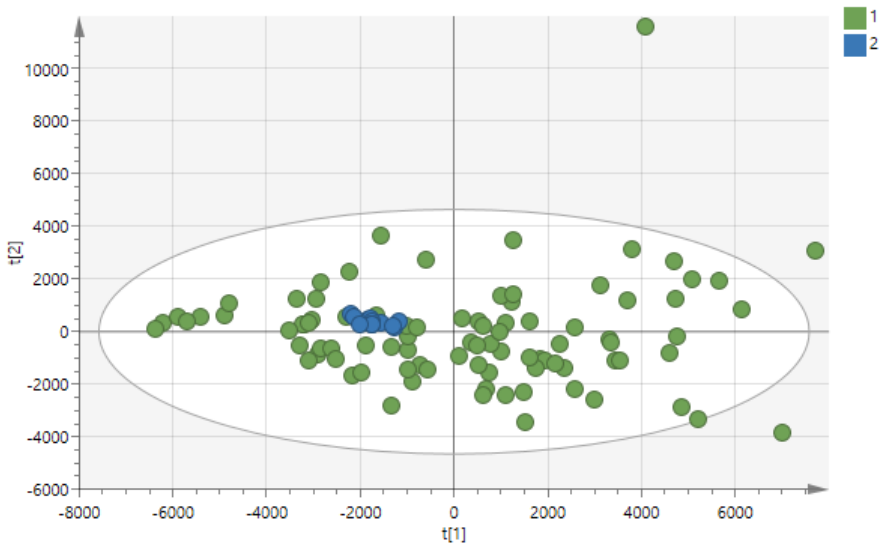
Unfortunately, it can be seen from the permutation testing that although the R^2Y and Q^2 values are promising, the model in its current form is certainly over fitted (see figure 4.10 above). Again, it can be postulated that the extent of this over-fitting would improve with a greater number of participants. Future work may find significant values for this model.

5 RESULTS: NEGATIVE MODE MASS SPECTROMETRY

This chapter details the results of negative mode mass spectrometry. The models discussed in the previous chapter are replicated. The baseline characteristics are presented in the previous chapter and in the Appendix as previously described.

It was demonstrated that following pre-processing of data, that the QC samples were well grouped on the PCA model shown below. Subsequent modelling was therefore undertaken.

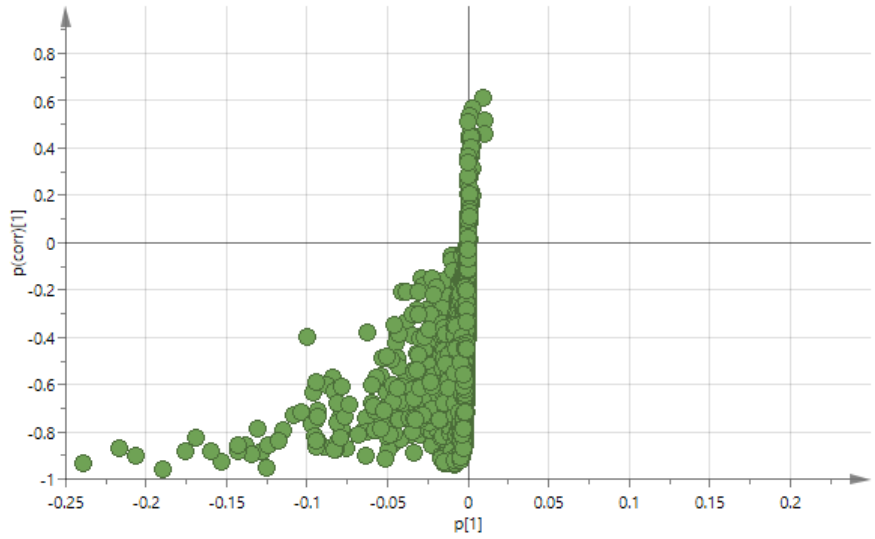
Figure 5.1: PCA of all samples following Pareto scaling



1 = Samples. 2 = QC samples.

There is good grouping of the QC samples. Indicating adequate experimentation.

Figure 5.2: S-plot representation of all samples



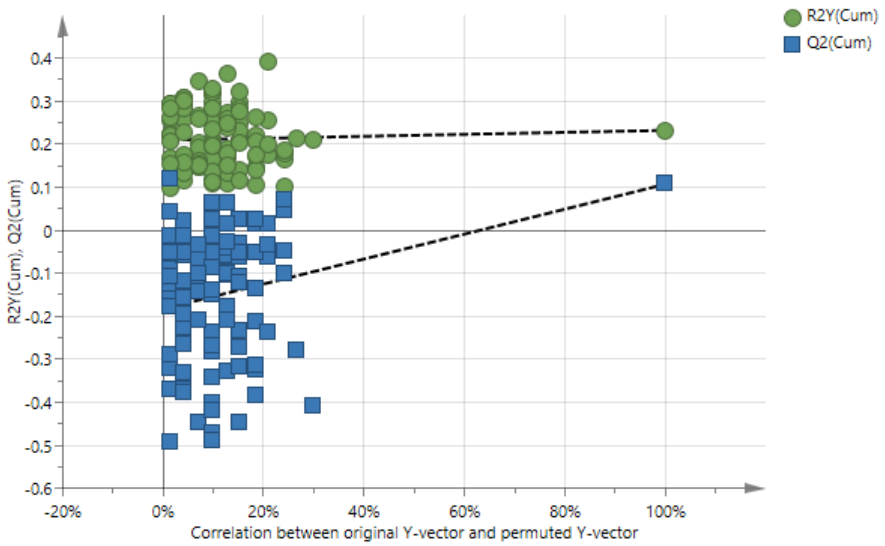
The results for each model are detailed below in Table X. As can be seen, the only statistically significant model was the broadest category that compared acute admission (regardless of final diagnosis) with study controls. Unlike in the positive mode mass spectrometry results, there is not a general trend of significance in any of the subsequent models. However, it was thought that should this model be suitable for analysis, any discriminatory metabolites may be useful for discussion.

Table 5.1: Summary of negative mode mass spectrometry OPLS-DA models

Model		**n**	**R^2Y**	**Q^2**	**p**
Disease Group	**Control Group**				
Acute admission	Study Controls	71	0.232	0.107	**0.021195**
All strokes	Study Controls	54	0.531	-0.87	1
Stroke & TIA*	Study Controls	71	0.186	0.0548	0.147
All ischaemic events**	Study Controls	63	0.194	0.0695	0.115
Acute ischaemic events	Study Controls	53	0.826	0.255	0.08785
Acute Stroke/TIA	Mimics	38	0.555	-0.134	1
Acute TIA	Subacute TIA	18	0.487	0.145	0.307
Ischaemic stroke	Haemorrhagic stroke	20	0.764	0.288	0.247785

To assess whether the Acute Admission Vs Control model would be suitable for analysis, permutation testing was performed with 300 permutations for scientific rigor. As can be seen in Figure 5.3 below, the results of the permutation test clearly demonstrate that this model is overfitted and any results subsequently found would not be statistically significant following false discovery rate analysis. Thus, metabolite identification was not performed.

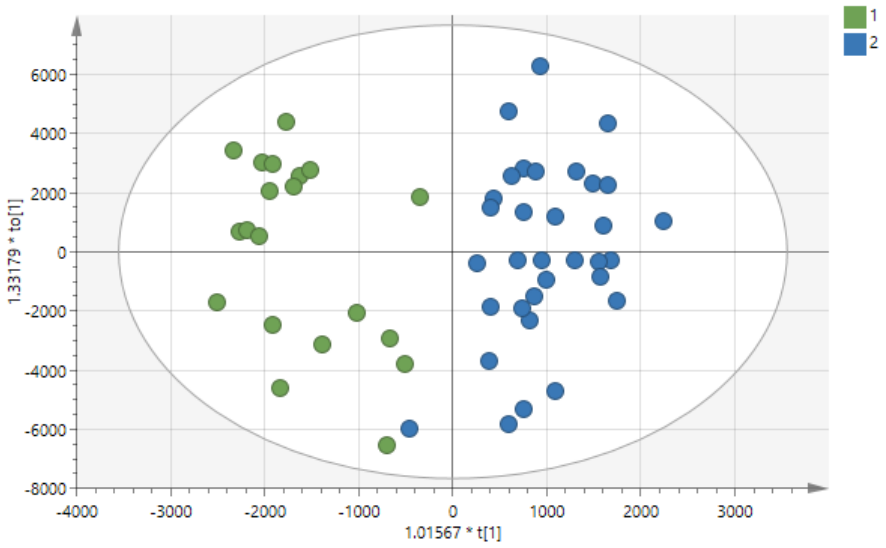
Figure 5.3: Permutation testing for the model Acute Admission Vs Controls



The permutation test generated a Q^2 value greater than the model. A marker of the model being over-fitted.

Despite the lack of robust statistically significant results, the appropriate OPLS-DA graphs are displayed below. It is not possible to graphically display models where the best model used only 1 principal component for analysis. For this reason, only those models with two or more principal components are displayed below.

