Proteomic Investigations and Biomarker Discovery in Transient Ischaemic Attack

A thesis by

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To my wife and family
whose love and support
made this thesis possible

“We are all visitors to this time, this place. We are just passing through. Our purpose here is to observe, to learn, to grow, to love... and then we return home.”

Australian Aboriginal Proverb

DECLARATION

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Manuscripts for submission:

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SUMMARY

Between 15-26% of ischaemic strokes are preceded by transient ischaemic attack (TIA) making accurate and timely diagnosis of TIA important for stroke prevention. However, TIA diagnoses are highly reliant on subjective history gathering and clinical assessments to differentially diagnose true TIA conditions from mimic presentations. Unfortunately, the subjective nature of TIA diagnosis has created a surprisingly high amount of variability between diagnoses made by physicians and specialist neurologists. Use of biomarker tests could offer an objective quantitative measuring tool that reduces inter-observer variation through the establishment of standardised quantitative measures and improved reproducibility. When used in combination with comprehensive clinical assessments and neurological imaging, biomarkers may offer a useful adjunct to assist a treating clinician to accurately and reliably interpret the clinical finding and confidently diagnose and treat a TIA or mimic condition. This thesis proposes a framework for undertaking an exploration of the human plasma proteome, and performs the very first proteomic pilot study that identifies candidate plasma protein biomarkers associated with TIA, which could also be used to distinguish from mimic presentations.

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LIST OF ABBREVIATIONS AND ACRONYMS

%	percentage
^{o}C	degrees Celsius
x g	x gravity
π	*pi*
Δ	change
µg	microgram
µL	microliter
µm	micrometre
2DE	two dimensional electrophoresis
ABCD2	age, blood pressure, clinical features, duration, diabetes
ACE	angiotensin converting enzyme
AngII	angiotensin II
ANOVA	analysis of variance
APOA	apolipoprotein A
APOB	apolipoprotein B
APOE	apolipoprotein E
AWGPN	Adelaide Western General Practice Network
BD	Becton Dickinson

BH_4	tetrahydrobiopterin
BP	band pass
BVA	biological variation analysis
C4A	complement component C4-A
CBF	cerebral blood flow
CBV	cerebral blood volume
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate detergent
CI	confidence interval
CID	collision induced dissociation
cm	centremetre
CNS	central nervous system
COMBAT	community-based rapid access TIA clinic
COX	cyclooxygenase
CSF	cerebrospinal fluid
CT	computed tomography
CTA	computed tomography angiography
CTP	computed tomography perfusion
CyDye	cyanine dye
Da	dalton
DALYs	disability adjusted life years
dH_2O	deionised water
DIA	differential in-gel analysis
DIGE	differential in-gel electrophoresis
DMF	dimethylformamide
DNA	deoxyribonucleic acid
DSA	digital subtraction angiography
DTT	dithiothreitol

DWI	diffusion weighted imaging
EBT	erichrome black T
ECG	electrocardiogram
eNOS	endothelial nitric oxide synthase
ESI	electrospray ionisation
EtOH	ethanol
FGA	fibrinogen alpha-chain
FGB	fibrinogen beta-chain
FIBG	fibrinogen gamma chain
FLAIR	fluid-attenuated inversion recovery
fMRI	functional magnetic resonance imaging
FPLC	fast protein liquid chromatography
FWHM	full width at half-maximum
GE	General Electric
GFAP	glial fibrillary acidic protein
GP	general practitioner
GP-SIS	general practitioner with a special interest in stroke
GRE	gradient-recalled echo magnetic resonance imaging
GS	gelsolin
H_2O	water
HbA1C	glycated haemoglobin
HCl	hydrochloric acid
HCV	healthy control volunteer
HDL-C	high density lipoprotein cholesterol
HEMO	hemopexin
HPLC	high performance liquid chromatography
HPPP	human plasma proteome project

HREC	human research ethics committee
hsCRP	high-sensitivity C-reactive protein
HUPO	human proteome organisation
ICH	intracranial haemorrhage
ICP	intracranial pressure
IEF	isoelectric focusing
IFN- γ	interferon gamma
IL	interleukin
INR	international normalised ratio
IPG	immobilised pH gradient
I.S.	internal standard
iTRAQ	isobaric tagging technology for relative and absolute quantitation
K_2EDTA	dipotassium ethylenediaminetetraacetic acid
kDa	kilodalton
KR	Keil rule- trypsin cleaves next to arginine or lysine but not before proline
LC	liquid chromatography
LDL-C	low density lipoprotein cholesterol
Lp-PLA2	lipoprotein-associated phospholipase A2
M	molar
MARS-Hu6	multiple affinity removal system – human 6 immunodepletion column
MBP	myelin basic protein
MCA	middle cerebral artery
mg	milligram
mL	millilitre
mm	millimetre
mM	millimolar
mmHg	millimetre of mercury

MPa	megapascal
mRNA	messenger ribonucleic acid
MRM	multiple reaction monitoring
MS	mass spectrometry
MS/MS	tandem mass spectrometry
MTT	mean transit time
MW	molecular weight
m/z	mass-to-charge ratio
n	sample size
NCCT	non-contrast heat computed tomography
NGS	next generation sequencing
NSE	neuron specific enolase
NHS	N-hydroxyl succinimidyl
nL	nanolitre
NL	non linear
nm	nanometre
nmol	nanomole
NSAID	non-steroidal anti-inflammatory drug
PAGE	polyacrylamide gel electrophoresis
PAI-1	plasminogen activator inhibitor 1
PBP	platelet basic protein
PCA	principal component analysis
PEEK	polyetheretherketone
PET	positron emission tomography
pH	hydrogen ion concentration
pI	isoelectric point
pmol	picomole

PMT	photo multiplier tube
ppm	parts per million
psi	pound-force per square inch
PWI	perfusion weighted imaging
r	radius
RAC	rapid assessment clinic
rt-PA	recombinant tissue plasminogen activator
S-100B	S100 calcium binding protein B
SAMP	serum amyloid P-component
SD	standard deviation
SDS	sodium dodecyl sulfate
SILAC	stable isotope labelling by amino acids in cell culture
SNP	single nucleotide polymorphism
TC	total cholesterol
TIA	transient ischaemic attack
TNF	tumour necrosis factor
TOF	time of flight
TOF-MRA	time of flight magnetic resonance angiography
TQEH	The Queen Elizabeth Hospital, Woodville South, Adelaide, SA
Trig	triglyceride
TUC	thiourea, urea, CHAPS, tris lysis buffer
UV	ultraviolet
V	volts
Vhrs	volt hours
v/v	volume per volume
w/v	weight per volume
ZA2G	zinc-alpha-2-glycoprotein

LIST OF APPENDICES

1 INTRODUCTION

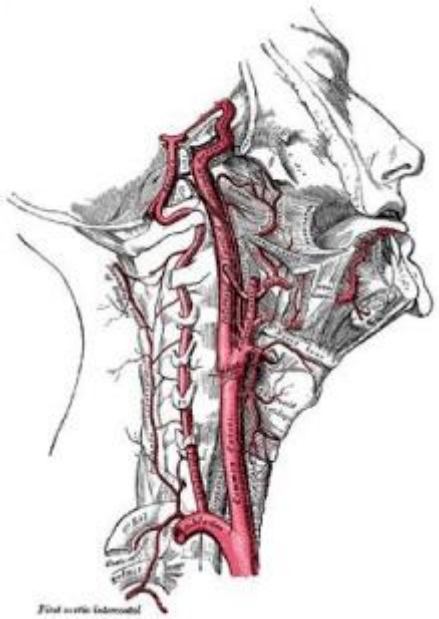
1.1 Basic Principles of Neuroanatomy and Cerebrovascular Circulation.

As an organ, the human brain comprises only two-percent of body weight, however, it receives 15-20% of total cardiac output, making it the most highly perfused organ in the body (Cipolla, 2009). The high metabolic requirements of the human brain (maintained by oxidative metabolism) underpins the need for this high portion of cardiac output and a consistent blood flow. The brain is enclosed by a rigid skull structure, which does not allow for expansion of either brain tissue or extracellular fluid without significantly harmful neurological complications or death. The brain controls intracranial pressure (ICP) whilst specifically regulating water and solute transport from the blood into the brain parenchyma to maintain an appropriate ionic balance that is conducive for neuronal function. Cerebral circulation is dependent on large arteries to provide a constant blood flow to neural tissue and protect cerebral microcirculation during fluctuations in arterial pressure. This chapter will explore structural and functional aspects of the human cerebral circulation before examining the pathophysiology of cerebrovascular diseases.

1.1.1 Arteries

Arterial blood supply to the human brain is provided by two pairs of large arteries, the left and right internal carotid and the left and right vertebral arteries (Figure 1). The internal carotid arteries supply the cerebrum, whilst the vertebral arteries merge to form the basilar artery whereby branches of both vertebral and basilar arteries supply blood for the cerebellum and brain stem. The proximal basilar artery connects to two internal carotid arteries and smaller communicating arteries to form an arterial circle at the base of the brain known as the circle of Willis. The circle of Willis contains three pairs of main arteries, the anterior, middle and posterior cerebral arteries, which divide into smaller arteries and arterioles that progressively become smaller until they penetrate brain tissue to supply blood to corresponding regions of the cerebral cortex.

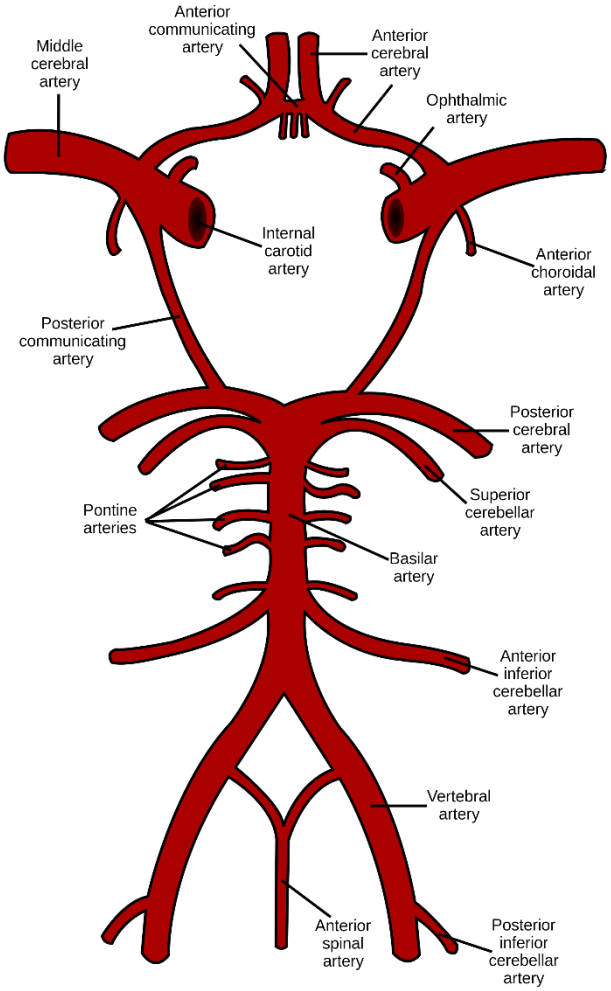
Figure 1: Right sided representation of the internal carotid and vertebral arteries. *Extracted from (Gray, 1918).*



Collateral circulation in the brain consists of vascular networks that preserve cerebral blood flow when primary blood vessels fail due to occlusion or constriction. The anastomotic loop of the circle of Willis allows for redistribution of blood flow via low-resistance connections to redistribute and provide primary collateral blood supply to

anterior and posterior circulations when extracranial or large intracranial vessels are occluded (Figure 2).

Figure 2: The Circle of Willis. *A circulatory anastomosis that supplies blood to the brain, which is comprised of an anterior communicating artery, left and right anterior cerebral, internal carotid, posterior cerebral and posterior communicating arteries, as well as the basilar artery. (Public domain)*

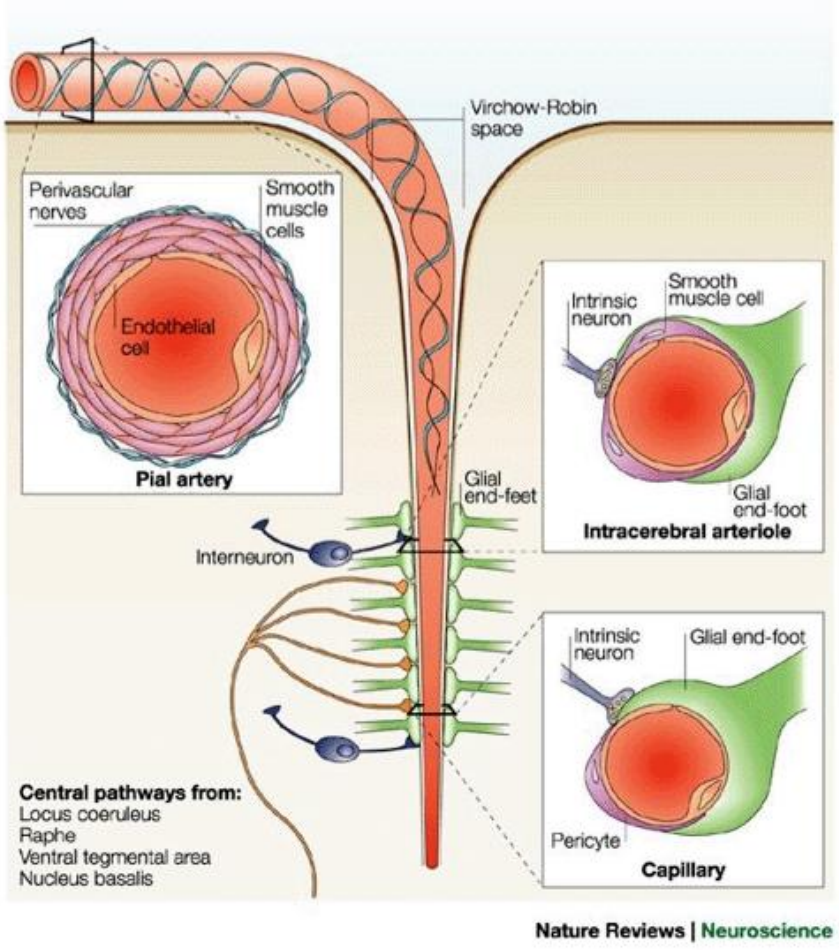


1.1.2 Cerebrovascular Architecture and the Neurovascular Unit

Pial vessels are intracranial blood vessels on the brain surface that are surrounded by cerebrospinal fluid (CSF) and give rise to smaller parenchymal arterioles that penetrate into brain tissue and eventually become surrounded by neurons and astrocytic vascular-

feet (Figure 3) (Jones, 1970; Rennels & Nelson, 1975). Communication between neurons, astrocytes and cerebral arterioles forms a neurovascular unit, which is able to finely regulate blood supply to match the energy and oxygen needs of activated neurons. This coupling of neurovascular units is so precise that it is used to map changes in neuronal activity from functional magnetic resonance imaging (fMRI) and positron emission tomography (PET) (Lecrux & Hamel, 2011). A key structural difference between pial vessels and the smaller penetrating parenchymal arterioles is that pial vessels form an extensive collateral network such that occlusion of a single pial vessel will not significantly reduce cerebral blood flow (Nishimura, Schaffer, Friedman, Lyden, & Kleinfeld, 2007). On the other hand, parenchymal arterioles are deeply penetrating singular vessels that are largely unbranched such that occlusion of an individual arteriole will result in a significant reduction in blood flow, causing brain tissue necrosis (infarction) to the surrounding local brain tissue (Nishimura et al., 2007).

Figure 3: Neurovascular architecture. *Pial arteries on the brain surface form penetrating parenchymal arterioles that supply the cerebral microcirculation including neurons and astrocytes known as the neurovascular unit. Extracted from (Iadecola, 2004).*



1.1.3 Endothelial modulation of tone and the control of cerebral blood flow.

Cerebrovascular endothelium is a highly specialised cell type in the brain that is involved in numerous physiological processes including the regulation of inflammatory and immune responses, thrombosis, adhesion, angiogenesis, and permeability (Zlokovic, 2008). The significance of endothelium is highlighted by the central role that endothelial dysfunction has in the pathogenesis of numerous cerebrovascular diseases including Alzheimer's disease, epilepsy, and stroke. Endothelium produces vasoactive mediators including nitric oxide, prostacyclin, and endothelium-derived hyperpolarising

factor, which significantly influence vascular tone and therefore influence cerebral blood flow (Faraci & Heistad, 1998). For example, nitric oxide is a prominent vasodilator produced by the endothelial nitric oxide synthase enzyme (eNOS). Nitric oxide production inhibits resting tone in pial arteries and parenchymal arterioles, thereby increasing cerebral blood flow through vasodilation (Faraci & Brian, 1994). A critical determinant of eNOS activity is the availability of the cofactor tetrahydrobiopterin (BH_4). Reduction in BH_4 availability has been linked to several pathologies including atherosclerosis, diabetes, and hypertension, whereby eNOS coupling is reduced, nitric oxide synthesis is decreased, and the endothelial tone modulation is disrupted.

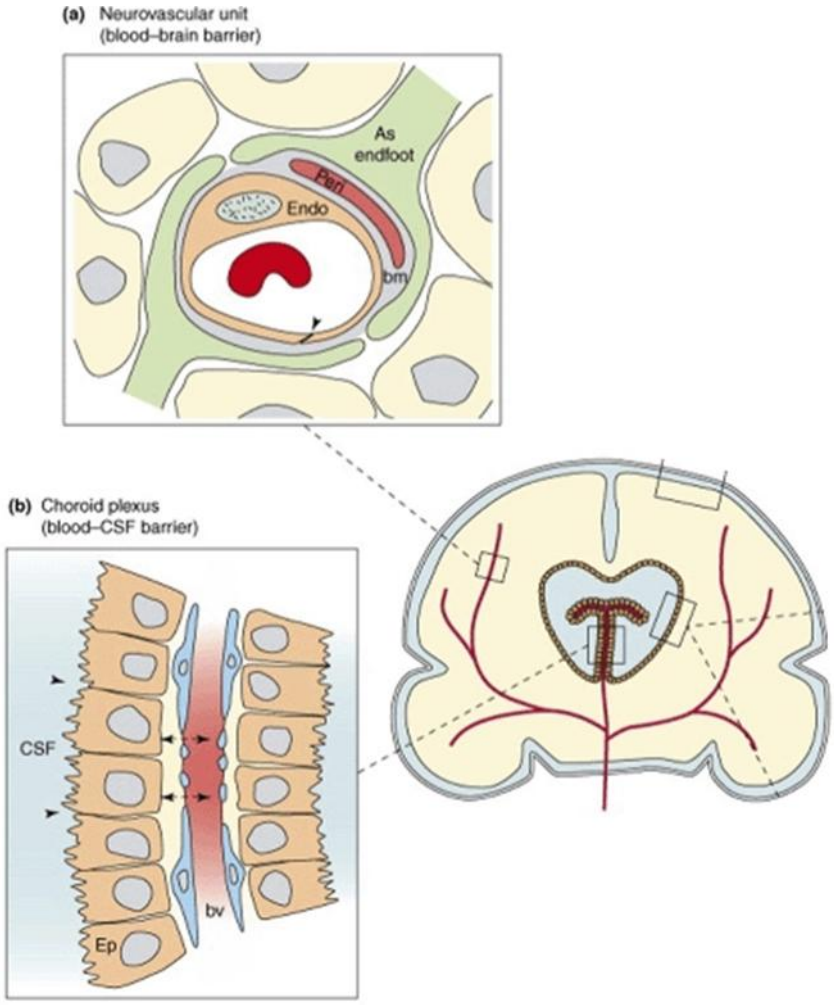
Given that the human brain uses approximately 20% of available oxygen for normal function, tight regulation of blood flow and perfusion is critical for survival. Autoregulation of cerebral blood flow is the ability of the brain to maintain a relatively constant blood flow despite changes in perfusion pressure. Cerebral blood flow is maintained at approximately 50 mL per 100 grams of brain tissue per minute (with a cerebral perfusion pressure between 60 – 160 mmHg). When the cerebral perfusion pressure drops below the 60 mmHg limit of autoregulation, an adaptive compensatory response to increase oxygen extraction from the blood occurs. If restriction to perfusion is prolonged and exceeds the ability for oxygen extraction to meet metabolic demands, then clinical signs or symptoms of cerebral ischaemia including dizziness, altered mental status and eventually irreversible tissue damage (infarction) will occur (Hossmann, 1994; Iadecola, 1998).

1.1.4 Barriers of the Central Nervous System

There are three main barriers in the brain: the blood-CSF barrier, the CSF-blood barrier, and the blood brain barrier (BBB). These barriers protect neurons from blood-borne substances, help maintain water homeostasis, and preserve an optimal ionic balance that is conducive for neuronal function (Choi & Kim, 2008; Saunders, Ek, Habgood, & Dziegielewska, 2008).

Cerebrospinal fluid is produced in the lateral third and fourth ventricles mainly by the choroid plexus and cerebral capillaries, where it functions as a cushion for the brain and spinal cord whilst providing nutrients to brain tissue (Skipor & Thiery, 2008). The capillaries of the choroid plexus are leaky and fenestrated, however, it is the tight junctions of the epithelial-like ependymal cells (the blood-CSF barrier) that surround the choroid plexuses, which restrict intercellular passage of molecules. The blood-CSF barrier plays an important role in establishing an osmotic gradient via ionic pumps, which contributes to CSF formation by the cells of the choroid plexus (Betz, Goldstein, & Katzman, 1994) (Figure 4).

Figure 4: Sites of blood-brain barrier interfaces. *(a) The blood–brain barrier is a barrier between the lumen of cerebral blood vessels and brain parenchyma. Arrowhead indicates luminal tight junctions that forms the physical barrier of the inter-endothelial cleft. Outside the endothelial cell is a basement membrane (bm) which also surrounds the pericytes (Peri). Around all these structures are the astrocytic endfeet processes from nearby astrocytes (as endfoot). All these structures together are often referred to as the neurovascular unit. (b) The blood–CSF barrier represents the barrier between choroid plexus blood vessels and the CSF. The choroid plexus blood vessels are fenestrated and form a non-restrictive barrier (small arrows); however, the epithelial cells (Ep) have apical tight junctions (arrowheads) that restrict intercellular passage of molecules. Extracted from (Saunders et al., 2008).*



The blood-brain barrier is formed by cerebral endothelium, and is the largest barrier in the brain. Cerebral endothelium comprises of polarised cells with apical tight junctions that limit passive diffusion of blood-borne solutes. Also located apically are transporters that actively regulate the flow of nutrients from the blood into the brain, whilst basolaterally located transporters function to remove toxic substances from the brain into the blood (Zlokovic, 2008). Hence the main function of the BBB is to tightly regulate the transport of nutrients and molecules into and out of the brain. Tight junctions and adaptor proteins of the BBB bind to the actin cytoskeleton, forming a continuous membrane that maintains a high trans-endothelial electrical resistance (Zlokovic, 2008). Oxygen and carbon dioxide freely diffuse through the BBB as well as small lipid-soluble molecules of < 400 Daltons (Da) (Pardridge, 2007). However, the high electrical resistance across tight junctions makes the BBB impermeable to hydrophilic molecules like glucose, amino acids and other essential nutrients. These molecules can only cross through carrier- and receptor-mediated transporters on the apical or basolateral endothelial cell membranes (Ueno, 2007). Many disease states including ischaemic stroke, hypertension and seizures result in dysregulation of tight junction proteins and transporter mechanisms (Hawkins & Davis, 2005).

1.1.5 Summary

The cerebrovascular circulatory system contains many unique structural properties that helps meet the high metabolic demands of neural tissue by maintaining consistent and uninterrupted perfusion of the brain. Additionally, the unique properties of the cerebral endothelium and tight junctions that form the blood-brain barrier restricts passage of molecules and maintains a low membrane conductivity which helps maintain an optimal ionic balance for neural function and water homeostasis in a space-limiting environment. Pathologies like ischaemic stroke damage these essential structural properties of cerebral circulation, resulting in brain injury.

1.2 Neuroimaging and evaluation of stroke

Neuroimaging refers to a group of essential non-invasive modalities used to visualise neural structures and assess the function of the brain. Neuroimaging modalities play a key role haemorrhagic and ischaemic stroke diagnoses, as well as in the selection of patients for acute stroke thrombolytic therapy. Given that patients with an ischaemic penumbra have a greater likelihood of responding to thrombolysis, detection of the penumbra by neuroimaging modalities is essential for guiding treatment options (Donnan & Davis, 2002). This review will explore two of the most common techniques: computerised tomography (CT) and magnetic resonance imaging (MRI), with a discussion of their respective advantages and disadvantages in stroke neuroimaging.

1.2.1 Concept of the ischaemic penumbra

The brain requires oxygen and glucose for normal neuronal metabolism and function. Cerebral blood flow (CBF) is maintained at approximately 50 mL per 100 grams of brain tissue per minute as previously described. It is universally accepted that if CBF drops to between 10 to 18mL/100grams/min then neuronal electrical activity stops, creating an ischaemic penumbra (Bandera et al., 2006). The ischaemic penumbra represents compromised hypoperfused neural tissue located peripherally to a developing infarction core. Tissue within the penumbra zone is still viable tissue and potentially salvageable if blood flow is quickly restored either by spontaneous clot fragmentation or therapeutic use of thrombolysis (Latchaw et al., 2003). If early recanalisation of the vascular occlusion and reperfusion does not occur or if collateral flow is not established to restore CBF to the ischaemic penumbra, then the infarction core will grow and progressively replace the penumbra (Mayer et al., 2000; Read et al., 2000). From a molecular perspective, neurons in the penumbra become depleted of ATP and switch to anaerobic glycolysis for energy production. This metabolic process generates lactate, which lowers intracellular pH and disrupts membrane sodium-potassium adenosine triphosphatase (Na+/K+ ATPase) pumps causing an uncontrolled influx of water into the intracellular space (cytotoxic oedema) resulting in membrane lysis and irreversible neuronal damage or death. Prolonged ischaemic conditions create a dysfunctional

blood-brain barrier where serum proteins and water diffuse into the interstitial space leading to coagulation necrosis and infarction (Nentwich & Veloz, 2012). If CBF drops below 10 mL/100 grams per minute, neuronal function and metabolism will only be maintained for several minutes, after which, if the occlusion is not removed and reperfusion not restored, deprived neural tissue will transform into an ischaemic infarction (Latchaw et al., 2003; Read et al., 2000).

1.2.2 Thrombolysis with Recombinant Tissue Plasminogen Activator

Intravenous thrombolysis with recombinant tissue plasminogen activator (rt-PA) is the only approved pharmacological therapy for treatment of patients with acute ischaemic strokes (H. Adams et al., 2005; Schellinger & Warach, 2004). According to the guidelines for early management of patients with acute ischaemic stroke, the time window of treatment with rt-PA is within 3 hours of ischaemic symptom onset, with a more selective eligibility criterion for patients to receive rt-PA treatment up to 4.5 hours post-symptom onset (Jauch et al., 2013; Nael & Kubal, 2016). However, as few as 3 to 8.5% of eligible ischaemic stroke patients actually receive thrombolysis due to lack of early medical evaluation as well as concern of causing an intracranial haemorrhage following aggressive thrombolytic therapy (Bambauer, Johnston, Bambauer, & Zivin, 2006). Neuroimaging modalities like MRI and CT play an important role in detecting infarction, excluding intracranial haemorrhage, identifying the site of arterial occlusion, and defining the infarct core and penumbra boundaries to safely direct thrombolysis therapy within the 3 to 4.5 hour timeframe (Furlan et al., 2006; Hacke et al., 2005; Jauch et al., 2013). Neuroimaging has shown that between 90 to 100% of patients with supratentorial arterial occlusion will have an ischaemic penumbra after the first 3 hours of stroke, however up to 80% of those patients will continue displaying viable penumbral tissue after 6 hours post-stroke (Hacke et al., 2004). These findings have challenged the existing clinical guidelines recommending the rigid time-dependent rt-PA treatment window for patient selection in favour of tissue-based evidence to guide therapy options (Hacke et al., 2004; Leiva-Salinas & Wintermark, 2010). Determining the extent of irreversibly damaged and viable brain tissue by delineating the infarct core from salvageable penumbral tissue using MRI and CT imaging modalities thus allows

thrombolytic treatment efficacy and clinical outcomes to improve (Donnan & Davis, 2002; Hacke et al., 2004; Leiva-Salinas & Wintermark, 2010).

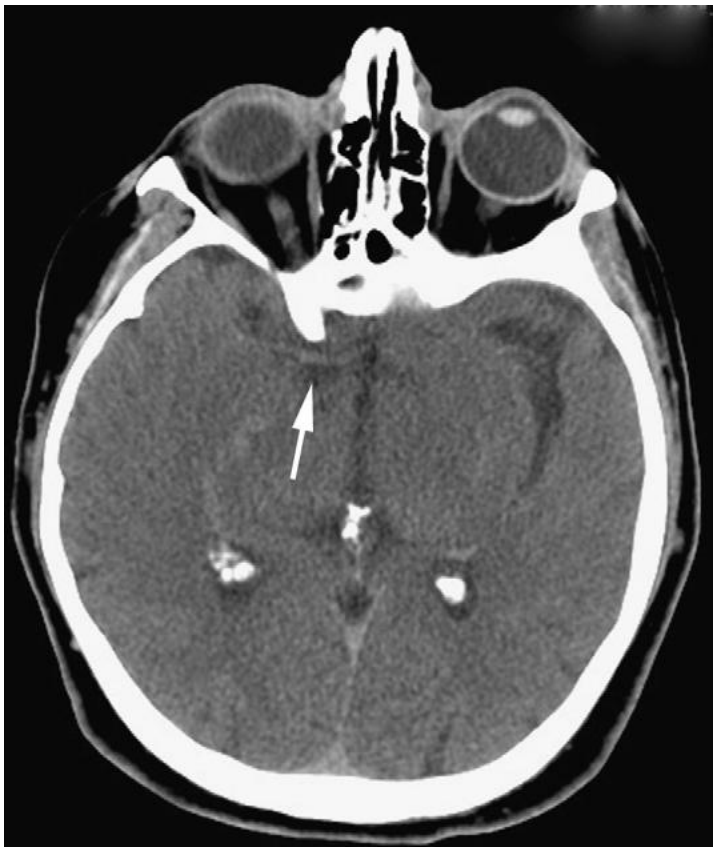
1.2.3 Computed Tomography

Brain computed tomography (CT) is frequently the primary choice in imaging acute stroke patients due to the inherent accuracy of excluding the presence of haemorrhage, as well as the relatively short imaging acquisition time and availability in most emergency department hospitals (Ginde, Foianini, Renner, Valley, & Camargo, 2008; Latchaw et al., 2009). Multimodal CT is a time efficient hyperacute stroke imaging sequence comprising of non-contrast head CT (NCCT), CT angiography (CTA), and CT perfusion (CTP) (Nentwich & Veloz, 2012; Scharf et al., 2006).

1.2.3.1 Non-Contrast Computed Tomography

Although not as sensitive as MRI to detect ischaemia, NCCT is regarded a first line imaging modality due to its widespread availability, short scan duration and lack of invasiveness to detect (or excludes) intracranial and subarachnoid haemorrhage as well as other stroke mimic conditions such as tumour or infections (Latchaw et al., 2009; Schellinger & Warach, 2004). Advances in NCCT resolution also allow for improved visualisation and identification of arterial occlusions of increased density (i.e. thrombus) and early imaging of ischaemic infarction, which can consequently guide treatment pathways (Figure 5) (H. P. Adams, Jr. et al., 2007). According to the American Heart Association 2010 guidelines for emergency cardiovascular care, it is recommended that NCCT is completed within 25 minutes of arrival to an emergency department and with result interpretation completed within an additional 20 minutes post-scan to determine the eligibility of potential acute ischaemic stroke patients to receive intravenous rt-PA (Jauch et al., 2010).

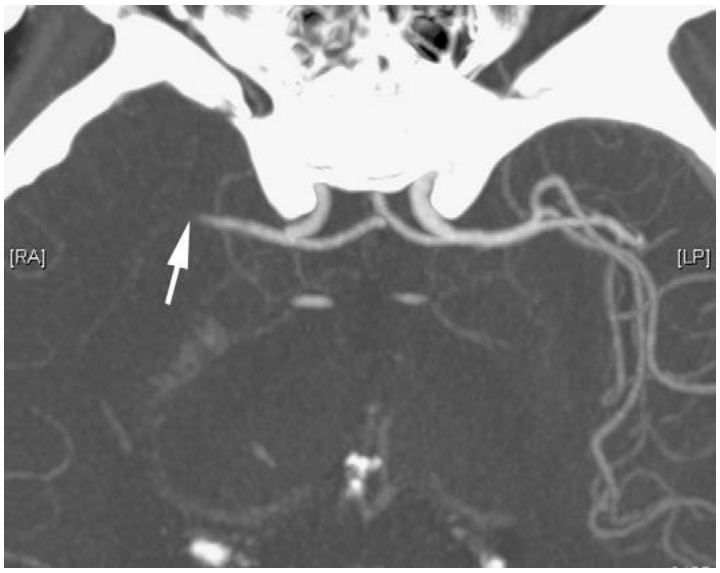
Figure 5: Representation of non-contrast brain CT in a patient with left hemiplegia. *Non-contrast brain CT displaying a right hyperdense signal (white arrow) indicating a thrombus occlusion of the right middle cerebral artery. Extracted from (Nentwich & Veloz, 2012).*



1.2.3.2 Computed Tomography Angiography

CT angiography evaluates the intra- and extra-cranial arterial circulation to identify the exact location of any intravascular occlusion and extent of narrowing/stenosis to potentially guide thrombolytic therapy (Figure 6) (Scharf et al., 2006). By using a non-ionic iodinated contrast material, CTA provides a qualitative 2- or 3-dimensional cerebral blood volume rendered image for assessment of tissue perfusion (Latchaw et al., 2009). CTA has been shown to have superior sensitivity to NCCT in detecting acute irreversible ischaemia and comparative to DWI-MRI with the exception of small ischaemic strokes (Latchaw et al., 2009).

Figure 6: Representation CT-angiography in a patient with acute left hemiplegia. *Displaying an intravascular thrombus (white arrow) in the right middle cerebral artery and the absence of downstream right MCA vessels. Extracted from (Nentwich & Veloz, 2012).*



1.2.3.3 Computed Tomography Perfusion

Computed tomography perfusion (CTP) provides information about brain perfusion through use of non-ionic iodinated contrast material that is intravenously injected (Eastwood, Lev, & Provenzale, 2003). Unlike CTA, by tracing the entry and washout of the contrast bolus, CTP can provide accurate quantitative measures of cerebral blood flow (CBF), cerebral blood volume (CBV) and mean transit time (MTT) required for blood to flow through brain tissue to assist in visualising and delineating the ischaemic penumbra from an irreversibly damaged ischaemic core (Scharf et al., 2006). However, CTP currently remains investigational due to the lack of a clinical criteria for use, as well as the lengthy repeat-scanning procedure required to identify perfusion deficits as the contrast material passes through brain tissue (Merino & Warach, 2010).

1.2.4 Multimodal Magnetic Resonance Imaging Stroke Protocol

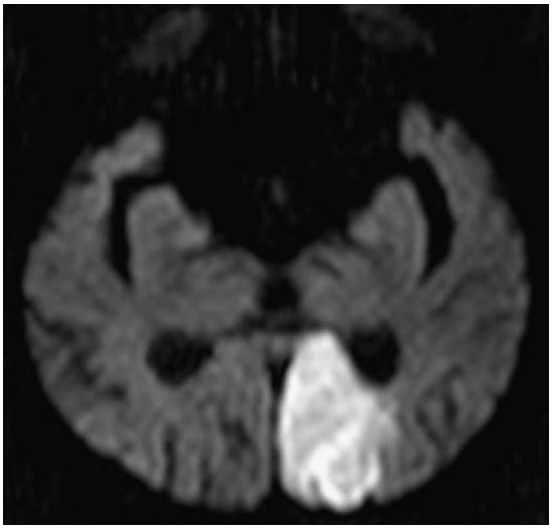
Magnetic resonance imaging has superior sensitivity in detecting acute ischaemic changes when compared with CT and hence is used as a first-line diagnostic modality for patients presenting with acute focal neurological deficits (Chalela et al., 2007). A typical multimodal MRI stroke protocol to evaluate and diagnose patients with suspected acute stroke consists of diffusion weighted imaging (DWI), T2 weighted fluid-attenuated inversion recovery (T2W/FLAIR), magnetic resonance angiography (MRA), perfusion-weighted imaging (PWI) and gradient-recalled echo (GRE) (Jauch et al., 2013). Multimodal MRI can be completed within 10 to 20 minutes making it an integral modality for diagnosis of acute ischaemic stroke within the 3 to 4.5-hour thrombolysis time window (Merino & Warach, 2010). Magnetic resonance imaging remains advantageous over CT due to the higher sensitivity and specificity for detection of acute stroke mimicking conditions including cerebral oedema, vascular malformations, infection, and inflammatory diseases, as well as the absence of ionising radiation exposure to the patient (Xavier, Qureshi, Kirmani, Yahia, & Bakshi, 2003). However, several major caveats of MRI include the higher cost and limited availability in many hospitals as well as several absolute contraindications including the presence of cardiac pacemakers or metallic implants that may be displaced by the magnetic field (Xavier et al., 2003). Furthermore, an estimated 5% of patients undergoing MRI are unable to complete the imaging process due to claustrophobia (Edelman & Warach, 1993).