Figure 5.4: OPLS-DA scores plot of Acute ischaemic events vs Controls

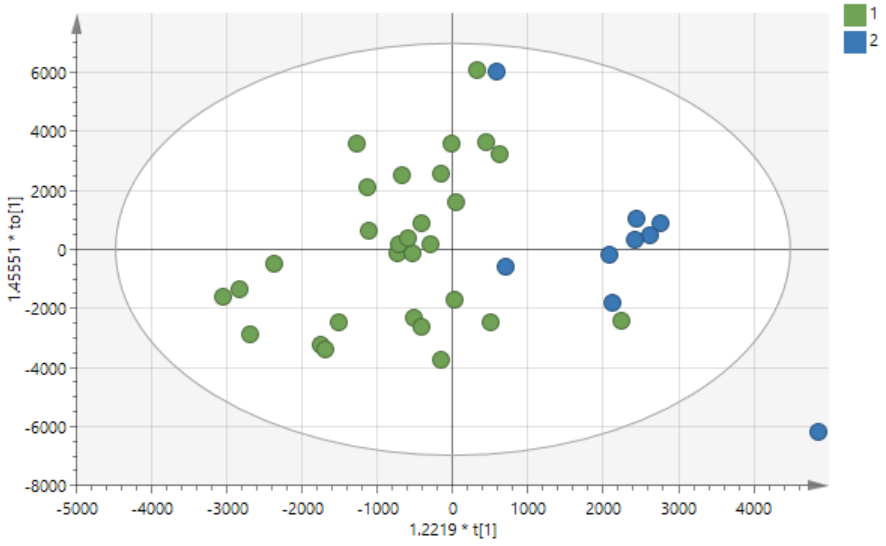


$R^2Y = 0.826$

$Q^2 = 0.255$

1 = disease group. 2 = controls

Figure 5.5: OPLS-DA scores plot of Acute Stroke/TIA vs Mimics

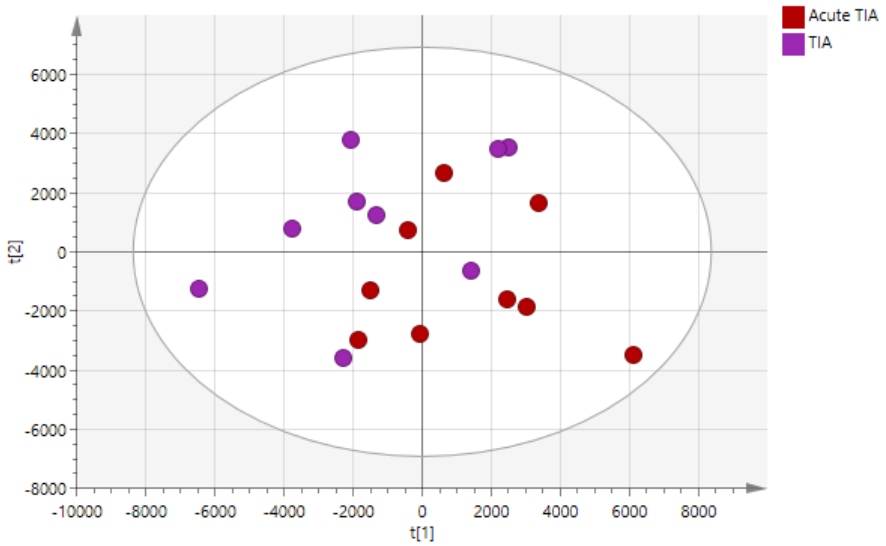


$R^2Y = 0.555$

$Q^2 = -0.134$

1 = disease group. 2 = controls

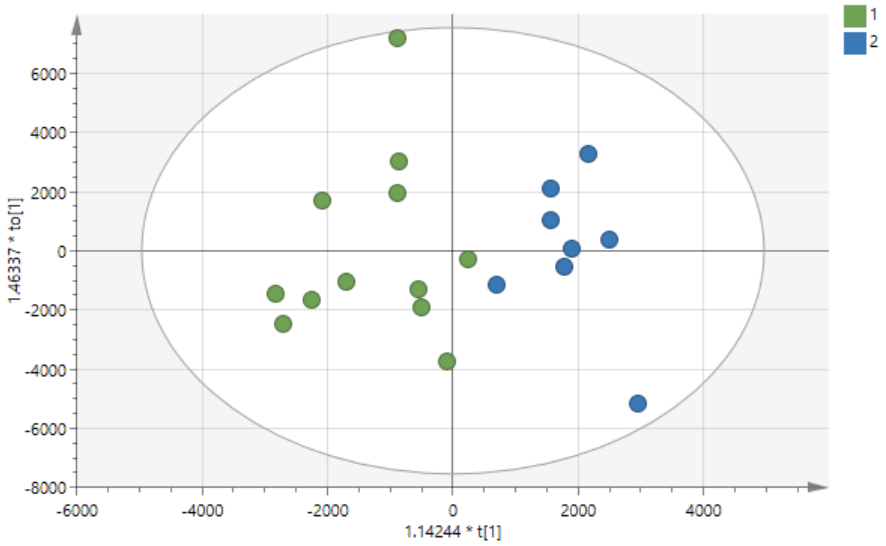
Figure 5.6: OPLS-DA scores plot of Acute TIA vs Subacute TIA



$R^2Y = 0.487$

$Q^2 = 0.145$

Figure 5.7: OPLS-DA scores plot of Ischaemic stroke Vs Haemorrhagic stroke



$R^2Y = 0.764$

$Q^2 = 0.288$

1 = ischaemic stroke 2 = haemorrhagic stroke

For completion of the mass spectrometry analysis, the same CA-PLS analysis was applied to the models. This was done for methodological completeness as it had already been decided that the Acute Admission vs Control model was not suitable for further analysis.

The results of the CA-PLS analysis are tabulated below. Although there are some improvements in the modelling parameters, the CA-PLS analysis does not produce a p-value for each model only for individual metabolites that are responsible for the overall statistical significance of each model shown in the OPLS-DA model.

Table 5.2: Updated model parameters following CA-PLS analysis (far right columns)

Model		**Parameters**				**CA-PLS**	
Disease Group	**Control Group**	**n**	**R^2Y**	**Q^2**	**p**	**R^2Y**	**Q^2**
Acute admission	Study Controls	71	0.232	0.107	**0.021195**	0.46	0.19
Stroke & TIA	Study Controls	71	0.186	0.0548	0.147	0.44	0.197
All ischaemic events	Study Controls	63	0.194	0.0695	0.115	0.37	0.0058
Acute ischaemic events	Study Controls	53	0.826	0.255	0.08785	0.45	0.06
Acute Stroke/TIA	Mimics	38	0.555	-0.134	1	0.508	0.02

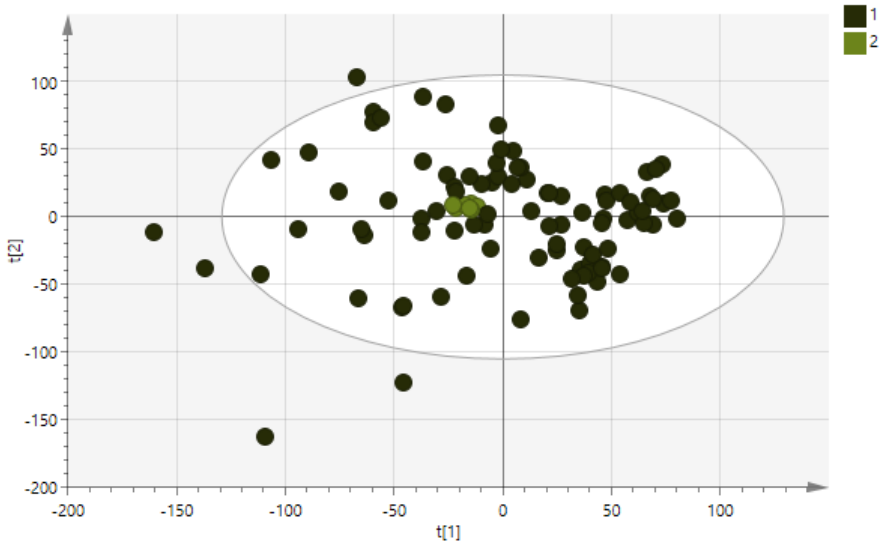
The yield of the negative mode mass spectrometry was less than was produced from the positive mode mass spectrometry. None of the clinically relevant models were statistically significant. Attempts to identify metabolites in an over-fitted yet statistically significant model that compares acute admissions to hospital with the non-acute control group without the benefit of further, clinically relevant models to reply upon for further analysis would not have been scientifically useful.

6 RESULTS: NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

This final results chapter reports on the NMR spectroscopy findings. The models seen in the previous chapters are reproduced here. Again, starting with the general acute admission vs control model and subsequently modelling a series of more clinically relevant questions in the hope of finding discriminatory metabolites for acute stroke diagnostics.

To begin with, the relationship between QC samples and patient samples was assessed by PCA analysis following data pre-processing. As seen in Figure 6.1 below, there is good grouping of the QC samples.

Figure 6.1: PCA of samples compared to quality controls.



1 = participant samples. 2 = QC samples. There is tight grouping of the QC samples indicating adequate methodology and results of NMR spectroscopy following pre-processing.

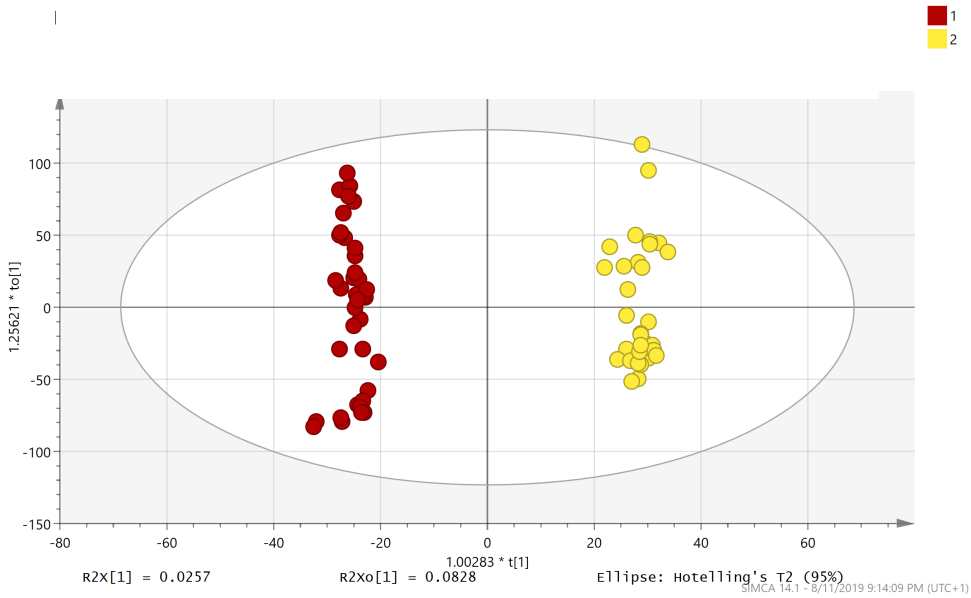
A similar table to what has been produced previously is shown below with the R^2Y and Q^2 values for each model run in the SIMCA software, alongside the p-values for each model.

Table 6.1: NMR model characteristics

Model		**n**	**R^2Y**	**Q^2**	**p**
Disease Group	**Control Group**				
Acute admission	Study Controls	70	0.992	0.228	**0.0353619**
All strokes	Study Controls	54	0.907	-0.0395	0.65
Stroke & TIA*	Study Controls	68	0.872	-0.01	1
All ischaemic events**	Study Controls	62	0.515	-0.0774	1
Acute ischaemic events	Study Controls	53	0.565	0.165	**0.0111173**
Acute Stroke/TIA	Mimics	39	0.804	-0.536	1
Acute TIA	Subacute TIA	17	0.975	0.156	0.7
Ischaemic stroke	Haemorrhagic stroke	20	0.819	-0.244	1

As seen in the table above, there are 2 statistically significant models. The first is the comparison between acute admissions and study controls. This model has 3 principal components and the corresponding OPLS-DA is shown in Figure 6.2.

Figure 6.2: OPLS-DA of the model Acute Admissions Vs Controls



$R^2Y = 0.992$

$Q^2 = 0.228$

1 = Acute Admissions, 2 = controls. Despite the appearance of a very clear separation, this a function of the supervised nature of OPLS-DA analysis which can represent graphical separation.

As mentioned in the positive mode mass spectrometry chapter, it is rare to find near perfect R^2Y values during the metabonomic analysis of uncontrolled and vastly heterogeneous human serum samples. The 'experimental' conditions of plant based metabonomics can simply not be replicated in untargeted, metabonomics studies. The Acute Admissions Vs Controls model displayed in the table above has an R^2Y value of 0.992 which is far higher than the Q^2 value. It is well known within metabonomic literature and analysis that when adding principal components to an OPLS-DA analysis the R^2Y value will continue to increase until it reaches one. This is not the case for the predictability measure Q^2 which will reduce in value once too many principal components have been added to the analysis. Indeed, it is this relationship between R^2Y and Q^2 that determines how many principal components should be added to each model.

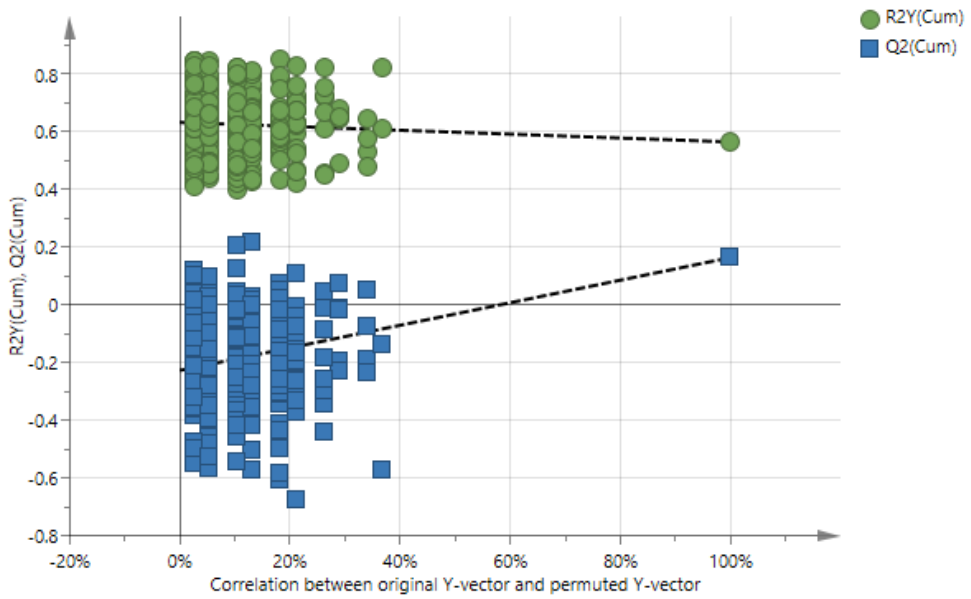
The methodology described above was used in determining how many principal components to add to the current analysis and it is not the function of human error that has yielded such a discrepancy between R^2Y and Q^2. Once the OPLS-DA analysis is complete, should there be a large discrepancy between the

two values, it is the function of the model being significantly over-fitted. Thus, despite the significant p-value, the model is not suitable for further metabonomic analysis.

Returning to the table above, there is one other statistically significant model, that of Acute Ischaemic Events Vs Controls with a p-value of 0.01111. This model is more compelling and has a relationship between R^2Y and Q^2 that is not immediately over-fitted. Thus, it was initially thought that there may be a case for exploring this model further.

To clarify whether the model was indeed suitable for metabolites identification a permutation analysis was performed using 300 permutations as had been applied to the models in the previous chapters. The permutation analysis is displayed in Figure 6.3 below.

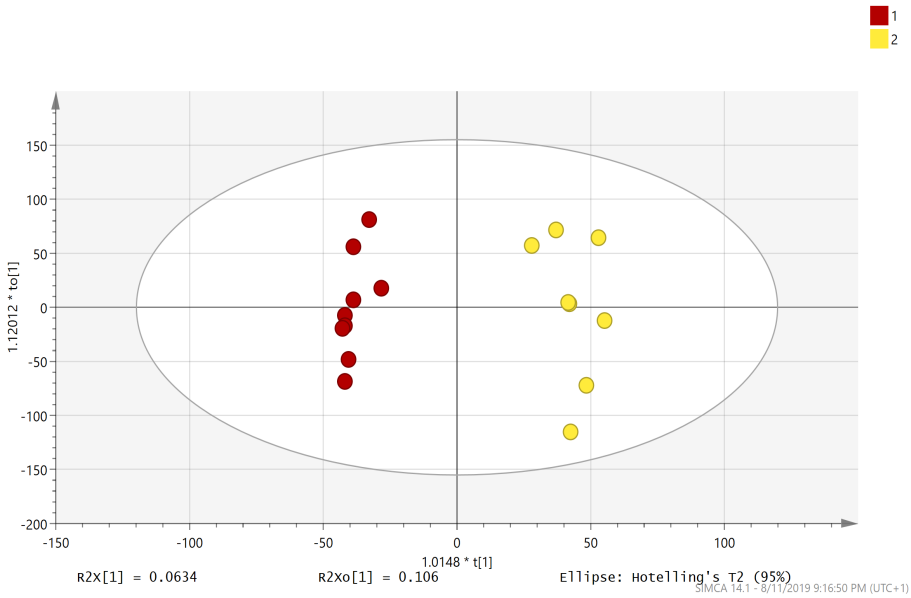
Figure 6.3: Permutation analysis for the model Acute Ischaemic Events Vs Controls



As is clearly represented in the permutation analysis, artificial Q^2 values that far exceed the model's original Q^2 value can be produced. In addition, there is downward trend from the permuted R^2Y values and the R^2Y value produced from the model. In a 'good' model, one would expect there to be a general upwards trend toward the model R^2Y value.

These factors clearly demonstrate that the Acute Ischaemic Event Vs Control model is also over-fitted. Albeit to a lesser degree than the Acute Admission Vs Control model. In addition to the over-fitted parameters, this model only has 1 principal component which makes the model weak for analysis. The over-fitting of models combined with the knowledge that CAPLS models in the negative mass spectrometry chapter did not improve results, further model analysis and metabolite elucidation was not performed on the NMR samples. For completeness, those models that had more than 1 principal component and allow graphical display of the scores plots are shown below.

Figure 6.4: OPLS-DA of Acute TIA vs Subacute TIA

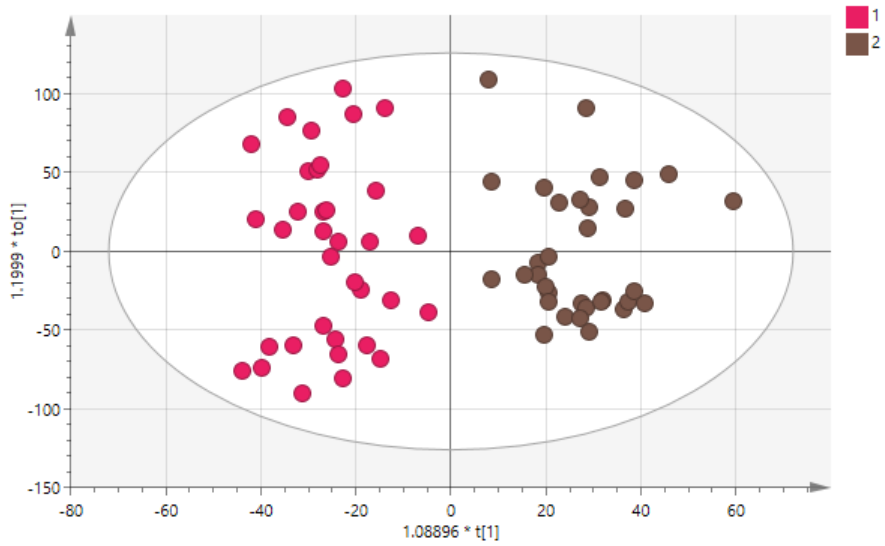


$R^2Y = 0.975$

$Q^2 = 0.156$

1 = Acute TIA, 2 = Subacute TIA.

Figure 6.5: OPLS-DA of Stroke and TIA Vs Study controls



$R^2Y = 0.872$

$Q^2 = -0.01$

1 = Stroke & TIA, 2 = controls.

It is unfortunate, but not entirely unsurprising, that the NMR results are negative in comparison to the MS results. It is known that NMR, whilst a more stable and reproducible technique does have a reduced metabolite detection rate compared to MS. It is possible to detect sphingomyelins by NMR spectroscopy for example, but the technique may not offer adequate resolution. This is the most likely reason for low yield results in the NMR analysis despite adequate methodology.

7 DISCUSSION

The search for a serological biomarker for acute stroke is not new and its potential discovery is well documented as one that would mark significant progress in the ability to provide comprehensive stroke care throughout the world (Mattila et al., 2016). It would allow not only timely diagnosis and acute treatment, but for those patients with stroke mimics it would facilitate appropriate triage. Additionally improving the care of patients who fall outside the diagnostic stroke pathway and improving healthcare resource allocation.

Over the last 5-7 years, there has been an increasing trend in using metabonomic techniques to identify putative diagnostic biomarkers for stroke (Qureshi et al., 2017). A line of scientific enquiry that has seen an even greater acceleration in the published literature over the last 2-3 years as evidenced by the literature review in this thesis. This is a significant step beyond the historic search for proteins that to date, have not proven clinical efficacy despite significant scientific exploration.

The results of this thesis largely fit with the discoveries of other metabonomic studies related to acute stroke diagnostics (Liu et al., 2017, Sun et al., 2017). Confidence is gained through the similarities found in the metabolic disturbances detected in serum following acute stroke and point to unifying underlying pathological mechanisms of altered sphingolipid metabolism (Sun et al., 2017). Although imperfect and impeded by recruitment numbers that would otherwise have driven down significance levels, this thesis does offer a new insight into the possibility of using dedicated lipidomic analysis in the search for a serological biomarker for acute stroke.

First, it is imperative to discuss the discovery of the elevated ganglioside found following positive mode mass spectrometry in patients presenting with an acute cerebral ischaemic event (despite the results of false discovery rate analysis) that includes patients with a diagnosis of either acute ischaemic stroke or an acute TIA. To the author's knowledge this is the first time that a ganglioside has been separated in serum related to acute stroke diagnostics. It is possible that its detection is related to the hyper-acute nature of blood sampling that was undertaken in this study compared to previous studies that mostly

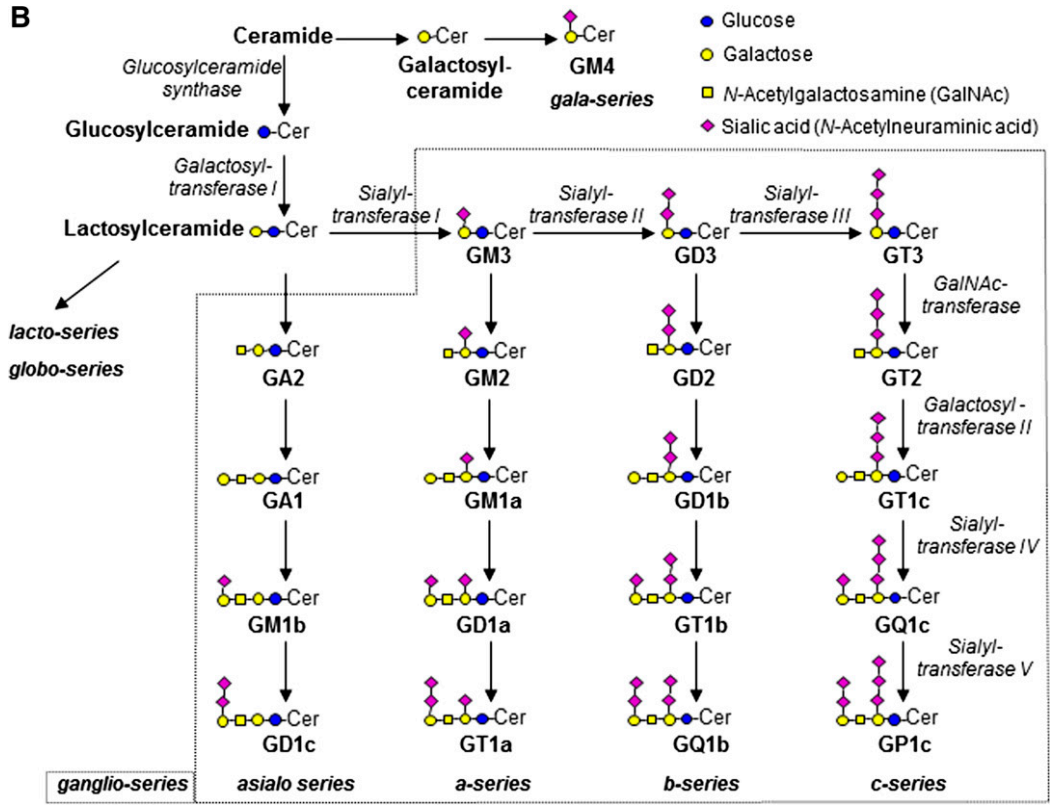
collected acute samples within 24 hours of symptomatic onset. Of all the results presented in this thesis, the ganglioside is the most interesting.