1.2.4.1 Diffusion Weighted Imaging

Diffusion weighted imaging (DWI) is used to depict areas of acute brain ischaemia by using the random motion of water within neural tissue (Nentwich & Veloz, 2012). In the immediate several minutes following a cerebrovascular occlusion, ischaemic conditions cause failure to ionic cell membrane pumps including the Na+/K+ -ATPase channel pump. Disruption of the ionic gradient allows a net influx of water into the intracellular space causing cytotoxic oedema and a net reduction of water molecules in this extracellular space (Astrup, Siesjo, & Symon, 1981). Diffusion weighted imaging detects the reduced extracellular volume and associated decrease in water diffusion in

ischaemic brain tissue as a hyper-intensity (Figure 7) (Srinivasan et al., 2006). The accuracy of DWI to detect physiological changes within 15 minutes of cerebrovascular occlusion and ischaemic injury onset has made this imaging modality a primary choice for diagnosis of acute ischaemic stroke (Wu, Nentwich, & al., 2011). Nevertheless, restricted water diffusion can also be mimicked by other cerebral pathologies including infections, tumours and inflammatory conditions causing false-positive DWI hyperintensities (Latchaw et al., 2009).

Figure 7: Representation diffusion weighted magnetic resonance imaging of a patient with a left occipital ischaemic stroke*. Hyperacute increase in signal intensity (white) indicating a left occipital ischaemic stroke. Extracted from (Nentwich & Veloz, 2012).*

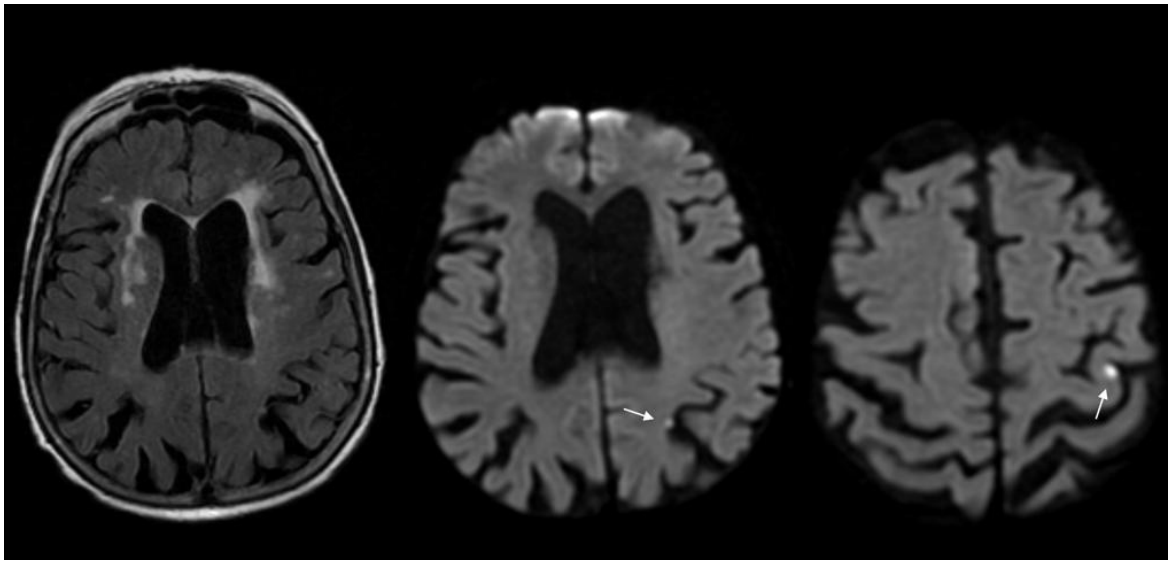


1.2.4.2 Fluid Attenuated Inversion Recovery Imaging

In contrast with DWI lesions that can be detected within minutes from ischaemic onset, T2-weighted (T2W) and fluid attenuated inversion recovery (FLAIR) imaging depicts ischaemic infarction as a hyperintense lesion within 3 to 8 hours following ischaemic stroke onset (Mohr et al., 1995; Nentwich & Veloz, 2012; Thomalla et al., 2009). FLAIR imaging involves the suppression of signal from cerebrospinal fluid to increase the sensitivity of detecting subacute ischaemic infarcts. It has been proposed that an MRI combination of a DWI-positive and FLAIR-negative mismatch have a greater than 90% probability of identifying acute ischaemic strokes that are within the 3 to 4.5 hours of symptom onset (Figure 8) (Thomalla et al., 2009). Therefore, to establish whether a

patient may be eligible for thrombolysis when timing of ischaemic symptom onset is unclear, by using the DWI-FLAIR mismatch, a patient with an acute ischaemic lesion detected on DWI but absent on FLAIR imaging is likely to be within the safe and efficacious 3-hour time window to receive rt-PA (Thomalla et al., 2009).

Figure 8: Neural imaging representation of a patient presenting with incoherent speech and left-sided facial droop of 5 minutes' duration. *Left image: A FLAIR image of a showing scattered periventricular and subcortical white matter hyperintensities representing subacute ischaemic lesions. Middle and right images: Diffusion weighted MRI showing two acute focal infarcts (white arrows) that were not identified by FLAIR imaging. Extracted from (Sorensen & Ay, 2011).*



1.2.4.3 Non-Contrast Time-of-Flight Magnetic Resonance Angiography

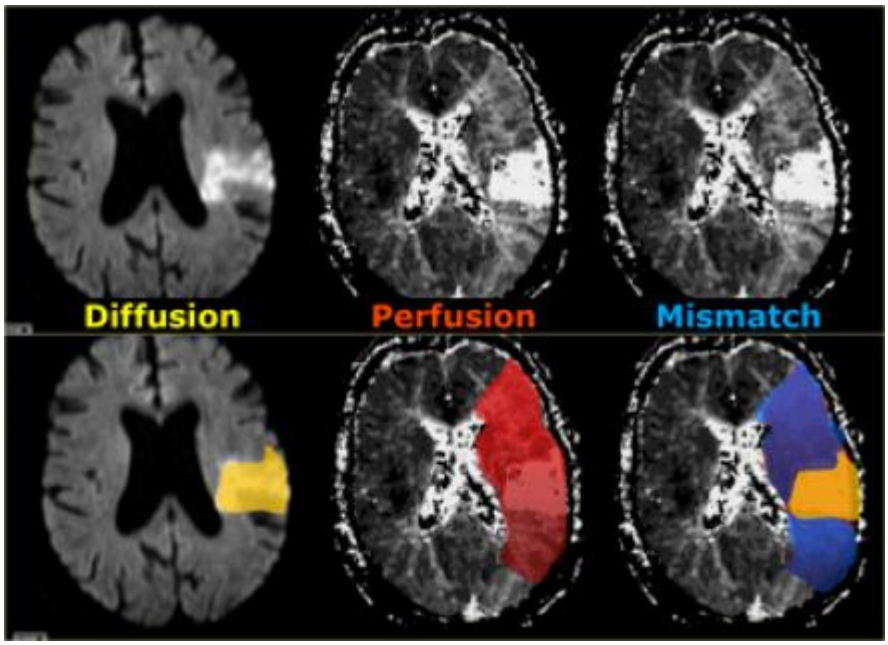
Digital subtraction angiography (DSA) generates high spatial resolution imaging of intracranial stenosis and vessel occlusion, and is considered the gold standard imaging modality (Nentwich & Veloz, 2012). However, DSA is the least desirable imaging modality for community or hospital-based settings as it is the most expensive, invasive and time consuming screening tool, which carries the greatest risks of all other imaging modalities (Nguyen-Huynh et al., 2008). The most commonly used intracranial imaging modality for evaluating the cranial vascular system is non-contrast time-of-flight magnetic resonance angiography (TOF-MRA). Specifically, TOF-MRA can generate

both 2D or 3D visualisations by applying repeat radiofrequency excitation pulses to a volume of brain tissue. Protons in the 'stationary' brain tissue become saturated and emit a low signal intensity while fresh inflowing unsaturated blood produces a high signal intensity between each excitation pulse (Miyazaki & Lee, 2008). Flow-dependent luminal imaging allows visualisation of intracranial vessels to detect vascular occlusion as well as the presence of stenosis in patients with ischaemic stroke (Gonzalez & Schaefer, 2006; Miyazaki & Lee, 2008). Several benefits of TOF-MRA include non-invasiveness, repeatability, and a lack of radiation exposure when compared with CTA (Nentwich & Veloz, 2012; Yoo, Pulli, & Gonzalez, 2011). However, a study comparing the accuracy of MRA and CTA to evaluate intracranial stenosis and occlusion in patients with acute ischaemic stroke and transient ischaemic attack indicated a lower sensitivity compared with CTA (70% vs 98%) and lower positive predictive power value when compared with CTA (65% vs. 93%) (Bash et al., 2005).

1.2.4.4 Perfusion Weighted Imaging

Perfusion weighted imaging (PWI) is an MRI modality that visualises capillary blood flow using an intravenous gadolinium-based bolus of contrast agent that depicts areas of brain tissue with reduced cerebral blood flow. While DWI positive-lesions have been associated with irreversibly damaged ischaemic tissue, PWI has shown clinical utility in identifying brain tissue that is dysfunctional because of reduced blood flow but potentially salvageable if flow is restored (Merino & Warach, 2010). The combination of DWI-PWI, specifically its mismatch (i.e. the volume difference between PWI and DWI) has been shown to identify patients with an ischaemic penumbra that will benefit from thrombolytic reperfusion (Figure 9) (Albers et al., 2006; Davis et al., 2008; Leiva-Salinas & Wintermark, 2010). Conversely, patients without a perfusion-diffusion mismatch (i.e. no difference between PWI and DWI volumes) indicates patients who do not have penumbral tissue either due to normalisation of a prior hypoperfusion or alternatively indicates complete infarction with total loss of penumbral tissue (Albers et al., 2006; Davis et al., 2008). Quantifying volume differences between DWI-PWI is essential to direct the appropriateness of thrombolytic treatment, whereby matching DWI-PWI indicates no clinical benefit to receiving thrombolytic therapy (Albers et al., 2006; Davis et al., 2008; Wu et al., 2011).

Figure 9: Representation of diffusion-perfusion mismatch and penumbra. *Left image: Diffusion imaging indicating irreversible tissue infarction (yellow). Middle image: Perfusion imaging indicating a region of hypoperfusion (red). Right image: Diffusion-perfusion mismatch (blue) indicating potentially salvageable brain tissue (penumbra) at risk of ischaemic infarction unless thrombolysis therapy is received. Extracted from (Srinivasan et al., 2006).*



1.2.4.5 Gradient-Recalled Echo

Intravenous thrombolysis treatment requires the exclusion of intracranial haemorrhage (ICH), which can be ruled out by non-contrast computed tomography (NCCT) or gradient-recalled echo magnetic resonance imaging (GRE-MRI) (Schellinger & Warach, 2004). Gradient-recalled echo is highly sensitive to haemoglobin-degradation products of deoxygenated blood that enters the parenchyma following ICH. It has been shown that when GRE is used in sequence with the multimodal MRI stroke protocol, it is as effective as NCCT in detecting acute ICH and superior to CT in detecting chronic haemorrhage (Kidwell et al., 2004). Experienced readers of GRE-MRI were shown to identify hyperacute ICH with 100% sensitivity and overall accuracy (Fiebach et al., 2004). This has made GRE-MRI an endorsed imaging modality by the American Heart

Association guidelines (class I, evidence level A) to distinguish ischaemic and haemorrhagic stroke for guiding reperfusion therapy (Jauch et al., 2013).

1.3 Significance of TIA diagnosis

Between 15-30% of strokes are preceded by a transient ischaemic attack (TIA) (Giles & Rothwell, 2007; Rothwell & Warlow, 2005). Transient ischaemic attack is recognised as a medical emergency, with the risk of stroke ranging between 10-20% in the following 90 days after symptom onset, with up to half of these patients having a stroke within the first 48 hours (Coull, Lovett, Rothwell, & Oxford Vascular, 2004; Giles & Rothwell, 2007; Johnston, Gress, Browner, & Sidney, 2000; Lovett et al., 2003). According to the prospective Existing Preventative Strategies for Stroke (EXPRESS) study, urgent specialist-provided assessment and treatment of TIA in either hospital-based or community-based GP clinical settings could prevent a significant proportion of strokes. Specifically, the use of rapid-access TIA clinics was associated with an 80% risk reduction of early recurrent stroke when compared with appointment-based specialist assessment and the delayed initiation of stroke prevention therapies (Rothwell et al., 2007).

Based on the previously reported rate of TIAs preceding stroke (Giles & Rothwell, 2007; Rothwell & Warlow, 2005), an estimated 7,500 – 15,000 Australians per year could potentially benefit from early TIA diagnostic assessment, management and treatment to reduce the risk of an imminent stroke. Early diagnosis of TIA and establishment of the prognostic risk of stroke in an accurate, timely and cost-effective way could significantly assist with the delivery of urgent stroke prevention therapies. The associated flow-on benefits of stroke prevention warrants research into strategies that could improve early recognition of TIA, and ultimately reduce mortality rates, long-term disability and the financial and economic burden associated with stroke.

1.3.1 Stroke statistics and economic burden

Stroke is the second single greatest cause of death and the leading cause of adult disability worldwide (Feigin et al., 2014). Annually, an estimated 16.9 million people will experience a stroke for the first time; with 5.9 million stroke-related deaths and a further 33.0 million stroke survivors (Feigin et al., 2014; Mukherjee & Patil, 2011). In Australia, there will be an estimated 50,000 new and recurrent strokes in 2016, with a further 440,000 stroke survivors, of which approximately one-third (131,000) will be living with long-term neurological impairments and physical disability (National Stroke Foundation, 2014; National Stroke Foundation, 2015). Since 1979, the rate of death from stroke in Australia has fallen by approximately 70% (Page, Lane, Taylor, & Dobson, 2012). This decline has been linked to improvements in medical treatments and in primary and secondary stroke prevention strategies; a trend that has been seen in high-income countries over the past four decades (Feigin et al., 2014; Rothwell et al., 2004). However, like most developed countries, the frequency of stoke events in Australia is anticipated to rise in line with the increased proportion of the ageing population over 65 years, which is expected to double over the next 40 years (United Nations, Department of Economic and Social Affairs, Population Division, 2015; Australian Institute of Health and Welfare, 2013).

From a health economics perspective, the direct annual cost of stroke is estimated to be $5 billion, of which the direct cost to the health sector is $881 million (National Stroke Foundation, 2013). Given that 30% of stroke survivors represent the working-age population under 65 years of age, the loss of healthy life or disability adjusted life years (DALYs) was estimated to be 285,158 years lost as a direct result of stroke-related disability and death. This stroke-associated burden of disease equates to a cost of approximately $49.3 billion per year (National Stroke Foundation, 2015). Although advancements in stroke treatment therapies has attracted much research focus and popular interest; improving the prevention of stroke through the management of risk factors has been recognised as a national health priority to help reduce the burden of stroke within the Australian population (National Health Priority Action Council, 2006).

1.3.2 Transient ischaemic attack definition

Transient ischaemic attack was previously defined by the World Health Organisation in 1978 as an episode of sudden focal neurological deficit lasting less than 24 hours and of vascular origin (Albers et al., 2002). However, with the advent of in neurological imaging technologies such as diffusion-weighted magnetic resonance imaging (DWI-MRI) and high resolution computed tomography (CT), between 21 - 48% of patients traditionally defined as having had a TIA (i.e. displaying symptoms lasting less than 24 hours) were shown to have suffered subclinical strokes with evidence of ischaemic infarction (Calvet et al., 2009; de Lau, den Hertog, van den Herik, & Koudstaal, 2009; Kidwell et al., 1999). The high occurance of brain infarctions that were misdiagnosed as TIA had prompted the American Stroke Association to adopt a new definition for TIA as being ‘a transient episode of neurological dysfunction caused by focal brain, spinal cord or retinal ischaemia, without acute infarction’, and recommending that all TIA patients undergo neuroimaging, preferably MRI scanning as an exclusion tool (Easton et al., 2009). Instead of basing diagnosis on an arbitrary time-frame, this revised definition of TIA improves the diagnostic accuracy by linking transient ischaemic symptoms with the associated tissue-based pathophysiology.

1.3.3 Pathophysiology and clinical features of TIA

A normal functioning adult human brain requires 50ml of blood to perfuse 100 grams of brain tissue every minute (Nagasawa et al., 1996). When blood flow is reduced to 50% or lower, neurological symptoms will develop (Mohr et al., 1997). The three most common aetiologies of a TIA comprise of atherosclerosis, thromboembolism, and cardio-embolic events (Calvet et al., 2009; Easton et al., 2009; Johnston et al., 2000). Classical risk factors of atherosclerosis such as abnormal lipid profile, diabetes, smoking or hypertension can lead to injury of the endothelial lining of blood vessels. These metabolic and cardiovascular risk factors have the potential to perpetuate a thrombogenic cascade through the contact of blood with pro-thrombotic sub-endothelial connective tissue (Galkina & Ley, 2009). Platelet activation and aggregation occurs in response to endothelial injury, and concurrently the coagulation cascade is upregulated to reinforce the formation of a platelet plug (Galkina & Ley, 2009). Monocyte

adherence enhances lipid accumulation, and over time results in the development of plaques that protrude into the arterial lumen, narrowing a blood vessel and restricting blood flow (Galkina & Ley, 2009; Libby, 2002). Destabilisation of carotid atherosclerotic plaques are hypothesised to occur by T-cell expression of the pro-inflammatory cytokines such as IFN-γ, IL-2, IL-3 and TNF within the plaque (Robertson & Hansson, 2006; Wolf, Zirlik, & Ley, 2015). Pro-inflammatory mediators have been associated with proliferation and infiltration of both plaque macrophages and circulating monocytes, as well as expression of matrix metalloproteinases to ulcerate and destroy a plaques extracellular matrix (Newby, 2015; Wolf et al., 2015). This is the hypothesised rationale for thrombus formation and the release of embolisms downstream that can occlude cerebral vessels thereby causing ischaemia (Libby, 2002; Newby, 2015). It is established that cardio-emboli account for up to 15-30% of ischaemic strokes (Ustrell & Pellise, 2010). Since cardiac thrombi spawn the majority of arterial emboli, cardiovascular disease remains an important determinant in the pathogenesis of TIA (Nadarajan, Perry, Johnson, & Werring, 2014).

Symptoms of a TIA will mimic those of an ischaemic stroke (for comprehensive review see (Nadarajan et al., 2014)). In overview, the cerebrovascular territory that is transiently occluded or compromised will generally correlate with the nature of the ischaemic symptoms. Patients that display evidence of anterior or posterior circulation compromise will commonly present with hemiparetic symptoms. More focal transient ischaemia such as carotid territory ischaemia will generally present with aphasia or transient monocular visual disturbance, while evidence of vertebral basilar ischaemia is most often associated with bilateral limb weakness, vertigo, hearing loss, and hemianopia or diplopia.

1.3.4 Stroke risk stratification

Use of the stroke risk stratification scoring system, the ABCD2 score can assist clinicians in assessing the early risk of stroke. Specifically, the ABCD2 score is calculated on a 7-point scale in patients presenting with a TIA, and comprises of five parameters: age > 60 years (1-point), blood pressure > 140/90 mmHg (1 point), clinical

features including unilateral weakness (2 points) or speech disturbance (1 point), duration of 10-59 minutes (1 point) or > 59 minutes (2 points), and diabetes (1 point). Use of this risk stratification tool has been included in the latest stroke guidelines as it is able to identify higher risk patients, where a score of ≥ 4 is considered high risk based on expert opinion level of evidence (National Stroke Foundation, 2010). The ABCD2 score identifies high-risk TIA patients with good sensitivity (i.e 92% of true TIAs identified), however there is poor associated specificity of the ABCD2 score (i.e. 33% specificity of non-TIAs correctly identified) (Ay et al., 2009; Quinn, Cameron, Dawson, Lees, & Walters, 2009). The ABCD2 risk stratification score does not adequately discriminate between transient ischaemic and non-ischaemic/mimic pathologies, and therefore is not a suitable stand-alone diagnostic tool (Kamal et al., 2015; Perry et al., 2011).

1.3.5 Neuroimaging for diagnosing TIA

Application of neuro-imaging technologies such as computerised tomography (CT) and DWI-MRI scanning of brain and vascular (both intra and extracranial) structures are integral to the process of differentially diagnosing cerebrovascular events through their respective detection sensitivities to intracranial haemorrhage (ICH) and acute ischaemic lesions (Burke, Kerber, Iwashyna, & Morgenstern, 2012; European Stroke Organization (ESO) Executive Committee). The recent TIA definition change has eliminated the ‘time-based’ 24-hour limit for a more accurate ‘tissue-based’ definition (Easton et al., 2009). Diagnosis of TIA therefore relies on the urgent application of neuro-imaging technologies to exclude the presence of cerebral ischaemic infarction, haemorrhage or other neurological pathologies (Chalela et al., 2007; Easton et al., 2009; Kamal et al., 2015; Perry et al., 2011). Imaging of brain and cerebral vasculature with MRI for evidence of DWI lesions and/or the presence of vessel occlusion has shown to be predictive of the risk for future stroke as well as increasing the diagnostic certainty of TIA by ruling out evidence of brain infarcts (Coutts et al., 2005; Souillard-Scemama et al., 2015). The combination of brain imaging with the ABCD2 clinical assessment produces higher sensitivity and specificity for detecting TIA and predicting the early risk of stroke (80% sensitivity, 73% specificity) (Ay et al., 2009). However, these neuroimaging modalities are unlikely to be gold standard tests for urgent TIA diagnosis

due to their inefficient image processing times, contraindications (i.e. electromagnetic interference to pacemakers and other metal-containing mechanical implants), high initial outlay and running costs that further limit accessablity, and limit availability in smaller hospitals as well as in regional and remote locations (Burke et al., 2012; National Stroke Foundation, 2010; Jensen, Chacon, Sattin, Aleu, & Lyden, 2008).

1.3.6 Challenges of TIA diagnosis

There are several challenges associated with the diagnosis and management of TIA. Firstly, to make a TIA diagnosis, clinical guidelines for TIA management have endorsed the need for clinical experts to collect a detailed history of the experienced symptoms from the presenting patient (National Stroke Foundation, 2010; Lavallee et al., 2007). However, TIAs frequently have atypical symptom presentations depending on which cerebral artery territory is compromised, with symptoms often resolving well before a patient seeks and receives a medical assessment (Albers et al., 2002; Easton et al., 2009; Fonseca & Canhao, 2011; Nadarajan et al., 2014). There is a risk that patients could distort, fail to recall, or fail to be aware of important details about the transient ischaemic symptoms they had experienced. Furthermore, patients may present with non-ischaemic conditions that can mimic the symptoms of a TIA including migraines, seizures, psychiatric disturbances, peripheral vertigo, pre-syncope, and other metabolic disorders. Several studies following up diagnoses of consecutive patients referred to TIA clinics indicate that anywhere between 22-60% of patients with suspected TIA will not have a final TIA diagnosis (Nadarajan et al., 2014; Prabhakaran, Silver, Warrior, McClenathan, & Lee, 2008). The most common conditions to mimic TIA included migraine, syncope, seizure, and benign paroxysmal positional vertigo (Amort et al., 2011; Nadarajan et al., 2014; Schrock, Glasenapp, Victor, Losey, & Cydulka, 2012). Mimic conditions need to be differentially diagnosed from TIAs and excluded from ongoing acute medical management as they are at low risk of stroke due to their non-ischaemic pathogenesis (Kamal et al., 2015).

The lack of a TIA-specific test has made it difficult for stroke clinicians to differentially diagnose TIA from an array of mimic conditions of non-ischaemic pathogenesis

(Johnston, 2002; Koudstaal, Gerritsma, & van Gijn, 1989). Depending on the clinical setting that a patient presents to (i.e. community GP, TIA clinic, or emergency department), between 10% – 48.5% of patients with suspected TIA reportedly have mimic conditions (Amort et al., 2011; Fallon et al., 2006; Sheehan et al., 2009). One study identified statistically significant differences (i.e. poor consistency) amongst fellowship-trained stroke neurologists in correctly diagnosing TIA, as in most cases, patients will present with normal neurological examination results, prompting a diagnosis to be based solely on a patient's account of the symptomatic events and past medical history (Castle et al., 2010). This highlights the subjectivity of clinical judgement that is ultimately required to make a TIA diagnosis. In contrast, cardiologists have tests such as ECG and the biomarker troponin to assist diagnosing myocardial infarction in patients presenting with acute chest pain. Stroke clinicians lack a specific set of clinical tests that can accurately diagnose TIA and prognosticate the future risk of stroke.

Given the limited utility of both the ABCD2 risk stratification score and neuro-imaging technologies to differentially diagnose TIA from mimic conditions, as well as the difficulty between stroke specialists to formulate a consensus on a TIA diagnosis, there exists a need for a troponin-equivalent test that can reliably, rapidly, and cost-effectively assist TIA diagnosis and stroke risk stratification (Amort et al., 2011; B. Cucchiara & Nyquist, 2011; Jensen et al., 2008; Nadarajan et al., 2014).

1.4 Candidate TIA Biomarkers

Recent studies report a reduced risk of stroke recurrence within the first year following transient ischaemic attack or minor ischaemic stroke diagnosis due to direct implementation of secondary prevention strategies including risk-factor control and antithrombotic treatment (Amarenco et al., 2016; Johnston et al., 2000; Lavallee et al., 2007; Lovett et al., 2003; Rothwell et al., 2007). Although interpretation of several conventional clinical risk factors and higher ABCD2 scores and are associated with recurrent stroke, a recent systematic review showed that the ABCD2 risk-stratification score does not reliably discriminate patients at low and high risk of early recurrent

stroke (Wardlaw et al., 2015). This section will review recent studies that have identified potential biomarkers that may help confirm the ischaemic aetiology and diagnose transient ischaemic attack (summarised in Table 1). I will also explore the current evidence of biomarkers that may help predict the risk of future ischaemic stroke and the emerging field of research of biomarker research to guide an appropriate choice of preventative pharmaceutical treatment.

1.4.1 Genetic biomarkers in TIA diagnosis

Genetic risk factors have a significant role in the aetiology of vascular pathologies. Plasminogen activator inhibitor 1 (PAI-1) is a critical regulator of the fibrinolytic system and the main endogenous inhibitor of the fibrinolytic tissue plasminogen activator (tPA) enzyme. A common 4G/5G insertion/deletion polymorphism at the nucleotide position -675 in the promoter region of the PAI-1 gene has been shown to increase the expression levels of PAI-1 and has consequently been associated with the pathogenesis and progression of thrombotic vascular events including myocardial infarction and stroke (Bentley, Peck, Smeeth, Whittaker, & Sharma, 2010; Onalan et al., 2008; Rallidis et al., 2010; Tjarnlund-Wolf, Brogren, Lo, & Wang, 2012). A study exploring the PAI-1 genotype of patients under the age of 60 years found that the homozygous 5G genotype was significantly associated with patients that had experienced a TIA or minor stroke (odds ratio = 2.10, 95% CI 1.01 – 4.30), whilst indicating a protective effect for carriers of the homozygous 4G genotype in this age group (odds ratio = 0.4, 95% CI = 0.2 – 0.9) (Endler et al., 2000). Genotyping platforms such as for the assessment of PAI-1 -675 4G/5G polymorphism could be of clinical utility for population-based screening and risk stratification to raise awareness, identify patients at increased risk of developing a TIA or minor stroke and provide opportunities for early intervention and implementation of an appropriate risk management strategy.

Elevated homocysteine is a known risk factor for atherosclerosis through increased oxidant stress, impaired endothelial function and promotion of thrombosis. Prospective studies have associated elevated plasma homocysteine levels with a two-fold increased risk of cardiovascular disease and to a lesser extent cerebrovascular disease (Guthikonda & Haynes, 2006). Exploration of a common C677T single nucleotide

polymorphism (SNP) in the gene for 5,10-methylenetetrahydrofolate reductase (5,10-MTHFR) has been associated with elevated homocysteine levels and has been proposed to be a candidate genetic risk factor for thromboembolic and arterial occlusive diseases (Lalouschek et al., 1999). A case-control study identified a trend associating carriers of the C677T MTHFR mutation with pro-thrombotic factor V Leiden mutation carriers in TIA and minor stroke patients when compared with control participants (odds ratio 2.75; 95% CI 0.83 – 9.17) (Lalouschek et al., 1999). Although further gene-association studies and sufficiently powered multi-centre validation studies are required to substantiate these findings, anecdotal evidence suggests the combined occurrence of mutations in C677T MTHFR and the hereditary-linked factor V Leiden may affect the prognostic outcomes of carriers, increasing their risk of developing TIA or minor stroke.

1.4.2 Protein biomarkers in TIA diagnosis

Genomic investigations play an important role in elucidating the genetic signatures of conditions like TIA by identifying disease-associated genes and predicting an individual's future risk of developing ischaemic disease. However, the altered expression level of a candidate genetic biomarker (as determined by a promoter region SNP) does not always correlate with the actual protein abundance in serum/plasma due to various regulatory feedback mechanisms and post-translational modifications (Ansong, Purvine, Adkins, Lipton, & Smith, 2008; Humphery-Smith, Cordwell, & Blackstock, 1997; Schramm et al., 2003). The emerging science of proteomics provides a highly sensitive and un-biased platform to accurately identify and quantify TIA-associated protein biomarkers in blood.

Earlier studies examining neurological injury have identified elevations in two glial-specific proteins: S100 calcium-binding protein B (S-100B) and glial fibrillary acidic protein (GFAP) (Herrmann, Vos, Wunderlich, de Bruijn, & Lamers, 2000). Both proteins are expressed by mature astrocytes that ensheath neurovascular vessels and have been associated with post-ischaemic release pattern. Specifically, venous blood concentrations of S100B and GFAP significantly correlate with the size of a brain lesion, suggesting a high sensitivity to infarction following an acute ischaemic stroke

(Herrmann et al., 2000). Nevertheless, both glial-specific proteins achieved peak serum concentration 4-days after stroke onset, indicating a 'tissue-leakage' delay which may not be suitable for an acute diagnostic setting.

Following acute middle cerebral artery infarction, two neuron specific markers; neuron-specific enolase (NSE) and myelin basic protein (MBP), have been shown to increase steadily in serum over 4 days post ischaemic stroke (Lamers et al., 2003). Delayed entry of NSE, S-100B and GFAP into systemic circulation may be explained by arterial occlusion, whereby suboptimal reperfusion of ischaemic tissue results in static 'biomarker-rich' blood downstream of the occlusion (Herrmann et al., 2000; Lamers et al., 2003). However, this limitation is not likely to be applicable in a TIA-biomarker model, as ischaemia is only transient (i.e. reperfusion ends a TIA), hence allowing for markers to enter systemic circulation a lot quicker. This may suggest a more immediate time-span to detect markers of brain injury post-TIA.

A recent prospective mass spectrometry-based proteomic study involving a small sample size of transient ischaemic attack, minor ischaemic stroke and controls sought to identify novel disease-associated biomarkers (George et al., 2015). Platelet basic protein (PBP), ceruloplasmin and complement component C8 gamma were identified as significantly upregulated in both TIA and minor ischaemic stroke cohorts, while PBP was the only successfully validated protein when compared with controls (i.e. patients with stroke-mimicking presentations such as migraine and seizure). Platelet basic protein is a granulocyte-derived protein involved in the inflammatory cascade and is released by platelets in response to injury (Kowalska, Rauova, & Poncz, 2010). Given the integral role of the inflammatory response to ischaemic conditions and atherosclerosis, this significantly high serum PBP measurement may highlight the inflammatory 'foot-print' that links TIA and minor ischaemic stroke (Brocheriou et al., 2011; George et al., 2015). Although this association may warrant further validation as a candidate TIA biomarker that could distinguish TIA from non-ischaemic 'stroke-mimic' conditions, there is a need for further exploration of PBP's role in the pathophysiology of TIA given the non-selective associations of PBP with other vascular pathologies including mimic conditions (Brocheriou et al., 2011).

1.4.3 Protein Biomarkers in TIA Prognosis

Prognostic biomarkers of TIA are likely to derive from the source of the arterial occlusion. Atherosclerotic plaque, but particularly active plaques at high risk of rupture and thromboembolic formation have attracted much interest as a source of biomarkers that could predict the risk of a future cerebrovascular attack. The added utility of exploring atherosclerotic plaque biomarkers is the ability to identify high risk patient that will likely benefit from endovascular intervention or surgery.

Lipoprotein-associate phospholipase A2 (Lp-PLA2) is an enzyme primarily associated with the atherogenic low-density lipoprotein and plays a key role in vascular inflammation and atherosclerosis, and has been described as a potentially useful plasma biomarker associated with cardiovascular disease (Carlquist, Muhlestein, & Anderson, 2007; Tellis & Tselepis, 2009). Two recent studies have identified an elevation in the plasma levels of Lp-PLA2 and soluble CD40 ligand (inflammatory mediator) in the acute period following TIA and minor stroke (J. Li, Wang, Lin, et al., 2015; Lin et al., 2015). Both studies were part of a randomised control trial and involved a sample size in excess of 3000 minor stroke and TIA patients. Elevated plasma levels of Lp-PLA2 and soluble CD40 ligand coupling was further associated with an increased risk of recurrent cerebrovascular events within a 90-day follow-up, suggesting a potential prognostic utility to identify TIA patients at high-risk of recurrence or imminent stroke while distinguishing from TIA patients at low-risk (J. Li, Wang, Lin, et al., 2015; Lin et al., 2015).

D-dimer is a by-product and marker of fibrinolysis, where elevated levels depict a hyperactive state of thrombus formation and breakdown (Fon et al., 1994; Zi & Shuai, 2014). A quantitative investigation of D-Dimer in TIA patients identified significantly elevated levels in both the acute (≤ 7 days) and post-acute (3 month) phases following a TIA (Fisher & Francis, 1990). However, patients taking warfarin or other anticoagulants negate any elevation in D-dimer concentration, and therefore limit the robustness of D-dimer sensitivity for use as a prognostic marker of brain ischaemia.

Elevated serum levels of high sensitive C-reactive protein (hsCRP), an acute phase inflammatory protein, has been associated with causation of active atherosclerotic plaque instability, rupture and atherothrombotic formation, as well as the prognosis of

ischaemic events (Arenillas et al., 2001; B. L. Cucchiara et al., 2009; Di Napoli et al., 2005; Muir, Weir, Alwan, Squire, & Lees, 1999; Ridker et al., 2005; Sotgiu et al., 2006; Winbeck, Poppert, Etgen, Conrad, & Sander, 2002; Yeh & Willerson, 2003). Specifically, TIA patients presenting within 24 hours of symptom onset with a hsCRP level greater that 4.1 mg/L have been shown to have an increased risk of further vascular events and recurrent stroke (Purroy et al., 2007). The effectiveness of aggressive secondary prevention therapies including aspirin and stain prescription as well as non-drug interventions that include weight loss, exercise and smoking cessation have all been shown to decrease CRP levels (Grad et al., 2009; Kennon et al., 2001; Okita et al., 2004; Ridker et al., 2001). This highlights the importance of rapid clinical measurement of hsCRP to guide secondary prevention strategies that reduce the risk of recurrent ischaemic events (Purroy et al., 2007). A recent study of TIA and non-cardioembolic ischaemic stroke patients found that an elevated hsCRP was an independent predictor of the one-year risk of developing a middle cerebral artery (MCA) stenosis ≥ 70% as well as experiencing a recurrent cerebrovascular ischaemic event (Gong et al., 2013). Studies by Purroy et al. and Gong et al. suggest a potential neuroprotective benefit in lowering the risk of stroke through use of anti-inflammatory interventions (Gong et al., 2013; Purroy et al., 2007). However, several major caveats of these studies limit the sensitivity and specificity of hsCRP to be used as a clinically viable prognostic TIA biomarker. Firstly, these studies lacked diagnostic homogeneity whereby TIA and ischaemic stroke patients were not separated for analysis. Secondly, relatively small sample sizes and an inability to eliminate the possibility of alternate triggers of acute-phase responses such as asthma, arthritis, and subclinical infections further increase the risk of a false-positive increase in hsCRP. There is a need for candidate TIA biomarkers to have the sensitivity and specificity to distinguish TIA from other inflammatory diseases to be of clinical utility.

Other novel sources of prognostic TIA biomarkers include antiphospholipid antibodies. These autoantibodies are directed at plasma proteins bound to phospholipid membranes and have been specifically associated with thrombosis, antiphospholipid antibody syndrome and atherosclerotic vascular disease (Bizzaro et al., 2007; Cohen, Berger, Steup-Beekman, Bloemenkamp, & Bajema, 2010; Forastiero et al., 2005). A recent study found that serum anti-phosphatidylserine/prothrombin IgG antibodies are independently associated with stroke or death in TIA patients that present within 48

hours of symptom onset, therefore suggesting potential utility as a biomarker of risk following TIA (Mullen et al., 2012). This suggest a potential use of antiphospholipid antibody biomarkers for risk stratifying patients following TIA, however, larger prospective studies are required to validate these findings.