Gangliosides are found in all tissues but are found in particularly high concentration within the brain, within neuronal membranes specifically, accounting for up to 6% of all brain lipids. They are also known to be relevant to neurological diseases beyond stroke which adds further weight to the results. For example, Guillain-Barre syndrome is known to be caused by an autoimmune response to cell surface gangliosides (Kaida et al., 2009) and a rare form of infantile onset epilepsy mainly found in Amish communities is also caused by a mutation in GM3 synthase (Simpson et al., 2004).

As previously discussed, it is not possible without further structural analysis to be certain of the exact ganglioside that may discriminate between patients with an acute cerebral ischaemic event and those who have not suffered a suspected acute neurological event. However, if the molecular weight of the compound is taken alone, according to the Human Metabolome Database, the ganglioside found in this analysis may correlate with the ganglioside, GM3. GM3 is known to be a component of cell membranes (Sonnino et al., 2006), produced in the endoplasmic reticulum and it is possible therefore that it is released from the blood brain barrier following acute cerebral ischaemia, or crosses the damaged BBB following release from ischaemic or infarcted neurons.

The biosynthetic pathway of gangliosides is shown below in Figure 7.1. As can be seen, GM3 is a basic, precursor ganglioside from which the more complex gangliosides are formed. Although GM3 accounts for <1% of all gangliosides within the brain and is the most prevalent ganglioside within peripheral tissues, the concentration is far lower than within the brain. It has been demonstrated in animal models that following middle cerebral artery occlusion although there is a transient rise in complex gangliosides (GD1a, GD1b and GT1b) during days 3-7 post infarct there is also an increase in GM3 expression at the infarct margin that corresponds with a decrease in the concentration of complex gangliosides (Whitehead et al., 2011).

Figure 7.1: Biosynthesis of Gangliosides



Gangliosides are composed of a ceramide moiety and a fatty acid chain with a branched sugar chain. GM3 represents one of the more basic precursor gangliosides that is used to produce more complex gangliosides that are far more prevalent in the brain than GM3. Reproduced from (Masson et al., 2015).

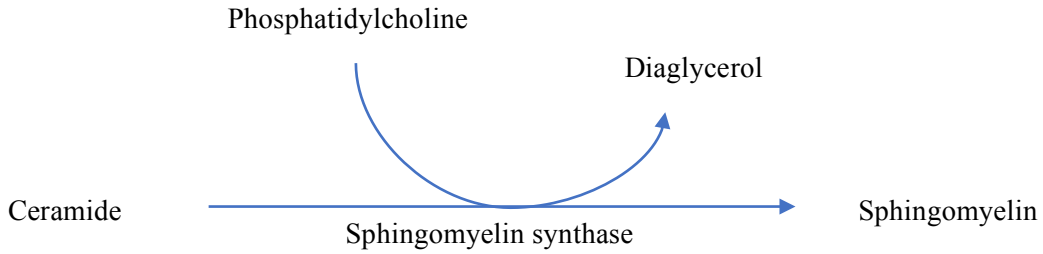
It has been postulated that the observed increase in GM3 post-infarct is secondary to the catabolic consumption of complex gangliosides during ischaemia / infarction (Whitehead et al., 2011) with the concomitant rise in GM3 a homeostatic response to replace the consumed complex gangliosides. The tentative results of this thesis lie in support of an upregulation of GM3, but follows a more acute timeline (within 6 hours of ischaemic insult) than those demonstrated in animal models. This may be due to an altered human response to ischaemic brain injury or it may represent the early detection of BBB / NVU dysfunction rather than the detection of upregulated GM3 at the outer aspect of the infarcted core seen in animal models (Whitehead et al., 2011). Furthermore, as the analysed group includes patients with

acute ischaemic stroke and acute TIA, it can be postulated that the release or upregulation of GM3 detected in serum is more likely to be related to tissue ischaemia than infarcted tissue alone.

The synthesis of gangliosides and their upregulation post-acute ischaemic events also explains the upregulation of ceramide (also of the sphingolipid class). Ceramide is the precursor molecule for the generation of all gangliosides (basic and complex) and its upregulation will follow that of GM3, particularly if GM3 is being produced in response to a catabolic reduction in complex gangliosides.

The remaining upregulated sphingolipids correlate with recognised pathways demonstrated well in animal models of acute stroke and traumatic brain injury. The early release of sphingolipids into blood, particularly sphingomyelin species, has been shown in rat models within 6 hours of cerebral insult. The proposed mechanism relies on the high concentration of sphingomyelin within oligodendrites of white matter tracts and (Sheth et al., 2015) and the cellular disruption that occurs following acute ischaemia or trauma. Moreover, a major source of ceramide production is generated by the sphingomyelinase breakdown of sphingomyelin. Thus, the upregulation of sphingomyelin may be two-fold: an acute release from blood brain barrier / NVU and cellular ischaemia as well as an attempt to maintain homeostasis via the ceramide/ganglioside biosynthetic pathway. The biosynthesis of sphingomyelin and PC is shown in Figure 7.3 below.

Figure: 7.2: Biosynthesis of sphingomyelin from PC and ceramide



The upregulation of phosphatidylcholines (PC) is unsurprising. PC is a phospholipid and a fundamental component of cell membranes and are additionally integral to cell signaling (Cole et al., 2012). PCs are generated de novo by 2 pathways. The principal pathway is by the CDP-choline pathway, whereby a dietary source of choline is converted to PC in 3 enzymatic steps which is possible in all nucleated cells (Cole et al., 2012). The second pathway is the progressive methylation phosphatidylethanolamine (PE). Methylation is the process of progressively adding a single carbon atom and three hydrogen atoms (a methyl group) to an organic substance. This is known as the PEMT pathway and is only significant in the hepatic synthesis of PC. Both pathways are dependent on the role of ER.

It is unclear why triglyceride levels were found to be low in those patients with an acute ischaemic event especially after covariate adjustment. However, there is evidence to suggest that those patients who suffer the worse clinical outcome following stroke do have low serum triglyceride levels (Dziedzic et al., 2004). Low levels may therefore be linked with ischaemia / infarct volume.

Finally, any future research on the potential link between acute cerebral ischaemia and raised serum ganglioside levels could have a significant impact on the diagnosis and management of TIAs. Currently, TIA diagnosis is reliant on an accurate clinical history, a neurological examination and the presumption that cerebral ischaemia is the causative mechanism of the described symptoms (Coutts, 2017). A blood test that could, within an acute time frame of 24-48 hours, reliably indicate the past occurrence of cerebral ischaemia, would be a major landmark in TIA diagnostics. Particularly, as there is documented and sustained inter-observer variation (kappa 0.65) in TIA diagnostics amongst senior neurologists (Kraaijeveld et al., 1984, Castle et al., 2010).

7.1 Conclusion

To conclude, although the results of this thesis have not definitively identified a diagnostic biomarker for acute stroke, those lipids that have been identified as upregulated follow a common pathological theme found in basic science stroke research in both animal and human models. Despite the results being dampened by the false discovery analysis, that is very likely linked with recruitment numbers, there is hope that the discovery of a sensitive diagnostic biomarker for acute ischaemic stroke (and TIA) is possible. There is further work to complete and with larger recruitment numbers, less restrictive inclusion criterion that will allow the metabolic differentiation of acute stroke from stroke mimics, such as seizures and brain neoplasms, it is likely that further insight into the pathological processes of cerebral ischaemia and infarction may be gained with the use of lipidomics. The separation of a ganglioside and the linked SM and PC pathways that explain the results found in positive mode mass spectrometry are important contributions to a growing body of metabolic knowledge of acute stroke pathophysiology and diagnostics. The greater the understanding of lipid metabolic disturbances following acute cerebral events the greater chance there is of identifying a highly sensitive diagnostic biomarker for acute stroke. The results of this thesis should be further examined in the clinical context of hyper-acute stroke and further explored in animal models to appreciate the origin and metabolic pathways of acute lipid disturbances following acute ischaemic cerebral events.

7.2 Limitations

There are some notable limitations to the current study which relate to the inability to discover a statistically significant biomarker, especially when a small number of other studies have reported positive results, even if not clinically validated.

The first is the unknown effect of the blood brain barrier on the likelihood of discovery. As has been discussed in earlier chapters, the blood brain barrier is known to undergo biphasic damage, initially in the acute stages following stroke. Increased permeability along a concentration gradient from blood to brain is proven scientifically and with the widespread use of contrast agents for CT and MRI scanning to detect enhancing brain tumours that also disrupt the blood brain barrier.

It is postulated in this thesis that such blood brain barrier disruption will also facilitate the movement of ischaemic metabolites, across a concentration gradient from brain parenchyma to blood. Current scientific knowledge has not advanced sufficiently to understand the effects of blood brain barrier dysfunction on metabolite efflux. It is possible that the blood brain barrier disruption that occurred in the stroke and TIA patients in this study was not sufficient to allow acute detection of significant metabolic disturbances. This may be a result of a highly effective central London HASU which is somewhat reflected in the moderate average NIHSS score of the included stroke participants. Had participants had greater neurological deficits and thus likely larger infarct cores and ischaemic penumbra volumes, perhaps the results would have been proven to be significant.

One difference between the study presented in this thesis and the current literature is the type of study controls used. Most other studies used healthy controls or those with a known acute cerebral event such as TIA. None have explicitly used patients with an established atherosclerotic burden. The choice to use such patients in this study was an attempt to advance the evidence base for acute stroke metabonomics and reduce the selection bias present in other studies. Any positive results, in such a

context, would have had far greater weight and greater likelihood of clinical applicability. Any acute stroke biomarker must be distinguishable from background atherosclerosis in real-world settings. It is likely that low recruitment numbers impacted on the ability to detect such biomarkers in this study but using patients with established atherosclerotic disease as a control group is an important consideration for future, larger studies.