1.4.4 Candidate Biomarkers of Response to Therapy

The value of biomarkers to diagnose and predict TIA is of major importance to initiate secondary prevention strategies. However, even if we are able to correctly diagnose TIA or identify patients at high risk of an imminent stroke, this doesn't change the clinical challenge of how to reduce the risk of stroke recurrence. Use of antiplatelet therapy is the main secondary prevention strategy for preventing recurrent TIA and ischaemic stroke, however, some patients will still experience ischaemic events despite continued antiplatelet therapy (J. Li & Wang, 2016). Genetic polymorphisms in the functional components of platelet aggregation including glycoprotein IIIa, COX-1, COX-2 and P2Y12 have shown to be predictive of antiplatelet treatment failure in patients with recurrent ischaemic vascular events including stroke (Topcuoglu, Arsava, & Ay, 2011). Other protein biomarkers including hsCRP, sCD40L and fibrinogen have been linked with antiplatelet ineffectiveness for treating cardiovascular disease, however, this association has yet to be explored in patients with TIA (Feher et al., 2006; Ge et al., 2012; Park et al., 2011).

A recent study identified an association between glycated albumin and the effects of single or dual antiplatelet treatments (i.e. aspirin and clopidogrel) in patients with TIA or minor stroke (J. Li, Wang, Wang, et al., 2015). Patients specifically responded well to dual antiplatelet therapies when glycated albumin was in low abundance, whilst patients with high glycated albumin abundance were at significantly high risk of haemorrhaging. Glycated albumin could hence be a predictive biomarker for selecting the optimal antiplatelet therapy strategy that is safe and efficacious following TIA (Jickling, 2015; J. Li, Wang, Wang, et al., 2015). This research forms part of an emerging field of pharmacoproteomics, which aims to bridge diagnostics and therapeutics by providing a

greater functional representation of patient-patient variation that will expand our understanding of drug discovery and personalised drug therapy (Jain, 2016).

Table 1: Potential diagnostic and prognostic biomarkers for TIA.

Biomarker type	Biomarker	Full name	Biological function	Reference
Genetic	PAI-1	Plasminogen activator inhibitor 1	-675 4G/5G polymorphism increases PAI-1 expression and inhibition of the fibrinolytic tissue plasminogen activator enzyme.	(Endler et al., 2000)
	5,10-MTHFR	5,10-methylenetetrahydrofolate reductase	C677T polymorphism increases homocysteine expression.	(Lalouschek et al., 1999)
Plasma protein (diagnostic)	S-100B	S100 calcium-binding protein B	Glial-derived protein, marker of blood-brain-barrier dysfunction.	(Herrmann, Vos, Wunderlich, de Bruijn, & Lamers, 2000)
	GFAP	Glial fibrillary acidic protein	Brain-specific filament protein maintaining astroglial cell structure.	(Herrmann, Vos, Wunderlich, de Bruijn, & Lamers, 2000)
	NSE	Neuron specific enolase	Cytosolic neuronal protein.	(Lamers et al., 2003)
	MBP	Myelin basic protein	Myelination of nerves.	(Lamers et al., 2003)
	PBP	Platelet basic protein	Granulocye-derived protein involved with neutrophil recruitment in the inflammatory cascade.	(George et al., 2015)
	CP	Ceruloplasmin	Multicopper enzyme involved with LDL oxidation and tissue damage.	(George et al., 2015)
	C8-gamma	Complement component C8 gamma	Cytolytic membrane attack complex	(George et al., 2015)
Plasma protein (prognostic)	Lp-PLA2	Lipoprotein-associate phospholipase A2	Vascular inflammation and atherosclerosis	(Lin et al., 2015)
	CD40	Soluble CD40 ligand	Inflammatory mediator post TIA.	(J. Li, Wang, Lin, et al., 2015)
	D-dimer	D-dimer	Fibrin degradation product following fibrinolysis	(Zi & Shuai, 2014)
	hsCRP	High sensitive C-reactive protein	Acute phase inflammation.	(Purroy et al., 2007)

1.5 Thesis objectives

This biomarker discovery research project is divided into three parts: 1) designing a human plasma proteomic method to detect novel biomarkers associated with TIA, 2) identify and quantify candidate TIA biomarkers using gel-based proteomics and mass spectrometry platforms, and 3) excluding an aspirin effect on candidate plasma TIA biomarkers using proteomics.

The specific aims were:

- To outline a study design and proteomic method that can detect differentially expressed plasma proteins between acute and follow-up time-points, as well as between TIA and mimic diagnoses (Chapter 2).
- To find differences in the plasma proteomes of TIA and mimic patients when compared with healthy volunteers by:
    - Identifying differentially abundant ‘candidate’ protein biomarkers associated with TIA and mimic diagnoses using 2D DIGE and mass spectrometry (Chapter 3).
    - Validate the proteomic detection method by measuring ‘candidate’ proteins using quantitative mass spectrometry (Chapter 3).
- Detect and identify differentially expressed plasma proteins associated with aspirin dosage in a healthy volunteer (Chapter 4).
- Concluding thoughts on significance and future directions of this PhD research study (Chapter 5).

2 STUDY DESIGN AND PROTEOMIC METHODS

2.1 Clinical Study Design

2.1.1 Patient Recruitment – *Community Based Rapid Access TIA Clinic*

A general practice clinic in the Western Adelaide area (Midwest Health, 678 Port Road, Beverley, South Australia 5009) was selected and established as the location for a community-based rapid access TIA clinic (COMBAT) by fellow PhD candidate Dr. Elaine S. Leung. The central location of this COMBAT clinic served to improve patient recruitment and assist engagement with general practitioners (GPs) from the Adelaide Western General Practice Network (AWGPN) to participate and refer any patient with a suspected TIA. Patients attending the COMBAT clinic were either referred by their GP or were self-referred. Patients included in the study were given a diagnosis of TIA as per the 2009 American Stroke Association TIA definition (Easton et al., 2009) that was determined by a GP with a special interest in stroke (GP-SIS) and a neurologist. Patients underwent stroke risk assessment using the ABCD2 stratification score, whereby scores ≥ 4 were referred to the Stroke Unit of The Queen Elizabeth Hospital (Adelaide,

Australia). Patients scoring < 4 were given an appointment at the TIA clinic within 48 hours, with bulk billing to ensure equal service provision for attending patients.

A pathway of recruitment, collection and storage is provided in Figure 10. Briefly, clinical examination involved assessments of heart rate and blood pressure, neurological and cardiovascular examination, and blood glucose testing. External investigations were arranged when clinically indicated and included:

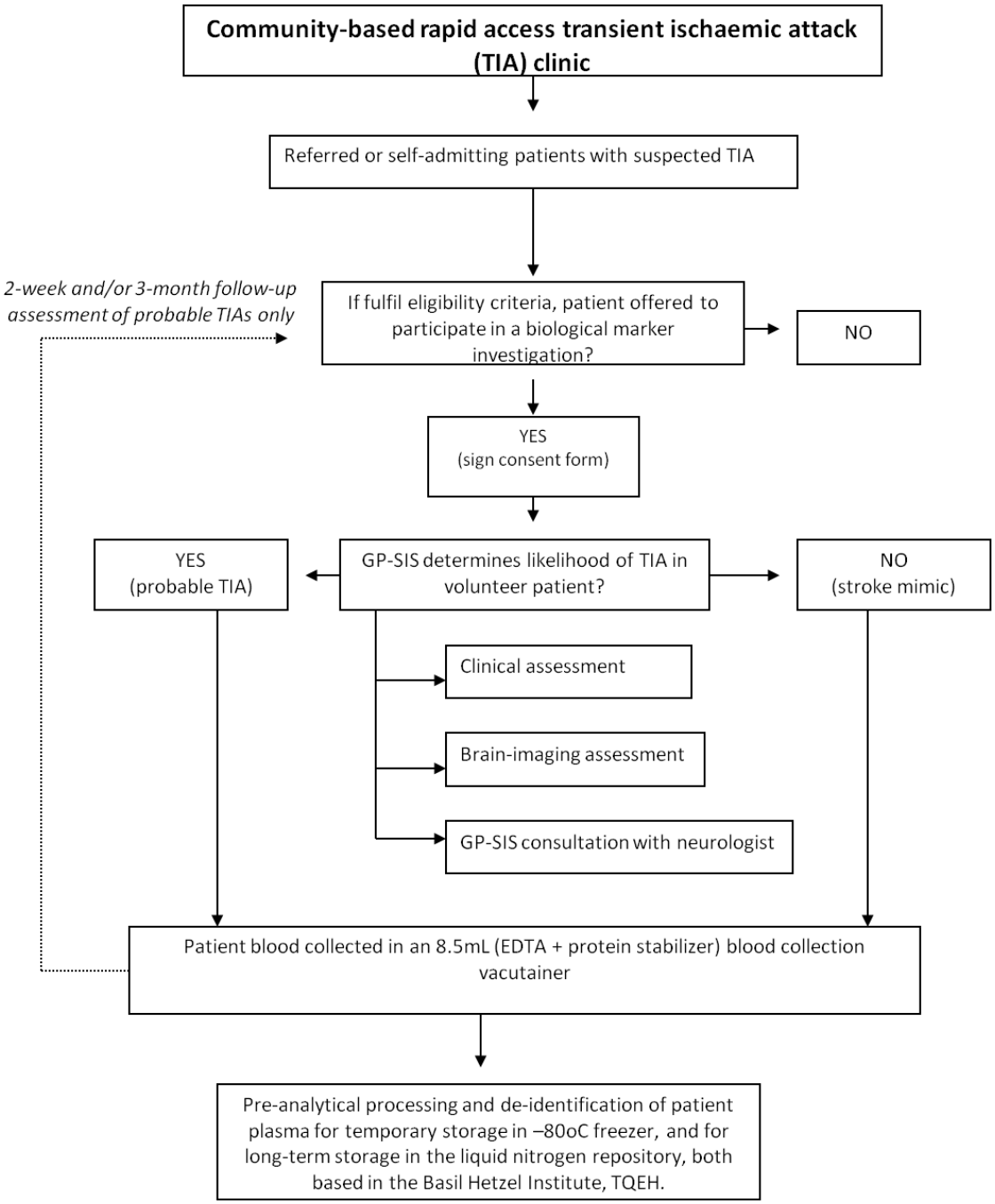
- Electrocardiogram (ECG).
- Blood testing:
    - Full blood count.
    - Urea/electrolytes/renal function.
    - Erythrocyte sedimentation rate.
    - C-reactive protein.
    - Blood glucose.
    - Lipids.
    - Cholesterol.
    - High-density lipoprotein.
    - Low-density lipoprotein.
    - Triglycerides.
- Carotid ultrasound scan for patients with carotid territory symptoms within 48-72 hours.
- Computer tomography (CT) brain scan within 24 hours.
- Magnetic resonance imaging (MRI).

Patients attending the COMBAT clinic were managed in accordance with evidence-based recommendations, including anti-platelet therapy, blood pressure lowering treatment, anti-coagulation therapy, cholesterol lowering treatment, diabetes management and/or lifestyle/behaviour change (National Stroke Foundation, 2010). Patients were then followed up 90-days later at the COMBAT clinic, with re-assessment

of recurrent TIAs or subsequent stroke, hospital admissions for other reasons, compliance with medications, and treatment targets including blood pressure, INR (for patients on warfarin), cholesterol level, HbA1C for diabetic control. At follow-up, patients received a second MRI scan to determine recurrence of stroke.

For the purposes of this proteomic TIA biomarker study, patients that presented within 10 days of symptomatic ischaemia onset without any previous diagnoses of TIA or stroke were recruited. Additionally, patients who were pregnant, terminally ill, experiencing dementia and/or other significant cognitive impairment were excluded from participating in this proteomic investigation. Two 8.5 mL K_2EDTA BD Vacutainers® of whole blood were sampled from the median cubital vein from each consenting TIA or mimic patient and collected at both acute presentation (≤10-days) and 90-day follow-up. Written informed consent was obtained from all participants and The Queen Elizabeth Hospital Ethics Committee approved this study (HREC Approval Number 2009123).

Figure 10: TIA patient recruitment, blood collection and storage pathway.

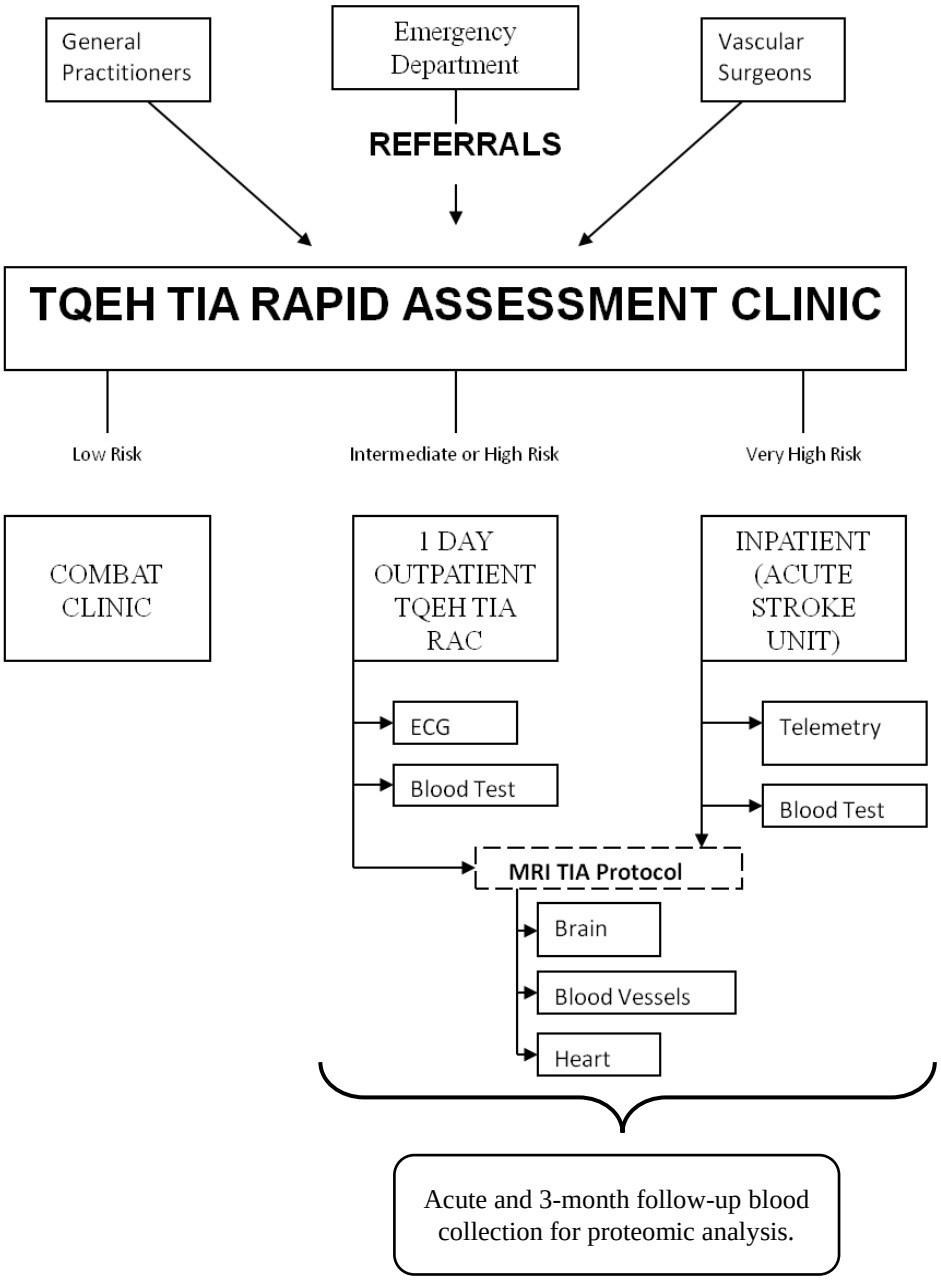


2.1.2 Patient Recruitment – *Hospital-Based Rapid Assessment Clinic*

All patients that presented to The Queen Elizabeth Hospital TIA Rapid Assessment Clinic (TQEH-RAC), with suspected TIA were invited to participate in this blood plasma proteomic TIA biomarker pilot study. Patients included for biomarker investigations were those that had had no past history of TIA or stroke that present to the TQEH TIA Rapid Assessment Clinic, and are given a first diagnosis of TIA as per

the 2009 American Stroke Association TIA definition (Easton et al., 2009). The diagnosis was determined after assessment by the TIA-clinic neurologist. Intermediate to high risk TIA patients that were referred to TQEH TIA Rapid Assessment Clinic were sourced from the 1-Day outpatient TQEH TIA Rapid Assessment Clinic, whilst very high risk TIAs were sourced from inpatients to the Acute Stroke Unit (Figure 11). Blood collection for the purposes of the proteomic TIA biomarker investigation was performed as per the COMBAT clinic blood collection guidelines as previously described. Written informed consent was obtained from all participants and The Queen Elizabeth Hospital Ethics Committee approved this study (HREC Approval Number 2009203).