The lack of separation between acute ischaemic stroke and TIAs is also worthy of discussion. It was hoped that the presence of an infarcted core in the acute stroke patients and its absence in TIA patients would lead to a significantly different metabolic profile. Unfortunately, this was not demonstrated. One factor that potentially influences these data is the use of an untargeted metabonomic approach. An approach made necessary by the unknown metabolic disturbances of the ischaemic/infarction continuum of TIA, acute ischaemic stroke and the metabolic profile of silent cerebral infarctions. This gap in knowledge means that analyses targeted at specific metabolite disturbances cannot be performed. Further studies that deepen scientific understanding of the metabolic differences between clinically significant acute cerebral infarctions and silent cerebral infarctions may cast some new light on which metabolites to target in future acute stroke metabonomic studies.

Despite efforts to reduce selection bias with the use of atherosclerotic controls and the recruitment of patients on admission to HASU prior to their known final diagnosis, some sources of selection bias remain. The inability to use acutely recruited patients as their own control with serial temporal blood tests meant that acute disturbances in metabolic profiles were less likely to be identified. As such, there is an inherent metabolic discrepancy between patients recruited acutely in the HASU and the non-stroke control group who have known atherosclerotic disease.

Although robust and validated efforts have been made to adjust for metabolic confounding factors in the statistical analysis, the nature of human metabonomic studies and the inability to adjust for all relevant factors, including diet remains an additional potential source of bias.

8 FUTURE WORK

This final section details the future work required to further explore lipid biomarkers for hyper-acute stroke.

8.1 All admissions to a hyper-acute stroke unit

The first step is to repeat a similar study but to include all patients assessed in a HASU and remove the exclusion criteria used in the current thesis. This would crucially include patients who have either a brain tumour or present to HASU secondary to a seizure. These are important conditions to include in any future studies, primarily because, in the case of seizure, it is a common presentation and in the case of brain tumours because there is known breakdown of the blood brain barrier.

From a basic science perspective, such a study would also ask some indirect questions about the known mechanisms of blood brain barrier dysfunction following acute stroke and the extent and/or type of blood brain dysfunction that occurs as part of a neoplastic process within the brain.

An additional crucial part of the follow up study, would be to ensure adequate funding for accurate, time-based (from acute event) temporal profile analysis of metabolic signatures. The advantage of this approach would be to clearly identify which metabolic disturbances accurately reflect acute ischaemia/infarct or haemorrhage. The temporal metabolic profile linked with disease severity and complications such as haemorrhagic transformation post ischaemic stroke, would be greatly beneficial if high recruitment numbers are achieved.

8.2 Specific TIA study

As part of this thesis, in response to low recruitment numbers and local policies that prevented temporal profile analysis, sub-acute TIA patients were recruited from a TIA clinic. This would be an important

recruitment arm in any follow-up study as the metabolic profile of TIA patients would be an interesting comparator with the acute temporal trend analysis described above.

Moreover, in addition to identifying a blood test for acute stroke, being able to accurately diagnose transient cerebral ischaemia based on a blood biomarker panel would be a significant step forward in the management and future risk profiling of TIA patients.

8.3 Silent cerebral infarctions

As discussed in the introduction, the AHA/ASA include silent cerebral infarction within the definition of stroke. A divergence from the WHO definition of stroke that requires an acute neurological deficit. To explore the pathophysiological similarities between acute stroke and silent cerebral infarctions further a study could be designed to compare acute stroke metabolic profiles with those patients who have suffered an acute silent cerebral infarction.

It is known for example that approximately 50% of patients who undergo aortic endovascular stenting will have multiple silent cerebral infarctions that can be diagnosed on DWI (Charbonneau et al., 2021). A comparison of pre- and post- surgical/interventional bloods with the metabolic profile of acute stroke patients would offer an interesting insight into the pathological differences between silent cerebral infarction and clinically detectable infarcts.

8.4 Acute mechanical thrombectomy study

The final arm of any acute stroke metabonomic study should also include the extraction of blood from the middle cerebral artery in those patients who undergo acute mechanical thrombectomy. Such an approach has the added advantage of taking blood closer to the site of ischaemic insult and therefore, it can be hypothesised that any metabolic disturbance will be evident to a greater degree. It would also be

necessary to identify the metabolic profile of heparinised-saline (which is continually infused through the micro-catheter during neuro-interventional procedures) so that it can be accounted for in the MS/NMR analysis.

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10 APPENDICES

10.1 Appendix I : Molecular masses of gadolinium chelates used as MRI contrast agents

Brand name	Generic name	Acronym	Molecular weight (kDa)
Omniscan	gadodiamide	Gd-DTPA-BMA	0.573
OptiMARK	gadoversetamide	Gd-DTPA-BMEA	0.661
Magnevist	gadopentetate dimeglumine	Gd-DTPA	0.939
MultiHance	gadobenate dimeglumine	Gd-BOPTA	1.058
Primovist	gadoxetic acid disodium salt	Gd-EOB-DTPA	0.682
Vasovist	gadofosveset trisodium	Gd-DTPA	0.958
ProHance	gadoteridol	Gd-HP-DO3A	0.558
Gadovist	gadobutrol	Gd-BT-DO3A	0.604
Dotarem	gadoterate meglumine	Gd-DOTA	0.559

*Table adapted from: Medicines and Healthcare Products Regulatory Agency Public Assessment Report(MHRA, 2007) and a cited table of molecular weights of contrast agents (Penfield and Reilly, 2007)

10.2 Appendix II: UPLC-MS sample preparation protocol

The lipid extraction methodology in detailed below:

1. Prepare sample aliquots of 100µl in Eppendorf tubes
2. Prepare a pooled sample by mixing 100µL of each sample in the same glass vial.
3. Aliquot the pooled sample in 10 samples, 100µL per tube
4. Randomise the sample order.
5. At this point samples can be stored in the freezer.
6. Defrost in batches of 26-28 samples
7. Include 1-2 blank samples (no biological sample) and 1-2 pooled sample in each batch (total batch 30 samples)
8. The aliquots of 100µl of sample are left to thaw for a few minutes
9. 700 µl of pre-chilled (-20C) organic solvent mixture (Methyl tert-butyl ether/methanol (3:1)) is added to the eppendorf tubes containing the thawed samples
10. Intense vortexing for 5minutes using the multimixer
11. Centrifuge for 30min (4^{o}C), 20,800G
12. Decant 600µL of supernatant in a new clean Eppendorf tube (avoiding the bottom aqueous layer)
13. Load the Eppendorf tubes with the decanted organic solvent open in the speedVac in order to evaporate the organic solvent. Use no temperature adjustment and maintain in the vacuum concentrator until completely dry (usually 2-3hrs)
14. Extracts are stored at -40 ^{o}C until reconstitution

Following lipid extraction, the lipid content is reconstituted with solvent to form a solution amenable for mass spectrometry analysis.

The protocol followed is detailed below:

1. 150µl of cooled (4^{o}C) organic solvent mixture (isopropyl alcohol/acetonitrile/water (3:1:1)) is added in the eppendorf tubes of the lipid extracts
2. Intense vortexing for 5min using the multimixer
3. Centrifuged for 30min (4oC), 20,800G
4. Decant 120µL of reconstituted sample in a new clean LCMS vial (avoiding the bottom pellet)
5. Prepare a pooled quality control (QC) sample by decanting and mixing 20µL of each reconstituted sample.

Following sample preparation, the reconstituted samples are randomised and placed in racks for mass-spectrometry analysis.

10.3 Appendix III: NMR sample and buffer preparation protocol

Plasma Buffer Preparation

Preparation of 0.142M $NaHPO_4$, 2mM NaN_3, 0.08% TSP solution for plasma/serum sample preparation (500mL)

Note: Reagents must be of analytical grade. 500mL of buffer is enough to prepare approximately 1500 plasma/serum samples

Reagents:

a) Disodium Hydrogen Phosphate Na_2HPO_4 (Sigma-Aldrich, 7558-79-4)

b) Deuterium Oxide D_2O (Sigma-Aldrich, 7789-20-0)

c) Sodium Azide NaN_3 (Sigma-Aldrich, 26628-22-8)

d) 3-(Trimethyl-silyl)propionic acid-d4 sodium salt, TSP (Sigma-Aldrich, 24493-21-8)

e) Potassium Hydroxide Solution KOH 45% w/w (Sigma-Aldrich, 1310-58-3)

f) Hydrochloric Acid HCl 37% w/w (Sigma-Aldrich, 7647-01-0)

Preparation:

1) Weigh out 5.32 g of Na_2HPO_4 into a small weighing boat.

2) Transfer the Na_2HPO_4 into a 500 mL volumetric flask.

3) Weigh 400 mg of TSP into another small weighing boat.

4) Transfer the TSP into the volumetric flask from step 2.

5) Add approximately 380 mL of H_2O to the volumetric flask dissolving the Na_2HPO_4 and TSP by capping the volumetric flask and shaking.

6) If no 4 % NaN_3 solution is available, carry out steps 7 - 9. Otherwise proceed to step 14.

7) Weigh 4 g of NaN_3 into a small weighing boat.

8) Transfer the NaN_3 into a 100 mL volumetric flask and fill with to 100 mL mark with H_2O.

9) Transfer the NaN_3 solution into a labelled reagent bottle. The bottle should be labelled 4 % NaN_3/H_2O SOLUTION and the also contain the date of reagent preparation.

10) Add 5 mL of 4 % NaN_3 solution to the volumetric flask from step 2.

11) Place a clean pH probe in the solution and add very small amounts of strong HCl solution or KOH solution while stirring until the pH probe reads 7.4.

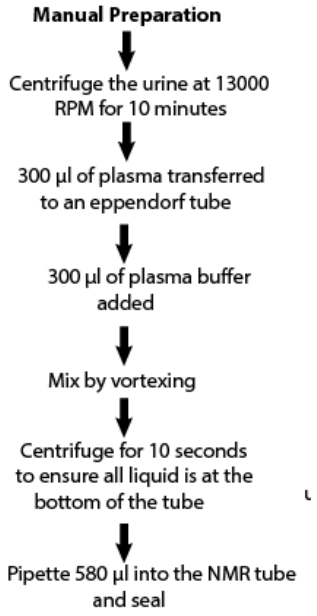
12) Add 100 mL of D_2O to volumetric flask.

13) Fill volumetric flask up to the 500 mL mark with H_2O and mix well by shaking.

14) Transfer the final solution into a labelled reagent bottle. The bottle should be labelled '0.142M $NaHPO_4$, 2mM NaN_3, 0.08% TSP solution, PLASMA/SERUM BUFFER' and also contain the date of reagent preparation

15) Store the reagent at 5 degree Celsius and keep for up to 12 months.