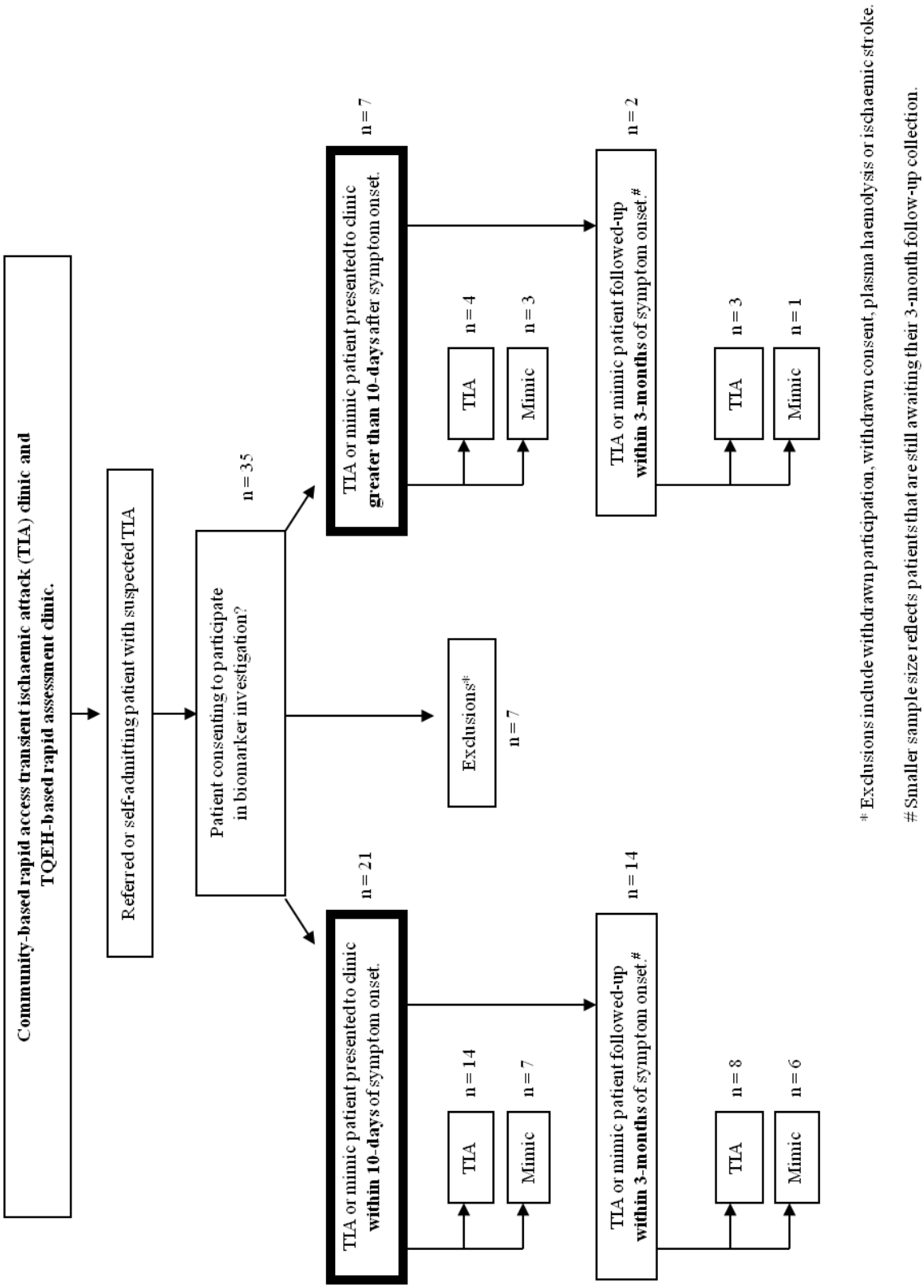
Figure 11: TQEH-RAC patient triage, recruitment, and blood collection pathway.



2.1.3 Patient Selection for Proteomic Investigations

A total of 35 consenting patients with suspected TIA were recruited from COMBAT and TQEH rapid assessment clinics between February 2010 and January 2011 (Figure 12). Fourteen patients were excluded from further investigations due either to presenting to clinic more than 10-days after ischaemic symptom onset, presence of plasma haemolysis, ischaemic stroke diagnosis, withdrawn participation or withdrawn consent. A total of 21 participant comprising 14 TIA's and 7 mimic conditions were eligible for participation. A total of 8 TIA and 6 mimic patient participants returned for a 3-month follow-up assessment and blood collection, with the best six TIA participants selected for proteomic analysis based on diagnostic certainty as confirmed by a GP with a special interest in stroke and two neurlogists. Blood samples were also obtained from six healthy control volunteers over the age of 40 years that were recruited from the wider Adelaide Metropolitan Community, without having any diagnosed co-morbidities and not taking prescription medications at the time of assessment (HREC Approval Number 2009123).

Figure 12: Total recruited patient blood plasma samples from COMBAT and TQEH-RAC.



2.1.4 Challenges of Proteomic Biomarker Discovery Using Human Plasma

Rapidly evolving proteomic technology offers researchers comprehensive analysis of proteomes to elucidate biomarkers of health and disease. However, there are bottle-necks associated with this type of biomarker research, which is limiting the success rate of candidate biomarkers making it into the clinical setting. Variation in biological sample collection, processing, storage, and analytical methods has plagued the biomarker discovery process and contributed to limited reproducibility and validation of results between different laboratories. This indicates the importance of pre-analytical considerations when handling biological samples, and the need to develop a standard operating procedure to minimise external sources of variation and bias. I aim to outline the necessary pre-analytical considerations and potential sources of variation with proteomic technologies in discovering protein biomarkers associated with TIA or any other disease.

2.1.4.1 Pre-analytical Considerations

Pre-analytical considerations refer to several phases in a biomarker study design workflow including the biological sample type, collection, handling, and storage till further analysis. A multi-institutional consortium has identified a lack of standard operating procedures in biomarker discovery workflow and the significance that even small differences in the pre-analytical processing method of samples between laboratories can have on the analytical outcomes of biomarker investigation (Tuck et al., 2009). I has been shown that the type of biological fluid sample collected (plasma, serum, cerebrospinal fluid or urine), type of additives used in specimen collection, sample processing times, temperature haemolysis, storage and the amount of freeze-thaw cycles are major sources of variation that can impact the analytical outcome of a biomarker investigation (Tammen, 2008). Further guidelines proposed by the International Society for Biological and Environmental Repositories provides a standard operating procedure that identifies and records the main pre-analytical factors that may

compromise the integrity of biological samples during the collection, processing and storage phases (Lehmann et al., 2012).

Due to the additional processing variable involving coagulation prior to extraction of serum, it was recommended by researches from the Human Plasma Proteome Project (HPPP) that plasma (via use of vacutainers containing anticoagulant) be the sample of choice for biomarker discovery investigations (Nanjappa et al., 2014; Rai et al., 2005; Tammen et al., 2005). Plasma represents a complete biological sample in comparison with serum, and is more readily accessible and requires a simple collection procedure when compared with other body fluids such as cerebrospinal fluid or urine (Tammen et al., 2005).

This study has adapted the following pre-analytical considerations to form the basis of a standardised and repeatable method of specimen collection for a TIA biomarker investigation:

- Comprehensive Patient/Volunteer Details (see Appendix Table 1), including:
    - Name/date of birth/sex/time and date of assessment.
    - Diagnosis.
    - List of co-morbidities.
    - Past medical history including previous symptoms suggestive of TIA, hypertension, diabetes, atrial fibrillation and hyperlipidaemia.
    - List of medications.
    - Time since ischaemic symptom onset.
    - Time since acute/initial assessment (for 3-month follow-up).
    - Ethnicity.
    - Menopausal status/menstrual cycle.
    - Fasting period.
    - Level of exercise participation (weekly).
    - Smoking status.
    - Level of caffeine and alcohol consumption (weekly).
- Blood sampling technique:

  - Sampling via median cubital vein.
  - Consistent time of day.
  - Use of tourniquet.
  - Seated posture.
- Blood collection:
  - Use of 8.5 mL K_2EDTA BD Vacutainer® with 1.8 mg EDTA anticoagulant per mL of whole blood for plasma extraction.
  - Post-collection vacutainer mixing/inversion rate.
  - Maintaining vacutainers between 15°C – 24°C prior to centrifugation.
- Handling and processing blood samples:
  - Centrifugation of blood within 30 minutes from collection (lag-time recorded).
  - Consistent centrifugation setting for 15 minutes at 2,000 x g (room temperature).
  - Gently pipette plasma out of vacutainer without disrupting buffy-coat.
  - Dispense plasma into low-protein-binding cryogenic vials in 1 mL aliquots.
  - Addition of 10 µL of Halt™ Protease and Phosphatase Inhibitor Cocktail into 1 mL plasma aliquots.
- Intermediate storage of extracted plasma:
  - Storage in portable liquid nitrogen dry vapour shipper (-150°C) during transport.
- Long-term storage:
  - Storage in a central liquid nitrogen repository.
- Bioinformation:
  - De-identification code labelling of plasma vials.
  - Code linkage database (password protected) for traceability and data retrieval.

- Patient/participant details including clinical test results stored electronically on a password-protected file, accessible on a department computer (backed up on University of Adelaide server).
- Storage of patient details checklists within a locked filing cabinet at research facility.

2.1.4.2 Analytical Considerations

The experimental methodology introduces several sources of variation to that can impact on the analytical outcomes of a biomarker investigation. Specifically, the use of immunodepletion, fractionation and sample enrichment/concentration alters sample composition by increasing the risk of medium-to-low abundant 'disease-specific' proteins forming complexes with depleted proteins. Human albumin is the most abundant blood plasma protein and contains high-affinity ligand-binding properties for endogenous and exogenous molecules (Fasano et al., 2005). Although immunodepletion of complex analytical samples is an essential step in the biomarker discovery method to improve detection of low abundant proteins, the removal of ligand-binding proteins like albumin increases the risk of lower abundant disease-specific proteins covalently bonding to and becoming co-depleted from the analytical sample (Granger, Siddiqui, Copeland, & Remick, 2005). Co-depletion is particularly pertinent consideration when exploring plasma for proteins associated with neurological conditions like transient ischemic attack and stroke. It is anticipated that disease-specific biomarkers derived from the central nervous system (CNS) are of exceptionally low abundance compared with the enormous complexity and wide dynamic range of blood plasma proteins that are derived from every vascularised organ system within the human body and spanning greater than 10 orders of magnitude (Farrah et al., 2014). The ability to accurately detect and quantify brain-derived proteins associated with transient cerebral ischaemia is a major challenge to the current proteomic technologies available (Parker, Pearson, Anderson, & Borchers, 2010). However, new targeted proteomic technologies such as multiple reaction monitoring (MRM) mass spectrometry methods have been shown to accurately measure undepleted plasma biomarkers with high quantitative sensitivity, robustness and reproducibility (Percy, Chambers, Yang, & Borchers, 2013). Such

platforms offer a solution to minimising the current analytical variation inherent with the current technology that requires sample preparation steps.

2.2 Proteomics Study Design and Methods

2.2.1 The proteomic approach

Advances in proteomic technologies now allow the mining of low abundance plasma proteins in search of TIA-associated biomarkers. The two main technologies used for the plasma proteomic analysis are two-dimensional gel electrophoresis (2DE) and mass spectrometry (MS). Two dimensional electrophoresis involves the separation of a complex mixture of proteins based on their isoelectric point and molecular weight. The use of CyDye$^{(TM)}$ Difference In-Gel Electrophoresis (DIGE) increases the accuracy of results by multiplexing samples and using an internal standard to control for gel-to-gel variability associated with conventional 2DE. Detection and quantification of differentially expressed protein spot features is performed by using DeCyder $^{(TM)}$ software algorithms, whereby spot intensity is normalised relative to the pooled internal standard and controls for variability in protein sample loading as well as the extinction coefficients of the CyDyes.

Mass spectrometry allows proteins to be identified from 2D gel spots or directly from complex sample mixtures. Proteins are digested into peptides using trypsin before undergoing ionisation with high voltage. Peptides of positive charges are drawn into the negatively charged mass spectrometer. This study utilises an Orbitrap mass spectrometer operated in the FTMS/ITMS mode, whereby a high resolution and high mass accuracy scan can be performed on the peptides entering the mass spectrometer to determine the charge state of ions so that singly charged ions (mostly background ions) are excluded and that an accurate mass measurement can be made on each peptide. For each Orbitrap mass/charge scan, the instrument identifies the top six multiply charged ions (i.e. peptides) and then fills the ion trap with peptides. All ions except for a 1 Da window around the target precursor ion (peptide) are removed from the trap by modulation of

the radio frequency and direct current voltages. The precursor ion is then accelerated into the collision gas helium present in the trap which increases the internal vibrational energy of the peptide. The increased energy causes the peptide backbone to fragment at the peptide bond. The collision energy used is sufficient to cause a single break in the majority of peptides so that a series of ions called b and y ions is produced. B ions retain the charge on the N-terminus of the fragment and y ions on the C-terminus. The b and y ions are scanned out of the ion trap producing a mass chromatogram from which the mass of each ion can be established (see Appendix Figures 3-8). The mass difference between each mass from the b and y ion series corresponds to the mass of each amino acid in the sequence. Software is used to compare the sequence with sequence databases to determine the protein from which the peptide originated.

2.2.2 Materials

Plasma Sample Collection, Processing, and Storage:

- 8.5 mL K_2EDTA BD Vacutainer® (Becton, Dickinson and Company, NJ, USA), with 1.8 mg EDTA per mL of whole blood.
- Halt™ Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific, Waltham, MA, USA).
- 2.0 mL Nunc® CryoFlex® vials (Signa-Aldrich, St Louis, MO, USA) with internal thread for cryogenic storage of plasma aliquots.

Multiple Affinity Removal System Liquid Chromatography:

- P-920 UPC-900 AKTA-Fast Protein Liquid Chromatography (FPLC) system with 0.50 mm orange polyetheretherketone (PEEK) sample-loop tubing (GE Healthcare, Piscataway, NJ, USA).
- Multiple Affinity Removal System® (MARS) Human -6 for serum/plasma proteins, 4.6 x 100 mm column (Agilent Technologies, Santa Clara, CA, USA).
- MARS Buffer A for loading, washing and equilibration of column (Agilent Technologies).

- MARS Buffer B for eluting the column of bound 'high-abundant' proteins (Agilent Technologies).
- 0.22 µm cellulose acetate spin filter (Agilent Technologies).
- 5 kDa molecular weight cut-off spin concentrators (Agilent Technologies).

Sample Preparation and Quantification:

- ReadyPrep™ 2D Cleanup Kit (BioRad, Hercules, CA, USA).
- EZQ® Protein Quantification Kit (Invitrogen, Carlsbad, CA, USA).
- Typhoon 9400 Variable Mode Imager (GE Healthcare).
- Methanol (Sigma-Aldrich).
- Acetic Acid (Sigma-Aldrich).

2D-Difference In-Gel Electrophoresis (DIGE):

- 5 nmol CyDye DIGE Fluor Minimal Labelling Kit (GE Healthcare).
- IPG Buffer (Ampholytes), pH 3-11 non-linear, (GE Healthcare).
- Immobiline DryStrip pH 3-11 NL, 24 cm gels (GE Healthcare).
- Mineral oil (Sigma-Aldrich).
- Strip holder cleaning solution (GE Healthcare).
- Whatman No. 1 filter paper (GE Healthcare).
- Ettan DALTsix gel caster with low fluorescence plates (GE Healthcare).
- Urea (Amresco, Solon, OH, USA).
- Thiourea (Scharlab, Barcelona, Spain).
- CHAPS (Amresco).
- DTT (Astral Scientific, NSW, Australia).
- Iodoacetamide (BioRad).
- Precision Plus Protein Unstained Standards (BioRad).

- Bromophenol Blue (Sigma-Aldrich).

Mass spectrometry:

- Trypsin Gold, Mass Spectrometry grade (Promega, Madison, WI, USA).
- Acetonitrile, LiChrosolv® hypergrade for LC-MS grade, 99.9% purity (Sigma-Aldrich).
- Formic acid, puriss. p.a. for mass spectrometry grade, 98% purity (Sigma-Aldrich).
- 2-Propanol, Chromasolv® Plus for HPLC grade, 99.9% purity (Sigma-Aldrich).
- Thermo LTQ Orbitrap XL mass spectrometer (Thermo Fischer Scientific).
- Nanospray ion source (Thermo Electron Corporation, San Jose, CA, USA).
- 300 µm ID x 5 mm C18 PepMap™ 100 precolumn (Dionex Corporation, Sunnyvale, CA, USA).
- 75 µm x 150 mm C18 PepMap™ 100 column (Nikkyo Technos, Tokyo, Japan).
- TripleTOF® 5600+ LC-MS/MS System (AB Sciex, Concord, Ontario, Canada).
- Eksigent nanoLC 400 system (AB Sciex).
- ProteoCol® Polar (3 µm particle size, 0.3 x 10 mm, SGE Analytical Science, Ringwood, Victoria, Australia).

2.2.3 Solutions

EZQ Protein Quantitation Assay:

- Rinse Buffer Solution: 10% (v/v) Methanol, 7% (v/v) Acetic Acid

Multiple Affinity Removal System Liquid Chromatography:

- 20% (v/v) Ethanol (Signa-Aldrich) in deionised H_2O.
- 0.02% (w/v) Sodium Azide (Sigma-Aldrich) in MARS Buffer A.

2D-Electrophoresis:

- DIGE labelling buffer: 7 M urea, 2M thiourea, 4% (w/v) CHAPS, 30 mM Tris, pH 8.5).
- IEF rehydration buffer: 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 0.5% (v/v) IPG buffer, 0.4% (w/v) DTT.
- SDS-equilibration buffer: 100 mM tris-HCl, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, pH 8.0.
- Equilibration buffer 1: 1% (w/v) DTT in SDS-equilibration buffer.
- Equilibration buffer 2: 4% (w/v) iodoacetamide in SDS-equilibration buffer, 0.003% (v/v) bromophenol blue solution.
- Agarose: 1% (w/v) low melting point agarose mixed in 1x running buffer.
- Running buffer (1x): 25 mM Tris, 192 mM glycine, 0.06% (w/v) SDS, pH 8.3.

Gel Staining and Imaging:

- Fixative solution for gel: 20% (v/v) methanol, 7.5% (v/v) acetic acid.
- Silver staining solutions:
- Fixative/stop solution: 30% (v/v) ethanol, 10% (v/v) acetic acid.
- Sensitiser solution: 0.006% (w/v) Erichrome Black T, 30% (v/v) ethanol.
- Destain: 30% (v/v) ethanol.
- Silver-stain: 0.25% (w/v) silver nitrate, 0.037% (v/v) formaldehyde.
- Developer: 2% (w/v) potassium carbonate, 0.04% (w/v) sodium hydroxide, 0.002% (w/v) sodium thiosulphate, 0.007% (v/v) formaldehyde.

Mass Spectrometry:

- HPLC Buffer A: 98% (v/v) H_2O, 2% (v/v) acetonitrile, 0.1% (v/v) formic acid.
- HPLC Buffer B: 20% (v/v) H_2O, 80% (v/v) acetonitrile, 0.1% (v/v) formic acid.
- 18.2 Megaohm deionised water (Cascada Water Purification System, Pall Life Sciences, Singapore).

2.2.4 EZQ Protein Quantification

To establish the protein content of plasma samples, EZY Protein Quantification assays were performed in accordance with manufacturer's instructions (Invitrogen). Specifically, 2 mg ovalbumin was reconstituted with 500 µL of dH2O to create a stock 'protein standard' solution of 4 mg/mL. Serial dilutions were subsequently made from this stock to give the following working standards of 2.0, 1.0, 0.5, 0.2, 0.1, 0.05, and 0.02 mg/mL.

Assay paper was inserted into the EZQ microplate cassette, and 1 µL of each standard (including a blank) and plasma sample was dispensed in triplicate onto the paper and allowed to dry completely. Spotted assay paper was then removed from the cassette and placed in 40 mL of methanol wash for 5 minutes with gentle agitation. After this washing step, the assay paper was air dried and placed in 35 mL of EZQ Protein Quantification Reagent and incubated for 30 minutes with gentle agitation on an orbital shaker (100 RPM). Following staining, the spotted assay paper was washed three times with 35 mL of Rinse Buffer Solution for a total of 2 minutes per wash.

The assay paper was imaged on a Typhoon 940 variable mode imager at 200 µm using a blue 488 nm laser, 520 BP emission filter and a photo multiplier tube (PMT) value of 550 V. Analysis of protein concentration was performed using Carestream Molecular Imaging Software (Version 5.0.6.20, Carestream Health Inc., Rochester, USA).

2.2.5 ReadyPrep 2D Clean-up

ReadyPrep 2D Protein Clean-up was performed on plasma samples as per the manufacturer's instructions to obtain the optimal reaction condition for 2D-DIGE (BioRad). This process involves the precipitation and concentration of sample proteins whilst removing ionic detergents, salts, lipids and nucleic acids. Exclusion of these contaminants increases the purity of protein samples as well as clarity of isoelectric focusing and 2D – electrophoretic separation by reducing streaking and background staining of artefacts. A total sample volume of 100 µL containing upto 500 µg of protein was added to a 1.5 mL microcentrifuge tube. A 300 µL aliquot of Precipitation Agent 1 was added to each protein sample followed by a 30 second vortex and an incubation on

ice for 15 minutes. Next, a 300 µL aliquot of Precipitation Agent 2 was added to each protein sample, followed by a 30 second vortex to mix the solutions. Samples were centrifuged at 18,000 x g for 5 minutes and the supernatant was removed, leaving behind a tight protein pellet. Samples were centrifuge again for 30 seconds with care taken to further remove any remaining supernatant from each sample.

A 40 µL aliquot of Wash Reagent 1 was added directly to each protein pellet followed by a 10 second vortex. Tubes were centrifuged at 18,000 x g for 5 minutes, with Wash Reagent 1 removed from each sample. A 25 µL aliquot of deionised water was added directly to the protein pellet and each sample was vortexed for 10 seconds. A 5 µL aliquot of Wash 2 Additive was combined with a 1 mL aliquot of Wash Reagent 2 (pre-chilled to -20^{o}C) and added to each sample, followed by a 1-minute vortex. Samples were incubated on ice for 30 minutes, with periodic vortexing performed for 30 seconds every 10 minutes during the incubation period. Following incubation, samples were centrifuged at 18,000 x g for 5 minutes and the supernatant was removed. A further centrifuge was performed again to remove any residual supernatant. The protein pellet was then left to dry at room temperature for 1 minutes until becoming translucent. At this stage, resuspension buffer was added to protein pellets to create a desired sample concentration. Resolubised samples were vortexed for 1 minute, left to incubate at room temperature for 5 minutes, and vortexed again for a further 1 minute. Samples were centrifuged a final time at 18,000 x g for 5 minutes before being pipette into a new microfuge tube and stored in -80^{o}C till use.

2.2.6 Multiple-Affinity-Removal System Chromatography

The multiple affinity removal system (MARS) column works on the principal of antibody-antigen interactions involving antibody-modified resins which selectively bind to and hence remove human albumin, immunoglobulin G, alpha-1 antitrypsin, immunoglobulin A, transferring, and haptoglobin from human biological fluid samples such as cerebrospinal fluid, serum or for the purposes of this study human plasma. Specific removal of the top 6 most abundant sample proteins eliminates approximately 85-90% of total plasma protein, allowing for enrichment and enhanced detection of low- to moderate abundance plasma proteins of potential clinical relevance to specific biological or disease processes. Evidence supports the use of immunodepletion

fractionation as an initial step in a biological fluid-based biomarker discovery method prior to protein separation and discovery techniques such as 2D-DIGE and LC- Tandem Mass Spectrometry (MS/MS) respectively (Smith et al., 2011).

Given that the MARS-Hu6 column uses a high-affinity binding technology, use of a gentler elution buffer such as 4 M magnesium chloride would likely not effectively elute all captured proteins therefore rapidly reducing both column life and consistency of immunodepletion between runs, and increase sample variation. Propriety buffer solutions (listed above) were used due to their optimised ability to elute high-affinity captured proteins as well as repeatability for up to 200 sample immunodepletions. Elution is achieved by using a low pH (acidic) solution of between 2.5 – 2.8 such a glycine buffer (acidic amino-acid) to dissociate hydrogen-bonding of capture antibodies bound to selective high-abundant proteins. Use of the wash buffer is therefore essential to equilibrate the pH of the column following elution due to the risk of affinity-binding antibodies deteriorating at low pH.

The upper pressure limit for use of the MARS-Hu6 4.6 x 100 mm column is 12 megapascals (MPa). Prior to attachment of the MARS-Hu6 column to the fast protein liquid chromatography (FPLC) system, both wash and elution buffers were degassed using a vacuum filtration system. Given that high pressure forces gas out of solution, there was a need to degas to ensure that air bubbles are not created within the FPLC pump circuitry as this can cause variable flow and pressure rates.

Fast protein liquid chromatography system uses an ultraviolet (UV) absorbance detector set at 280 nm, and a conductivity meter. Conductivity meter detects electrical (salt) conductivity thereby recording how the mobile (liquid) phase conductivity changes as different sample components are eluted from the column. Different compounds will absorb different amounts of light in the UV and visible ranges. A beam of UV light is shone through the analyte after it is eluted from the column. The amount of light absorbed will depend on the amount of the compound that is passing through the beam.

Polyetheretherketone (PEEK) tubing is the recommended sample-loop tubing for connection between the injection valve of the FPLC system and the MARS column due to its inert polymer that can withstand high pressures of upto 7,000 psi (483 bar). Orange PEEK tubing of 0.05 mm diameter was selected with tubing length measured to equate to the total loading volume of sample to be depleted. The optimal binding capacity for the 4.6 x 100 mm MARS-Hu6 column is between 30-40 µL of neat human plasma. This volume is to be diluted 1:5 with Buffer A, making the total diluted sample-loop volume between 150 µL – 200 µL. Volumes exceeding this range increase the risk of capture antibody over-saturation, with the effect of high-abundant proteins entering the flow-through fraction. Hence, a ratio of 30 µL of neat plasma to 120 µL of Buffer A (i.e. 150 µL total diluted sample) was selected as the optimal PEEK sample-loop tubing volume for each MARS immunodepletion run. The length of 0.5 mm diameter PEEK tubing required to hold the total volume of 150 µL was calculated as follows:

$\pi\, r^2$ x *length* = tubing volume

$\therefore \pi.0.025^2$ x *length* = 0.150 mL

$\therefore$ 0.001964 cm^2 x *length* = 0.150 mL

$\therefore$ *length* = 0.150 mL / 0.001964 cm^2

$\therefore$ Needed *76.37 cm* of PEEK tubing to hold 150 µL sample volume.

To ensure that the entire 150 µL volume capacity of PEEK sample-loop tubing was consistently filled between each immunodepletion run, it was essential to have extra sample volume (i.e. >150 µL). This was to ensure that the sample-loop can fill entirely by allowing some volume over-flow go to waste, as well as the need to fill extra dead-space volumes in-between tubing connections to prevent air-bubbles remaining within the system and disrupting flow and pressure rates. Hence, 40 µL of neat plasma was diluted with Buffer A at 1:5 (i.e. 40 µL plasma : 160 µL Buffer A = 200 µL volume).

2.2.6.1 Sample Preparation

To ensure removal of particulate matter, each thawed and diluted plasma sample was centrifuged using polypropylene spin filters with 0.22 µm pore-size cellulose acetate filter membranes (Agilent Technologies) at 16,000 x g. A 100x stock solution of Halt™ Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific) (see Appendix Table 2) was added to diluted plasma samples at a final cocktail concentration of 10 µL/mL to minimise enzymatic digestion, degradation and modifications of plasma proteins during the forthcoming immunodepletion steps.

By injecting 200 µL of each 1:5 diluted plasma sample through the injection-valve connection of the FPLC system, 150 µL of diluted sample was consistently loaded in the sample-loop tubing for immunodepletion (Table 2). Thirty-six plasma samples were depleted in duplicate using this method (i.e. 72 immunodepletion runs) to provide enough depleted plasma protein for quantification and 2D-DIGE analysis.

A working solution of 0.02% Sodium Azide in Buffer A (0.002 g : 10 mL Buffer A) was made and manually injected into the column to fill the void volume. Sodium Azide solution has antibacterial properties which helps maintain the sterility of the column when returned to refrigeration storage between immunodepletion runs.

Table 2: Fast protein liquid chromatography configuration for MARS-Hu6 plasma depletion.

	Time (min)	Buffer B (%)	Flow-rate (mL/min)	Pressure (MPa)	Loop Status	Purpose
Purge	10	20% EtOH, then Buffer A in Pump A, and Buffer B in Pump B.	1.0	0.03	Closed	Purging of LC system without column attached
Attach MARS-Hu6 4.6 x 100 mm column to FPLC						
Equilibrate	4	0	1.0	0.03	Closed	Column washout/equilibration
Injection of 200 µL sample to fill 150 µL capacity PEEK tubing						
Step 1	2.5	0	0.5	0.05	Open	Injection of plasma
Step 2	3.5	0	0.5	0.05	Closed	Flow-through fraction collection
Step 3	7.0	100	1.0	0.07	Closed	Elution fraction
Step 4	11.0	0	1.0	0.06	Closed	Re-equilibration

2.2.6.2 Depleted Plasma Sample Preparation

All 36 plasma samples were immunodepleted twice, with each yielding three flow-through fractions containing plasma sample devoid of the top-6 most abundant plasma proteins, as previously described. Flow-through fractions from all 72 immunodepletion runs underwent centrifugal concentration using 5 kDa molecular weight cut-off spin concentrators (Agilent Technologies) at 2,000 x g for 30 minutes at 4^{o}C to reduce their volumes. Concentrated sample volumes were recorded and quantified using EZQ® protein quantification assay, according to the manufacturer's instructions as described in section 2.2.4 (Table 3). All 72 depleted plasma samples were purified using ReadyPrep™ 2D Protein Clean-up Kit (BioRad) according to the manufacturer's instructions as described in section 2.2.5, followed by re-suspension in 2D DIGE labelling buffer (TUC: 2 M Thiourea, 7 M Urea, 4% CHAPS, 30 mM Tris, pH 8.5) to an anticipated concentration of 10 mg/mL. Matching (duplicate) concentrated samples were then pooled together (i.e. Sample #'s 1 & 37, 2 & 38 etc.). However, the potential for incomplete precipitation during protein clean-up can contribute to significant protein loss. Therefore, a 1 µL aliquot from the 36 'combined' post-2D-clean-up samples was taken for re-quantification purposes (Table 3), whilst the remaining volume of the 36 cleaned-up/re-suspended samples in TUC lysis buffer were stored in -80^{o}C until required for DIGE CyDye labelling. Quality control experiments were performed by one- and two dimensional electrophoresis separation of crude plasma, depleted flow-through and eluted fractions to validate this immunodepletion method (Figures 13 and 14).

Figure 13: Quality control one-dimensional electrophoresis separation of un-depleted plasma, MARS flow-through and eluted fractions. *Twenty-five micrograms of plasma protein separated by molecular weight on 10% SDS-PAGE.*

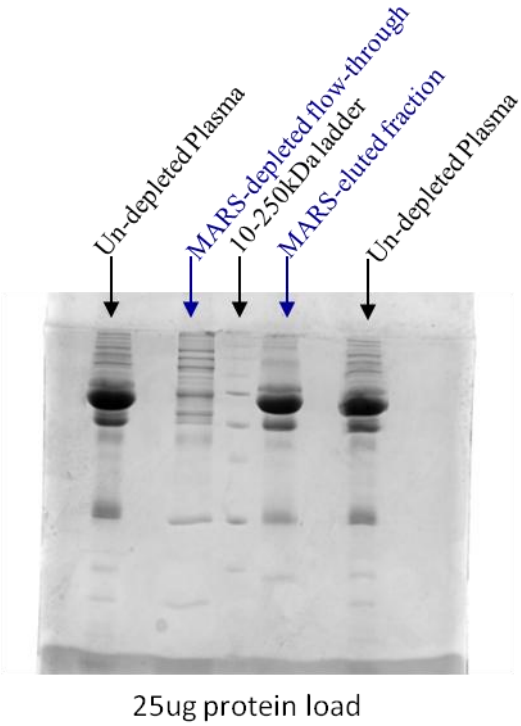
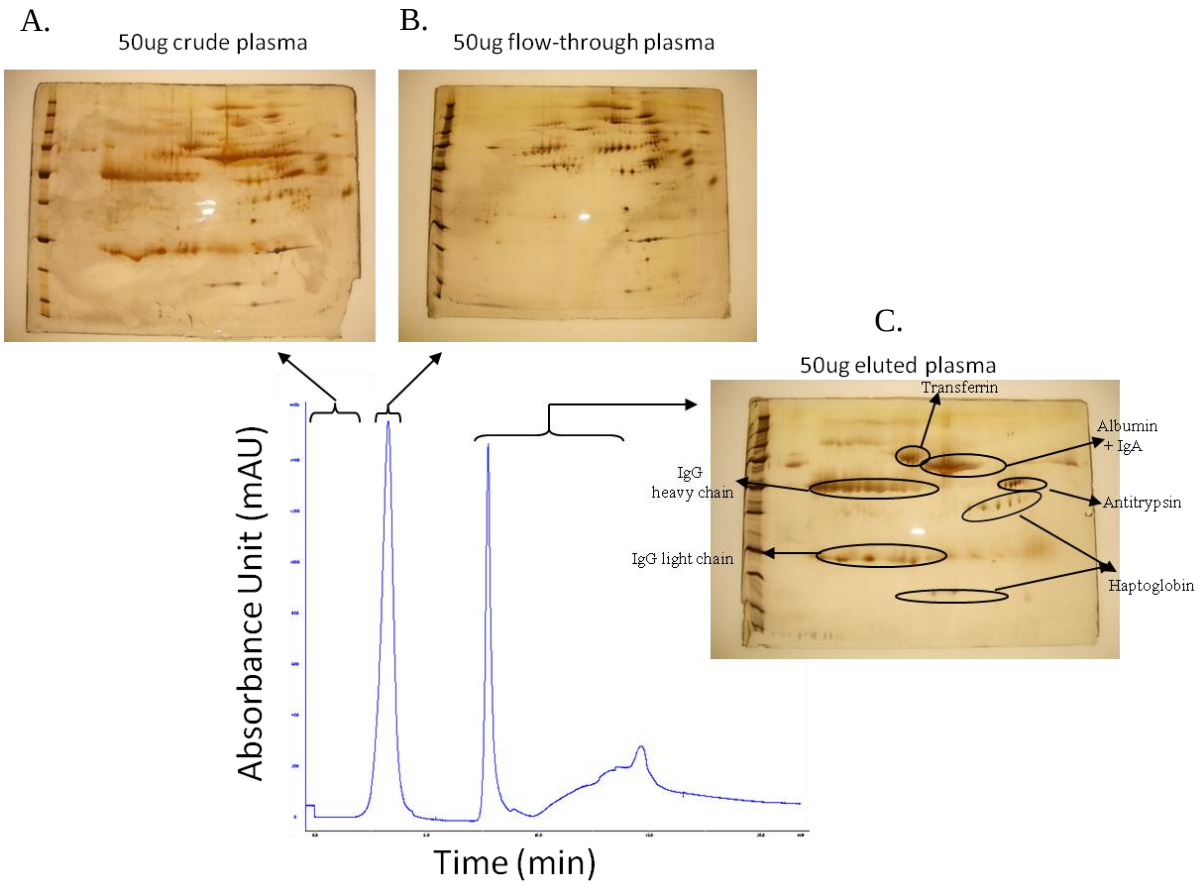


Table 3: EZQ protein quantification assays of depleted plasma samples. *EZQ Protein Quantification Assays for MARS Hu6 immunodepleted TIA, Mimic, and Healthy Control Volunteer plasma samples, with associated post- 2D-cleanup and TUC re-suspension concentrations. T= TIA patient, M=Mimic patient, H= Health Control Volunteer, i = initial/acute sample, f = 3-month follow-up sample.*

Sample Number	Depletion Volume (µL)	Depletion Quantity (µg)	Sample Number	Depletion Volume (µL)	Depletion Quantity (µg)	Combined Protein Quantity (µg)	Post - 2D-Cleanup Protein Re-suspension [mg/mL]
1 (T1i)	33.4	344.55	**37 (T1i)**	33.5	200.91	545.46	15.75
2 (T1f)	45.1	469.12	**38 (T1f)**	38.3	341.50	810.62	10.63
3 (M1i)	41.5	349.67	**39 (M1i)**	27.5	252.86	602.53	15.31
4 (M1f)	53.8	253.71	**40 (M1f)**	50.0	342.19	595.90	14.57
5 (H1i)	60.8	218.51	**41 (H1i)**	36.4	213.15	431.66	18.82
6 (H1f)	59.1	297.89	**42 (H1f)**	44.2	252.80	550.69	14.06
7 (T2i)	59.1	306.36	**43 (T2i)**	70.0	305.64	612.00	13.40
8 (T2f)	40.2	315.09	**44 (T2f)**	47.0	223.84	538.93	13.64
9 (M2i)	40.0	304.35	**45 (M2i)**	45.0	258.84	563.19	14.79
10 (M2f)	45.2	331.99	**46 (M2f)**	61.5	304.65	636.64	12.84
11 (H2i)	45.1	261.72	**47 (H2i)**	45.6	183.81	445.53	12.84
12(H2f)	51.5	213.40	**48 (H2f)**	65.1	290.32	503.72	12.56
13 (T3i)	45.6	160.30	**49 (T3i)**	45.8	257.63	417.93	16.23
14 (T3f)	47.1	211.76	**50 (T3f)**	54.4	346.85	558.61	11.92
15 (M3i)	52.5	229.46	**51 (M3i)**	57.0	339.12	568.58	11.66
16 (M3f)	54.4	207.68	**52 (M3f)**	63.4	347.93	555.61	14.12
17 (H3i)	55.2	182.45	**53 (H3i)**	60.0	447.44	629.89	10.73
18 (H3f)	50.0	266.18	**54 (H3f)**	63.0	261.03	527.21	10.26
19 (T4i)	36.9	160.11	**55 (T4i)**	47.0	339.52	499.63	8.24
20 (T4f)	66.5	203.15	**56 (T4f)**	75.8	394.82	597.97	7.96
21 (M4i)	55.6	175.88	**57 (M4i)**	58.6	338.35	514.23	12.55
22 (M4f)	50.2	225.42	**58 (M4f)**	57.3	394.28	619.70	12.75
23 (H4i)	50.0	156.62	**59 (H4i)**	55.8	197.37	353.99	11.94
24 (H4f)	45.0	212.79	**60 (H4f)**	50.0	257.28	470.07	10.58
25 (T5i)	60.0	264.96	**61 (T5i)**	60.0	304.80	569.76	9.74
26 (T5f)	57.2	313.20	**62 (T5f)**	61.9	317.02	630.22	13.26
27 (M5i)	58.8	280.79	**63 (M5i)**	40.0	279.21	560.00	16.84
28 (M5f)	49.1	382.29	**64 (M5f)**	60.6	262.31	644.6	13.59
29 (H5i)	52.1	379.48	**65 (H5i)**	53.2	262.23	641.71	12.06
30 (H5f)	53.4	365.84	**66 (H5f)**	61.3	220.07	585.91	14.01
31 (T6i)	46.8	320.28	**67 (T6i)**	52.0	256.47	576.75	12.15
32 (T6f)	44.1	254.70	**68 (T6f)**	50.0	181.61	436.31	16.67
33 (M6i)	50.0	194.04	**69 (M6i)**	50.0	288.05	482.09	18.64
34 (M6f)	27.4	305.34	**70 (M6f)**	34.5	267.06	572.40	14.09
35 (H6i)	50.0	121.20	**71 (H6i)**	51.1	227.59	348.79	18.80
36 (H6f)	43.2	291.60	**72 (H6f)**	52.6	293.98	585.58	11.38

Figure 14: Quality control two dimensional electrophoresis separation of crude plasma, flow-through and eluted plasma following MARS Hu6 immunodepletion. *Immunodepletion using fast protein liquid chromatography system and separation using 2D- electrophoresis with (A) indicating crude plasma, (B) indicating flow-through fraction devoid of top-6 most abundant proteins, and (C) indicating elution of the top-6 most abundant proteins. Protein samples were separated in the first dimension by isoelectric focusing using pH 3-11 non-linear gradient followed by separation by molecular weight in the second dimension using 24 cm 12.5% linear polyacrylamide gels. EBT silver staining was used to resolve protein features in polyacrylamide gels.*



2.2.7 2D-DIGE

2.2.7.1 Introduction

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) is a gel-based proteomic technique developed by O'Farrell et al., which is widely used to analyse and quantify complex protein specimens derived from cells, tissues, as well as from biological fluids such as urine, cerebrospinal fluid, serum or plasma (O'Farrell, 1975). Protein separation occurs by electrophoresis in two dimensions based on unique protein properties of charge and molecular mass.