A flow diagram of NMR tube sample preparation following buffer preparation



10.4 Appendix IV: Brain injury review search terms

First performed on 13/11/2016 and updated 17/03/2018

1. biomarker and (serum or serol* or blood)
2. (stroke or cerebrovascular accident or brain injury)
3. 1 and 2
4. (biomarker and (serum or serol* or blood) and (stroke or cerebrovascular accident or brain injury) and acute)
5. remove duplicates from 4
6. limit 5 to english language

10.5 Appendix V : Informed Consent From

Centre number:
Study number:
Participant number:

Informed Consent Form

Project: Metabolic Biomarker Responses in Acute Cerebral Events (M-BRACE)

Name of researcher: Mr A P Coupland

Principle Investigator: Professor Alun Davies

Please initial each box

1. I confirm that I have read and understand the patient information sheet dated ...7/5/16..... version ....1.3..... for the above study and I have had the opportunity to ask questions which have been answered fully.	
2. I understand that my participation is voluntary and I am free to withdraw, without giving any reason, without my medical care or legal rights being affected. 2a. Should I lose the capacity to consent during the recruitment period, I understand that data and samples stored whilst I had capacity will be kept.	
3. I understand that sections of my medical notes may be looked at by responsible individuals from Imperial College London/Imperial College Healthcare NHS Trust or from regulatory authorities where it is relevant to my taking part in research. I give permission for these individuals to access my records that are relevant to this research.	
4. I agree for my samples to be used in future ethically approved studies.	
5. I agree to take part in the above study and for my GP to be informed of my involvement	

Name of Participant (Printed)	**Date**	**Signature**
Name of person taking consent (Printed)	**Date**	**Signature**

1 copy for participant; 1 copy for researcher; 1 copy for hospital notes

10.6 Appendix VI: Participant Information Sheet

Metabolic Biomarker Responses in Acute Cerebral Events

[The 'M-BRACE' study]

You are being invited to take part in a research study that will form part of a PhD thesis. In order for you to decide whether to take part, it is important you understand what the research is about, why it is being done and what it will involve.

Please take time to read through this information sheet carefully and discuss it with others, if you wish.

Please do ask further questions if you would like more information about the study.

Thank you for taking the time to read this information sheet, we hope you find it interesting and useful.

What is the purpose of the study?

The aim of this study is to understand more about stroke and improve its diagnosis. In the future we hope to develop a blood test that can diagnose a stroke. To do this we must perform research on patients who *do* and *do not* have a stroke.

We are collecting blood samples from patients admitted to Charing Cross Hospital with a suspected stroke who are over the age of 45. We need 12mls of blood (less than 3 teaspoons) to store in the laboratory for further testing. We are collecting samples from 450 patients. The first 100 patients recruited to the study will have 24mls of blood taken to allow for an interim analysis.

This blood has already been taken when you arrived in the hospital, along with your usual blood tests.

If after reading this information leaflet, if you do not wish to be included in the study, the extra 12mls of blood will be discarded and you will not be included in the study.

If you agree, we will use your blood to help develop a blood test that can diagnose a stroke quickly and accurately.

Why have I been chosen?

You have been chosen for inclusion in this study because you have been admitted to the Charing Cross Hospital with the possibility of having a stroke and you are over 45 years of age.

Do I have to take part?

It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part, you are still free to withdraw at any time and without giving a reason.

A decision to withdraw at any time, or a decision not to take part, will not alter the standard of care you receive.

What will happen to me if I take part?

You will receive normal treatment, like all patients admitted to Charing Cross Hospital with your condition. If you take part:

- The extra blood samples that have been taken on your admission to hospital, will be stored and analysed at our laboratories at South Kensington.
- We will access some of your medical notes for research purposes. All data will be held confidentially.
- You may be one of 50 patients who are invited to provide 4 further blood tests over the next 48 hours. If you are one of these patients, you will be informed.

What do I have to do?

You should comply with all treatment as directed by the Stroke team. You will not need to alter your treatment in any way.

What are the benefits of taking part?

There are no direct benefits of taking part, but you will help with cutting edge research into stroke. Help us as doctors and scientists better understand what happens in the body after a stroke. With this information we hope to get one step closer to developing a blood test that can diagnose stroke.

What are the possible disadvantages / risks of taking part?

There are few disadvantages. You will need to spend a small amount of time with a researcher who will explain the study in more detail and ask questions about your health and wellbeing. We will also ask you to sign a consent form.

A small number of patients will need more than one blood test (5 in total). This will mean having 4 further blood tests taken using a small needle over the next 48 hours. If you are one of these patients, you will be told during the consent process. With each blood test there is the risk of a small amount of bruising around the needle puncture site.

Exclusion criteria

Unfortunately, you cannot be enrolled in the study if:

- You are being treated for cancer
- You have had a stroke in the last 6 months
- You are taking medication in another clinical trial
- You have epilepsy
- You are a pre-menopausal woman

If any of these apply to you, please let the researcher know.

What happens when the research study stops?

After the research study we will provide you with a summary of our findings. No novel treatment methods will be offered following this research. Your blood samples may be kept for further study but will eventually be destroyed.

Incidental Findings

Should our research identify a significant problem other than stroke, we will communicate with you by letter and suggest appropriate steps to ensure full clinical investigation.

What if something goes wrong?

It is unlikely that this study will be associated with something going wrong with your treatment, as it will not impact the standard of care you receive.

Imperial College London holds insurance policies which apply to this study. If you experience serious and enduring harm or injury as a result of taking part in this study, you may be eligible to claim compensation without having to prove that Imperial College is at fault. This does not affect your legal rights to seek compensation.

If you are harmed due to someone's negligence, then you may have grounds for a legal action. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you have been treated during the course of this study then you should immediately inform the Investigator (Mr Alexander Coupland at a.coupland@imperial.ac.uk).

The normal National Health Service complaints mechanisms are also available to you. If you are still not satisfied with the response, you may contact the Imperial Joint Research Compliance Office.

Important information

If you agree to take part in the study, all the data we generate about you and other patients in the study will be kept confidentially.

With your permission, we would like to inform your GP of your inclusion in the study.

If you lose capacity to consent to further involvement in the study during the 48 hour recruitment period, we will store information and blood we have already collected, but we will not collect further data without consulting a close family friend or relative.

We will present our results at conferences and publish research papers. You will not be identified.

At the end of the study, all participants will receive a summary of our findings.

10.7 Appendix VII: Participant Withdrawal Form

Centre Number: Study Number:

Participant Identification Number for this study:

PARTICIPANT WITHDRAWAL FORM

Study: Metabolic Biomarker Responses in Acute Cerebral Events (MBRACE)

Name of Researcher: Mr Alexander Coupland

I_____would like to withdraw from the above named study.

_____	_____	_____
Name of participant	Date	Signature
_____	_____	_____
Name of researcher	**Date**	**Signature**

10.8 Appendix VIII : Regained capacity information sheet

Metabolic Biomarker Responses in Acute Cerebral Events

[The 'M-BRACE' study]

You have been recruited into the above named research study on admission to hospital. This was done in conjunction with one of your close friends or relatives, or following the advice of a nominated doctor. At the time, you were unable to make an informed decision due to the nature of your condition.

You do not have to continue to take part. In order for you to decide whether to take part, it is important you understand what the research is about, why it is being done and what it will involve.

Please take time to read through this information sheet carefully and discuss it with others, if you wish.

Please do ask further questions if you would like more information about the study.

Thank you for taking the time to read this information sheet, we hope you find it interesting and useful.

What is the purpose of the study?

The aim of this study is to understand more about stroke and improve its diagnosis. In the future we hope to develop a blood test that can diagnose a stroke. To do this we must perform research on patients who *do* and *do not* have a stroke.

We are collecting blood samples from patients admitted to Charing Cross Hospital with a suspected stroke who are over the age of 45. We need 12mls of blood (less than 3 teaspoons) to store in the

laboratory for further testing. We are collecting samples from 450 patients. The first 100 patients recruited to the study, will have 24mls of blood taken to allow for an interim analysis.

This blood has already been taken when you arrived in the hospital, along with your usual blood tests.

If after reading this information leaflet, if you do not wish to be included in the study, the extra 12mls of blood will be discarded and you will not be included in the study.

If you agree, we will use your blood to help develop a blood test that can diagnose a stroke quickly and accurately.

Why have I been chosen?

You have been chosen for inclusion in this study because you have been admitted to the Charing Cross Hospital with the possibility of having a stroke and you are over 45 years of age.

Do I have to take part?

It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part, you are still free to withdraw at any time and without giving a reason.

A decision to withdraw at any time, or a decision not to take part, will not alter the standard of care you receive.

What will happen to me if I take part?

You will receive normal treatment, like all patients admitted to Charing Cross Hospital with your condition. If you take part:

- The extra blood samples that have been taken on your admission to hospital, will be stored and analysed at our laboratories at South Kensington.
- We will access some of your medical notes for research purposes. All data will be held confidentially.
- You may be one of 50 patients who are invited to provide 4 further blood tests over the next 48 hours. If you are one of these patients, you will be informed.

What do I have to do?

You should comply with all treatment as directed by the Stroke team. You will not need to alter your treatment in any way.

What are the benefits of taking part?

There are no direct benefits of taking part, but you will help with cutting edge research into stroke. Help us as doctors and scientists better understand what happens in the body after a stroke. With this information we hope to get one step closer to developing a blood test that can diagnose stroke.

What are the possible disadvantages / risks of taking part?

There are few disadvantages. You will need to spend a small amount of time with a researcher who will explain the study in more detail and ask questions about your health and wellbeing. We will also ask you to sign a consent form.

A small number of patients will need more than one blood test (5 in total). This will mean having 4 further blood tests taken using a small needle over the next 48 hours. If you are one of these patients, you will be told during the consent process. With each blood test there is the risk of a small amount of bruising around the needle puncture site.

Exclusion criteria

Unfortunately, you cannot be enrolled in the study if:

- You are being treated for cancer
- You have had a stroke in the last 6 months
- You are taking medication in another clinical trial
- You have epilepsy
- You are a pre-menopausal woman

If any of these apply to you, please let the researcher know.

What happens when the research study stops?

After the research study we will provide you with a summary of our findings. No novel treatment methods will be offered following this research. Your blood samples may be kept for further study but will eventually be destroyed.

Incidental Findings

Should our research identify a significant problem other than stroke, we will communicate with you by letter and suggest appropriate steps to ensure full clinical investigation.

What if something goes wrong?

It is unlikely that this study will be associated with something going wrong with your treatment, as it will not impact the standard of care you receive.

Imperial College London holds insurance policies which apply to this study. If you experience serious and enduring harm or injury as a result of taking part in this study, you may be eligible to claim compensation without having to prove that Imperial College is at fault. This does not affect your legal rights to seek compensation.

If you are harmed due to someone's negligence, then you may have grounds for a legal action. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you have been treated during the course of this study then you should immediately inform the Investigator (Mr Alexander Coupland at a.coupland@imperial.ac.uk).

The normal National Health Service complaints mechanisms are also available to you. If you are still not satisfied with the response, you may contact the Imperial Joint Research Compliance Office.

Important information

If you agree to take part in the study, all the data we generate about you and other patients in the study will be kept confidentially.

With your permission, we would like to inform your GP of your inclusion in the study.

If you lose capacity to consent to further involvement in the study during the 48 hour recruitment period, we will store information and blood we have already collected, but we will not collect further data without consulting a close family friend or relative.

We will present our results at conferences and publish research papers. You will not be identified.

At the end of the study, all participants will receive a summary of our findings.

10.9 Appendix IX: Regained Capacity Withdrawal Form

Centre Number: Study Number:

Participant Identification Number for this study:

PARTICIPANT WITHDRAWAL FORM

Study: Metabolic Biomarker Responses in Acute Cerebral Events (MBRACE)

Name of Researcher: Mr Alexander Coupland

I understand that I was included in the above named study, prior to giving consent, as agreed by the Research Ethics Committee under Section 32 of the Mental Capacity Act.

I have now received further information about the study from a researcher and I do not wish to be involved.

I_____would like to withdraw from the above named study.

10.10 Appendix X: Consultee Declaration Form

Centre Number: Study Number:

Participant Identification Number for this study:

CONSULTEE DECLARATION FORM

Study: Metabolic Biomarker Responses in Acute Cerebral Events (MBRACE)

Name of Researcher: Mr Alexander Coupland

Please initial box

I _____ have been consulted about _____'s participation in this research project. I have had the opportunity to ask questions about the study and understand what is involved. ☐

☐

In my opinion he/she would have no objection to taking part in the above study.

I understand that I can request he/she is withdrawn from the study at any time, without giving any reason and without his/her care or legal rights being affected. ☐

I understand that relevant sections of his/her care record and data collected during the study may be looked at by responsible individuals from Imperial College London or from regulatory authorities, where it is relevant to their taking part in this research. ☐

I agree to their GP or other care professional being informed of their participation in the study. ☐

Name of Consultee	Date	Signature

Relationship to participant: _____

Person undertaking consultation (if different from researcher):		
Name	Date	Signature
Researcher		Date
	Signature	

When completed: 1 (original) to be kept in care record, 1 for consultee; 1 for researcher site file

10.11 Appendix XI: Consultee Information Sheet

Metabolic Biomarker Responses in Acute Cerebral Events

(MBRACE)

Information for Consultee

Version 1.1 Date 09/05/16

Introduction

We feel your relative/friend is unable to decide for himself/herself whether to participate in this research.

To help decide if they would join the study, we would like to ask your opinion. We ask you to consider what you know of their wishes and feelings, and to consider their interests. Please let us know of any advanced decisions they may have made about participating in research. These should take precedence.

If you decide your relative/friend would have no objection to taking part, we will ask you to read and sign the consultee declaration on the last page of this information leaflet. We will keep you fully informed during the study so you can let us know if you have any concerns or you think your relative/friend should be withdrawn.

Whether your friend/relative takes part in the research or not, will not alter the standard of care they receive.

If you are unsure about taking the role of a consultee you may seek independent advice.

We will understand if you do not wish to take on this responsibility.

The following information is the same as would have been provided to your relative/friend.

What is the purpose of the study?

The aim of this study is to understand more about stroke and improve its diagnosis. In the future we hope to develop a blood test that can diagnose a stroke. To do this we must perform research on patients who *do* and *do not* have a stroke.

We are collecting blood samples from patients admitted to Charing Cross Hospital with a suspected stroke who are over the age of 45. We need 12mls of blood (less than 3 teaspoons) to store in the laboratory for further testing. We are collecting samples from 450 patients. The first 100 patients recruited to the study will have 24mls of blood taken to allow for an interim analysis.

This blood has already been taken when your friend/relative arrived in the hospital, along with their usual blood tests.

If after reading this information leaflet, you do not think your friend/relative would agree to be included in the study, the extra blood will be discarded.

If you do think they would consent, we will use their blood to help develop a blood test that can diagnose a stroke quickly and accurately.

Why has my friend/relative been chosen?

They have been chosen for inclusion in this study because they have been admitted to the Charing Cross Hospital with the possibility of having a stroke and are over 45 years of age.

Does my friend/relative have to take part?

It is up to you to decide whether or not your friend/relative takes part. If you do decide they would take part, you will be given this information sheet to keep and asked to sign a consultee declaration form.

A decision to withdraw at any time, or a decision not to take part, will not alter the standard of care your friend/relative receives.

What will happen to my friend/relative if they take part?

They will receive normal treatment, like all patients admitted to Charing Cross Hospital with the same condition. If they take part:

- The extra blood samples that have been taken on admission to hospital, will be stored and analysed at our laboratories at South Kensington.
- We will access some of your friend/relative's medical notes for research purposes. All data will be held confidentially.
- Your friend/relative may be one of 50 patients who are invited to provide 4 further blood tests over the next 48 hours. If they are one of these patients, you will be informed.

What does my friend/relative have to do?

They should comply with all treatment as directed by the Stroke team. They will not need to alter their treatment in any way.

What are the benefits of taking part?

There are no direct benefits of taking part, but your friend/relative will help with cutting edge research into stroke. Help us as doctors and scientists better understand what happens in the body after a stroke. With this information we hope to get one step closer to developing a blood test that can diagnose stroke.

What are the possible disadvantages / risks of taking part?

There are few disadvantages. You will need to spend a small amount of time with a researcher who will explain the study in more detail and ask questions about the health and wellbeing of your friend/relative. We will also ask you to sign a consultee declaration form.

A small number of patients will need more than one blood test (5 in total). This will mean having 4 further blood tests taken using a small needle over the next 48 hours. If your friend/relative is one of these patients, you will be told during the declaration process. Each blood test carries a small risk of bruising around the needle puncture site.

What happens when the research study stops?

After the research study we will provide you with a summary of our findings. No novel treatment methods will be offered following this research. Your blood samples may be kept for further study but will eventually be destroyed.

Incidental Findings

Should our research identify a significant problem other than stroke, we will communicate with you by letter and suggest appropriate steps to ensure full clinical investigation.

What if something goes wrong?

It is unlikely that this study will be associated with something going wrong with your friend/relative's treatment, as it will not impact the standard of care they receive.

Imperial College London holds insurance policies which apply to this study. If your friend/relative experiences serious and enduring harm or injury as a result of taking part in this study, they may be eligible to claim compensation without having to prove that Imperial College is at fault. This does not affect their legal rights to seek compensation.

If your friend/relatives is harmed due to someone's negligence, then they may have grounds for a legal action. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way your friend/relative has been treated during the course of this study then you should immediately inform the Investigator (Mr Alexander Coupland at a.coupland@imperial.ac.uk).

The normal National Health Service complaints mechanisms are also available to you and your friend/relative. If you are still not satisfied with the response, you may contact the Imperial AHSC Joint Research Compliance Office.

Important information

If you agree that your friend/relative can take part in the study, all the data we generate about them and other patients in the study will be kept confidentially.

With your permission, we would like to inform your friend/relative's GP of their inclusion in the study.

We will present our results at conferences and publish research papers. Your friend/relative will not be identified.

At the end of the study, all participants will receive a summary of our findings.

10.12 Appendix XII: Consultee Withdrawal Form

Centre Number: Study Number:

Participant Identification Number for this study:

CONSULTEE WITHDRAWAL FORM

Study: Metabolic Biomarker Responses in Acute Cerebral Events (MBRACE)

Name of Researcher: Mr Alexander Coupland

I_____would like to withdraw

Mr/Mrs/Miss _____from the above named study.

_____	_____	_____
Name of Consultee	Date	Signature

Relationship to participant:

_____

_____	_____	_____
Name of Researcher	**Date**	**Signature**

Appendix XIII: Participant lost capacity, consultee declaration form (for expected temporal trend blood sampling)

Centre Number: Study Number:

Participant Identification Number for this study:

CONSULTEE DECLARATION FORM

Study: Metabolic Biomarker Responses in Acute Cerebral Events (MBRACE)

Name of Researcher: Mr Alexander Coupland

Please initial box

I _____ have been consulted about _____'s continued participation in this research project. I have had the opportunity to ask questions about the study and understand what is involved. ☐

In my opinion he/she would have no objection to taking part in the above study. ☐

I understand that I can request he/she is withdrawn from the study at any time, without giving any reason and without his/her care or legal rights being affected. ☐

I understand that relevant sections of his/her care record and data collected during the study may be looked at by responsible individuals from Imperial College London or from regulatory authorities, where it is relevant to their taking part in this research. ☐

I agree to their GP or other care professional being informed of their participation in the study. ☐

Name of Consultee	Date	Signature

Relationship to participant:

Person undertaking consultation (if different from researcher):		
Name	Date	Signature

Researcher		Date
	Signature	

When completed: 1 (original) to be kept in care record, 1 for consultee; 1 for researcher site file

10.13 Appendix XIII: Participant lost capacity, consultee information sheet

(for expected temporal trend blood sampling)

Metabolic Biomarker Responses in Acute Cerebral Events

(MBRACE)

Information for Consultee

Version 1.0 Date 09/05/16

Introduction

Your relative/friend agreed to be included in the above named research study on admission to hospital. He was selected as 1 of 50 patients who will have 4 further blood tests.

Your relative/friend is no longer able to decide for him/herself whether to have the further blood tests taken. To help decide if they would continue to be involved in the study, we would like to ask your opinion.

We ask you to consider what you know of their wishes and feelings, and to consider their interests. Please let us know of any advanced decisions they may have made about participating in research. These should take precedence.

If you decide your relative/friend would have no objection to taking part, we will ask you to read and sign the consultee declaration on the last page of this information leaflet. We will keep you fully

informed during the study so you can let us know if you have any concerns or you think your relative/friend should be withdrawn.

Whether your friend/relative takes part in the research or not, will not alter the standard of care they receive.

If you are unsure about taking the role of a consultee you may seek independent advice.

We will understand if you do not wish to take on this responsibility.

The following information is the same as would have been provided to your relative/friend.

What is the purpose of the study?

The aim of this study is to understand more about stroke and improve its diagnosis. In the future we hope to develop a blood test that can diagnose a stroke. To do this we must perform research on patients who *do* and *do not* have a stroke.

We are collecting blood samples from patients admitted to Charing Cross Hospital with a suspected stroke who are over the age of 45. We need 12mls of blood (less than 3 teaspoons) to store in the laboratory for further testing. We are collecting samples from 450 patients. The first 100 patients recruited to the study will have 24mls of blood taken to allow for an interim analysis.

This blood has already been taken when your friend/relative arrived in the hospital, along with their usual blood tests.

If after reading this information leaflet, you do not think your friend/relative would agree to be included in the study, the extra blood will be discarded.

If you do think they would consent, we will use their blood to help develop a blood test that can diagnose a stroke quickly and accurately.

Why has my friend/relative been chosen?

They have been chosen for inclusion in this study because they have been admitted to the Charing Cross Hospital with the possibility of having a stroke and are over 45 years of age.

Does my friend/relative have to take part?

It is up to you to decide whether or not your friend/relative takes part. If you do decide they would take part, you will be given this information sheet to keep and asked to sign a consultee declaration form.

A decision to withdraw at any time, or a decision not to take part, will not alter the standard of care your friend/relative receives.

What will happen to my friend/relative if they take part?

They will receive normal treatment, like all patients admitted to Charing Cross Hospital with the same condition. If they take part:

- The extra blood samples that have been taken on admission to hospital, will be stored and analysed at our laboratories at South Kensington.
- We will access some of your friend/relative's medical notes for research purposes. All data will be held confidentially.
- Your friend/relative may be one of 50 patients who are invited to provide 4 further blood tests over the next 48 hours. If they are one of these patients, you will be informed.

What does my friend/relative have to do?

They should comply with all treatment as directed by the Stroke team. They will not need to alter their treatment in any way.

What are the benefits of taking part?

There are no direct benefits of taking part, but your friend/relative will help with cutting edge research into stroke. Help us as doctors and scientists better understand what happens in the body after a stroke. With this information we hope to get one step closer to developing a blood test that can diagnose stroke.

What are the possible disadvantages / risks of taking part?

There are few disadvantages. You will need to spend a small amount of time with a researcher who will explain the study in more detail and ask questions about the health and wellbeing of your friend/relative. We will also ask you to sign a consultee declaration form.

A small number of patients will need more than one blood test (5 in total). This will mean having 4 further blood tests taken using a small needle over the next 48 hours. If your friend/relative is one of these patients, you will be told during the declaration process. Each blood test carries a small risk of bruising around the needle puncture site.

What happens when the research study stops?

After the research study we will provide you with a summary of our findings. No novel treatment methods will be offered following this research. Your blood samples may be kept for further study but will eventually be destroyed.

Incidental Findings

Should our research identify a significant problem other than stroke, we will communicate with you by letter and suggest appropriate steps to ensure full clinical investigation.

What if something goes wrong?

It is unlikely that this study will be associated with something going wrong with your friend/relative's treatment, as it will not impact the standard of care they receive.

Imperial College London holds insurance policies which apply to this study. If your friend/relative experiences serious and enduring harm or injury as a result of taking part in this study, they may be eligible to claim compensation without having to prove that Imperial College is at fault. This does not affect their legal rights to seek compensation.

If your friend/relatives is harmed due to someone's negligence, then they may have grounds for a legal action. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way your friend/relative has been treated during the course of this study then you should immediately inform the Investigator (Mr Alexander Coupland at a.coupland@imperial.ac.uk).

The normal National Health Service complaints mechanisms are also available to you and your friend/relative. If you are still not satisfied with the response, you may contact the Imperial AHSC Joint Research Compliance Office.

Important information

If you agree that your friend/relative can take part in the study, all the data we generate about them and other patients in the study will be kept confidentially.

With your permission, we would like to inform your friend/relative's GP of their inclusion in the study.

We will present our results at conferences and publish research papers. Your friend/relative will not be identified.

At the end of the study, all participants will receive a summary of our findings.

10.14 Appendix XIV: Reasons for participant exclusion

Date	Time	Reason for exclusion
13/07/2016	1030	Pre-menopausal
14/07/2016	1130	Seizure
14/07/2016	1430	Did not consent
20/07/2016	1125	wake-up stroke
20/07/2016	1130	wake-up symptoms
25/07/2016	1000	wake up symptoms
25/07/2016	1510	>6 hours onset
03/08/2016	1200	wake up
03/08/2016	1430	<45
04/08/2016	1145	active malignancy
08/08/2016	1015	wake up symptoms
08/08/2016	1230	thrombolysis given at Northwick Park
09/08/2016	1330	thrombolysis given. Transfer for thrombectomy
08/09/2016	905	<45
08/09/2016	1100	Pre-menopausal
12/08/2016	1430	Thrombectomy transfer; thrombolysis given
14/08/2016	1230	wake-up stroke
14/09/2016	1410	Transfer from SMH. Bloods taken at SMH.
14/09/2016	1415	Transfer for thrombectomy. Bloods taken prior to arrival
15/09/2016	1015	wake up stroke
15/09/2016	1245	Symptomatic onset yesterday
15/09/2016	1500	Seizure
16/09/2016	1000	wake up stroke
19/09/2016	1230	wake up stroke
19/09/2016	1810	Temp 38.4
22/09/2016	1500	Previous stroke last 6 months
26/09/2016	1330	<45
28/09/2016	1225	None English speaking
28/09/2016	1300	Malignancy
28/09/2016	1315	No time of onset
08/10/2016	1000	Insufficient blood
08/10/2016	1300	Insufficient blood
24/10/2016	1100	Insufficient blood
11/11/2016		Head injury
11/11/2016		>6 hours onset
16/11/2016	1400	Previous stroke last 6 months
17/11/2016	1930	Previous stroke last 6 months
17/11/2016	1945	Sepsis
21/11/2016	1300	insufficient blood
28/11/2016	1200	<45

28/11/2016	1600	thrombolysis given. Transfer for thrombectomy
29/11/2016	1045	Known malignancy
01/12/2016	1905	did not consent
01/12/2016	1920	insufficient blood
01/12/2016	2020	insufficient blood
07/12/2016	1500	Previous stroke last 6 months
07/12/2016	1800	Insufficient blood
09/12/2016	1030	<45
09/12/2016	1250	Thrombectomy transfer; thrombolysis given
15/12/2016	1520	Unknown onset time
15/12/2016	1715	Malignancy
03/01/2017	1025	wake up stroke
03/01/2017	1130	wake up stroke
09/01/2017	1345	<45
16/01/2017	1330	Wake up
16/01/2017	1335	Insufficient blood
16/01/2017	1400	Thrombectomy transfer; thrombolysis given
16/01/2017	1600	Previous stroke last 6 months
22/01/2017	1500	<45
23/01/2017	1500	insufficient blood
23/01/2016	1200	sepsis
24/01/2016	1315	Seizure
01/02/2017	1030	wake up
01/02/2017	1400	<45
01/03/2017	1100	<45
02/03/2017	1200	Thrombectomy transfer; thrombolysis given
02/03/2017	1200	Bloods already taken
03/03/2017	1400	Refused
08/03/2017	1430	Active Ca
13/03/2017	1145	<45
13/03/2017	1500	Insufficient blood
14/03/2017	1130	<45
14/03/2017	1230	Malignancy
27/03/2017	1345	Previous stroke last 6 months
17/04/2017	1200	Sepsis
17/04/2017	1300	<45
18/04/2017	1045	Thrombectomy transfer; thrombolysis given
24/04/2017	1200	Thrombectomy transfer; thrombolysis given

10.15 Appendix XV: Baseline characteristics for matched models

Acute event Vs Mimic

	Ischaemic events (n= 29)	Control (n= 10)	p-value
Age (mean)	70	68	0.76
Sex (m)	18	4	0.28
BMI (mean)	26	26	0.99
Smoking status	4	2	0.99
Statin use	13	3	0.48
Anti-platelet use	11	2	0.45

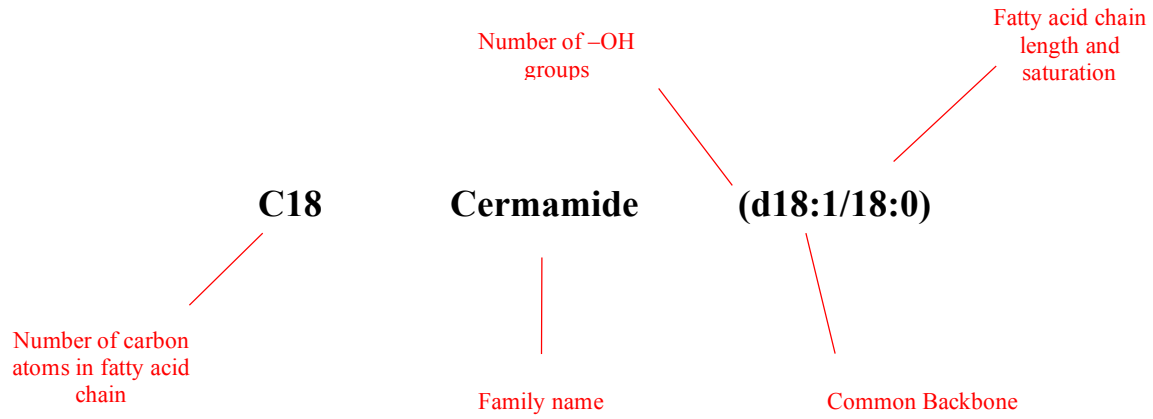
Acute TIA Vs Subacute TIA

	Ischaemic events (n= 9)	Control (n= 9)	p-value
Age (mean)	71	70	0.79
Sex (m)	6	6	0.99
BMI (mean)	28	27	0.79
Smoking status	0	1	0.99
Statin use	4	4	0.99
Anti-platelet use	4	5	0.99

Ischaemic stroke Vs Haemorrhagic stroke

	Ischaemic events (n= 12)	Control (n= 8)	p-value
Age (mean)	71	65	0.33
Sex (m)	6	6	0.37
BMI (mean)	26	23	0.23
Smoking status	2	2	0.99
Statin use	5	4	0.99
Anti-platelet use	4	3	0.99

10.16 Appendix XVI: Cayman's Lipid Nomenclature



11 ADDITIONAL DOCUMENTATION

Below are the grants/fellowships, posters and publications achieved that are relevant to this thesis.

Published abstracts

Qureshi M, Coupland A, Vorkas P, Jenkins I, Holmes E, Davies AH. Metabolic profiling of ischaemic stroke. *International Journal of Stroke*. 2016; 11: 4S

Published papers

Coupland A, Thapar A, Qureshi MI, Jenkins H, Davies AH. The definition of stroke. *Journal of the Royal Society of Medicine*. 2017; 110(1): 9-12

Qureshi MI, Vorkas PA, Coupland A, Jenkins IH, Holmes E, Davies AH. Lessons from metabonomics on the neurobiology of stroke. *The Neuroscientist*. 2016.

Presentations

Coupland A. In search of metabolic biomarkers for acute cerebral events. Imperial Network of Excellence in Vascular Science Launch Meeting. Imperial College London, March 2018.

Coupland A, Milinis K, Thapar A, Lobotesis K, Davies AH. A systematic review of thrombus composition following mechanical thrombectomy for acute ischaemic stroke. Charing Cross Symposium, London, April 2017

Stather P, Muscara F, on behalf of the VERN Collaborators. Audit of Best Medical Therapy for Referrals to Vascular Clinic. Oral presentation, Vascular Society of Surgeons of Great Britain and Ireland, Manchester, December 2016 *(Oral)*

Dattani N, Stather P, on behalf of the VERN Collaborators. Peripheral Arterial Disease: a multicentre study of primary and secondary care practices. Oral presentation, Vascular Society of Surgeons of Great Britain and Ireland, Manchester, December 2016 *(Oral)*

Qureshi M, Coupland A, Vorkas P, Jenkins I, Holmes E, Davies AH. Metabolic profiling of ischaemic stroke. UK Stroke Forum. November 2016

Grants/Fellowships

Graham-Dixon Charitable Trust, Research Grant, 2016, £5870

Imperial Private Healthcare Clinical Research Fellowship (annual salary and contributions towards tuition fees)