The first dimension of protein separation involves isoelectric focusing (IEF), where by proteins are separated in a pH gradient according to their isoelectric point (pI). Proteins are amphoteric molecules which carry positive, negative and/or zero net charge in amino-acid side-chains. Under IEF conditions, proteins will move towards the pole opposite to their charge, through a gel matrix containing an immobilised pH gradient until it reaches the point in which the pH of the proteins' isoelectric point is reached. Therefore, the pI is the specific pH at which a protein no longer has a net electric charge due to either de-protonation (for positively charged proteins) or protonation (for negatively charged proteins). Isoelectric focusing concentrates proteins at their pI and separates proteins on the basis of small charge differences.

The second dimension involves protein separation polyacrylamide gel electrophoresis according to a proteins molecular mass. Specifically, protein separation is performed in polyacrylamide gels containing sodium dodecyl sulphate (SDS). The intrinsic electric charge of sample proteins is not a factor in the separation by molecular mass due to the presence of SDS in the sample and the gel. SDS is an anionic (negatively charged) detergent, whereby large amounts of SDS form complexes with proteins at a ratio of 1.4:1. SDS masks the charge of the proteins themselves and the formed anionic complexes have a roughly constant negative charge per unit mass. Additionally, the reducing agent dithiothreitol (DTT) is added to break disulfide bonds forming between cysteine residues of proteins, preserving the structural integrity by allowing proteins to

unfold completely. When protein samples are treated with both SDS and a reducing agent (DTT), the degree of electrophoretic separation within a polyacrylamide gel depends largely on the molecular weight of the protein.

This technique was further developed into the two-dimensional difference in-gel electrophoresis (2D-DIGE) method (Unlu, Morgan, & Minden, 1997). The DIGE method utilises fluorescent cyanine dyes (CyDyes; Cy2, Cy3, Cy5) which contain a highly reactive N-hydroxyl succinimidyl (NHS) ester group for use in protein labelling. Specifically, the NHS-ester covalently bonds via an amide linkage to epsilon-amino groups of lysine side-chains located in every protein molecule. This process is called minimal labelling, whereby the stoichiometry of the reactive CyDye to protein ratio is kept to a minimum so that each CyDye labels a single lysine per protein molecule. CyDye's poses a single positive charge which replaces the lysine positive charge, thereby having no effect on a protein's isoelectric point or 1st dimensional isoelectric focusing. Likewise, all three CyDyes have an approximate molecular weight of 0.5 kDa, and therefore a negligible effect on 2nd dimension protein separation. Up to three CyDye labelled protein samples can be separated within one polyacrylamide gel, thereby allowing multiplexed quantitative comparisons through the use of selective excitation wavelengths to illuminate three separate protein profile images within the same gel.

2.2.7.2 DIGE minimal Labelling

Minimal DIGE labelling was performed according to the instructions of the manufacturer (GE Healthcare). CyDye DIGE Fluor minimal dyes (Cy2, Cy3, and Cy5) were warmed to room temperature for 5 minutes and reconstituted with 50 µL of fresh anhydrous DMF, vortexed vigorously for 30 seconds and then centrifuged to give a 100 pmol/µL working solution concentration. To label each 50 µg protein sample, 200 pmol of each Cydye was needed, equating to 2 µL of working solution. Each 50 µg protein sample was quantified as having a concentration between 7.92 – 18.82 mg/mL at pH 8.5 in DIGE labelling buffer (Table 4). Cy3 and Cy5 dyes were used interchangeably to label acute and follow-up plasma samples in all 18 subjects (see Table 6 for dye-swap details), while Cy2 was used as a pooled internal standard label. The pooled internal standard contained an equal quantity of protein sample from all 36 plasma samples used

in the DIGE study (i.e. 18- acute and 18-follow-up, see Table 5 for calculations). Every protein from all plasma samples in this study was represented in the Cy2 labelled pooled internal standard and therefore included in all 18x 2D DIGE gels to provide a reliable quantitative way of measuring Cy3 and Cy5 labelled samples (i.e. acute vs. follow-up) as a ratio to its associated spot within the pooled internal standard. This comparison of Cy3 and Cy5 with the Cy2 pooled internal standard within the same gel removes gel-to-gel variation and associated risks of protein loss and sample variation between isoelectric focusing and electrophoretic separation steps, as well as variability in gel preparation and running conditions. Given that this study involves the preparation and running of 18 individual 2D-DIGE gels, the potential for gel-to-gel variation would be very high if using a traditional 2D-electrophoresis approach.

Fifty micrograms of each acute and follow-up plasma protein sample was mixed with 2 µL of 100 pmol/µL reconstituted Cy3 or Cy5 dye working solution. An equal proportion of plasma protein (30 µg) from all 36 plasma samples was pooled and mixed with 43.2 µL of 100 pmol/µL reconstituted Cy2 dye working solution such that 50ug of Cy2 pooled internal standard could be loaded onto 18-DIGE gels including an allowance for three extra/repeat gels and pipetting error (see Table 5 and Table 6). Samples mixed with CyDye were left to incubate on ice for 30 minutes in the dark to allow the covalent bonding of the NHS-ester component of CyDye with lysine amino acids present in plasma proteins. This labelling reaction was stopped by the addition of 10 mM lysine (at a ratio of 1 µL lysine: 1 µL mixed labelled sample volume) for a further 10 minutes on ice and in the dark. Lysine outcompete plasma proteins for CyDye, thereby quenching the minimal labelling reaction. Labelled protein samples representing acute and follow-up plasma for each patient/volunteer were pooled together with an equal quantity of internal standard and stored in 18 respective microcentrifuge tubes for storage in the dark at -80^{o}C (i.e. 6-TIAs, 6-Mimics, 6-Healthy Controls, each with 50 µg of acute and 50 µg of follow-up plasma samples labelled with Cy3 and Cy5 interchangeably, and 50 µg of Cy2 labelled pooled internal standard, making a combined total protein quantity of 150 µg per DIGE) (see Table 6 for labelling order).

Table 4: Minimal CyDye labelling quantities and volumes for Cy3 and Cy5 dyes.

Diagnosis	Sample Type	Depleted Plasma Protein Concentration [mg/mL]	Protein Quantity for Minimal Labelling (µg)	Volume of Protein Sample used for Minimal Labelling (µL)
TIA 1	Acute	15.75	50	3.175
	Follow-Up	10.63	50	4.702
Mimic 1	Acute	15.31	50	3.266
	Follow-Up	14.57	50	3.432
Healthy Control 1	Acute	18.82	50	2.656
	Follow-Up	14.06	50	3.557
TIA 2	Acute	13.40	50	3.732
	Follow-Up	13.64	50	3.666
Mimic 2	Acute	14.79	50	3.380
	Follow-Up	12.84	50	3.893
Healthy Control 2	Acute	12.84	50	3.894
	Follow-Up	12.56	50	3.982
TIA 3	Acute	16.23	50	3.080
	Follow-Up	11.92	50	4.193
Mimic 3	Acute	11.66	50	4.289
	Follow-Up	14.12	50	3.541
Healthy Control 3	Acute	10.73	50	4.661
	Follow-Up	10.26	50	4.875
TIA 4	Acute	8.24	50	6.070
	Follow-Up	7.96	50	6.283
Mimic 4	Acute	12.55	50	3.985
	Follow-Up	12.75	50	3.920
Healthy Control 4	Acute	11.94	50	4.187
	Follow-Up	10.58	50	4.725
TIA 5	Acute	9.74	50	5.134
	Follow-Up	13.26	50	3.770
Mimic 5	Acute	16.84	50	2.969
	Follow-Up	13.59	50	3.679
Healthy Control 5	Acute	12.06	50	4.145
	Follow-Up	14.01	50	3.570
TIA 6	Acute	12.15	50	4.114
	Follow-Up	16.67	50	2.999
Mimic 6	Acute	18.64	50	2.683
	Follow-Up	14.09	50	3.548
Healthy Control 6	Acute	18.80	50	2.660
	Follow-Up	11.38	50	4.396

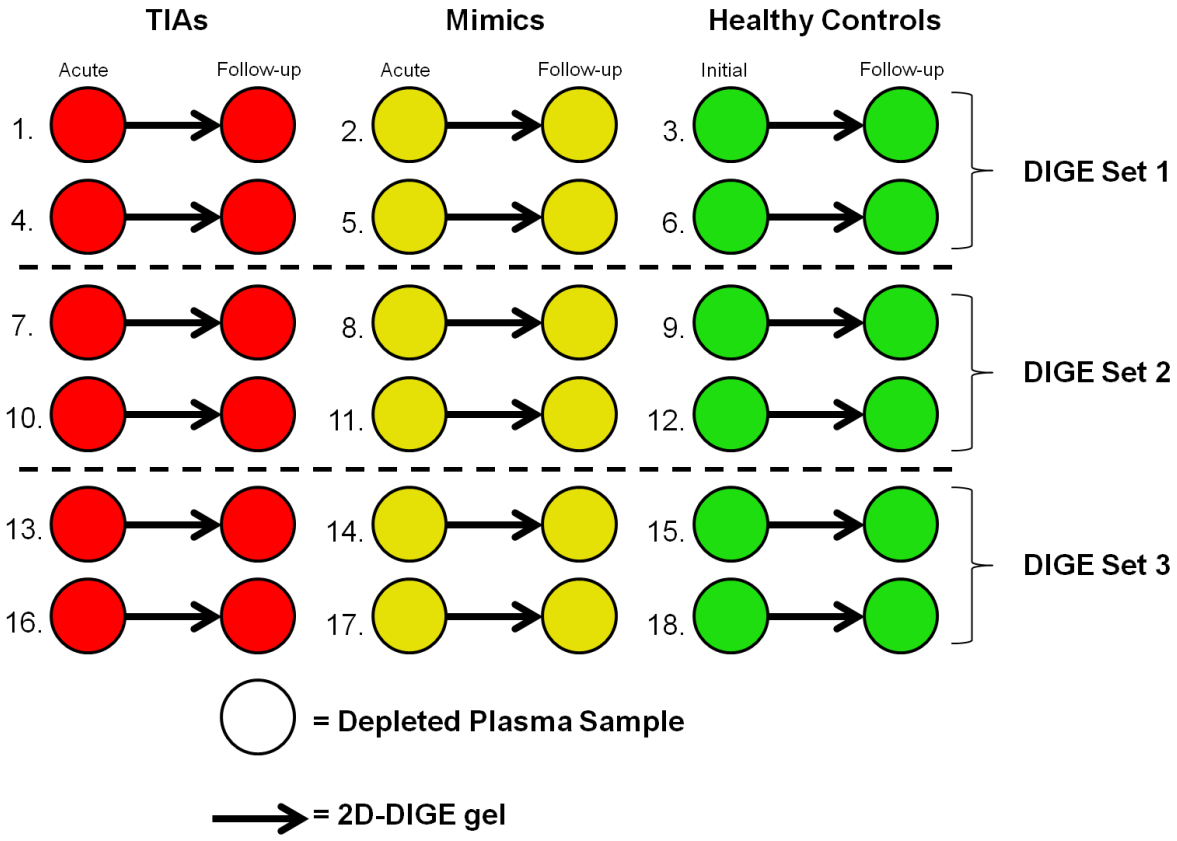
Table 5: Minimal CyDye labelling quantities and volumes for Cy2 pooled internal standard. *To allow for 50 µg of Cy2 labelled pooled internal standard to be loaded on all 18 x 2D-DIGE gels including an allowance for three extra/repeat 2D-DIGE gels and pipetting error, 30 µg of plasma protein was required from all 36-samples to be pooled and labelled.*

Diagnosis	Sample Type	Depleted Plasma Protein Concentration [mg/mL]	Protein Quantity for Minimal Labelling (µg)	Volume of Protein Sample used for Minimal Labelling (µL)
TIA 1	Acute	15.75	30	1.905
	Follow-Up	10.63	30	2.822
Mimic 1	Acute	15.31	30	1.960
	Follow-Up	14.57	30	2.059
Healthy Control 1	Acute	18.82	30	1.594
	Follow-Up	14.06	30	2.134
TIA 2	Acute	13.40	30	2.239
	Follow-Up	13.64	30	2.199
Mimic 2	Acute	14.79	30	2.028
	Follow-Up	12.84	30	2.336
Healthy Control 2	Acute	12.84	30	2.336
	Follow-Up	12.56	30	2.389
TIA 3	Acute	16.23	30	1.848
	Follow-Up	11.92	30	2.517
Mimic 3	Acute	11.66	30	2.573
	Follow-Up	14.12	30	2.125
Healthy Control 3	Acute	10.73	30	2.796
	Follow-Up	10.26	30	2.924
TIA 4	Acute	8.24	30	3.641
	Follow-Up	7.96	30	3.769
Mimic 4	Acute	12.55	30	2.390
	Follow-Up	12.75	30	2.353
Healthy Control 4	Acute	11.94	30	2.513
	Follow-Up	10.58	30	2.836
TIA 5	Acute	9.74	30	3.080
	Follow-Up	13.26	30	2.262
Mimic 5	Acute	16.84	30	1.781
	Follow-Up	13.59	30	2.208
Healthy Control 5	Acute	12.06	30	2.488
	Follow-Up	14.01	30	2.141
TIA 6	Acute	12.15	30	2.469
	Follow-Up	16.67	30	1.800
Mimic 6	Acute	18.64	30	1.609
	Follow-Up	14.09	30	2.129
Healthy Control 6	Acute	18.80	30	1.596
	Follow-Up	11.38	30	2.636

Table 6: TIA Biomarker DIGE Labelling Order. *5 nmol CyDye DIGE Fluors Cy2, Cy3, and Cy5 are each reconstituted in 50 µL of dimethylformamide (DMF) giving a final working solution concentration of 100 pmol/µL. 2 µL of each CyDye equating to 200 pmol is labelled with 50 µg of depleted plasma protein. I.S.= Internal Standard.*

Gel#	Cy2 I.S.	Cy3	Cy5	DIGE Set
1	200 pmol	200 pmol 'TIA 1' Follow-up	200 pmol 'TIA 1' Acute	#1
2	200 pmol	200 pmol 'Mimic 1' Follow-up	200 pmol 'Mimic 1' Acute	#1
3	200 pmol	200 pmol 'HCV 1' Follow-up	200 pmol 'HCV 1' Acute	#1
4	200 pmol	200 pmol 'TIA 2' Acute	200 pmol 'TIA 2' Follow-up	#1
5	200 pmol	200 pmol 'Mimic 2' Acute	200 pmol 'Mimic 2' Follow-up	#1
6	200 pmol	200 pmol 'HCV 2' Acute	200 pmol 'HCV 2' Follow-up	#1
7	200 pmol	200 pmol 'TIA 3' Follow-up	200 pmol TIA 3' Acute	#2
8	200 pmol	200 pmol 'Mimic 3' Follow-up	200 pmol 'Mimic 3' Acute	#2
9	200 pmol	200 pmol 'HCV 3' Follow-up	200 pmol 'HCV 3' Acute	#2
10	200 pmol	200 pmol 'TIA 4' Acute	200 pmol 'TIA 4' Follow-up	#2
11	200 pmol	200 pmol 'Mimic 4' Acute	200 pmol 'Mimic 4' Follow-up	#2
12	200 pmol	200 pmol 'HCV 4' Acute	200 pmol 'HCV 4' Follow-up	#2
13	200 pmol	200 pmol 'TIA 5' Follow-up	200 pmol 'TIA 5' Acute	#3
14	200 pmol	200 pmol 'Mimic 5' Follow-up	200 pmol 'Mimic 5' Acute	#3
15	200 pmol	200 pmol 'HCV 5' Follow-up	200 pmol 'HCV 5' Acute	#3
16	200 pmol	200 pmol 'TIA 6' Acute	200 pmol 'TIA 6' Follow-up	#3
17	200 pmol	200 pmol 'Mimic 6' Acute	200 pmol 'Mimic 6' Follow-up	#3
18	200 pmol	200 pmol 'HCV 6' Acute	200 pmol 'HCV 6' Follow-up	#3
100 pmol CyDye Required Volume	*36 µL*	*36 µL*	*36 µL*	
Allowance for 3x Repeat Gels	*6.0 µL*	*6.0 µL*	*6.0 µL*	
Aspirin DIGE gel sub-study	*2.0 µL*	*2.0 µL*	*2.0 µL*	
10% Pipetting Error	*4.4 µL*	*4.4 µL*	*4.4 µL*	
Total volume	*48.4 µL*	*48.4 µL*	*48.4 µL*	

Figure 15: 2D-DIGE Running Order. An *internal standard comprising of an equal quantity of every sample in the study was included in each 2D-DIGE.*



2.2.7.3 First Dimension Isoelectric Focusing

Isoelectric focusing was performed according to the manufacturer's guidelines (GE Healthcare), with a capacity for six DIGE gels to be processed at a time (see Figure 15 and Table 6 for DIGE running order). A total combined CyDye-labelled protein quantity of 150 µg from each of the 18 patients/volunteers was diluted into 475 µL of IEF rehydration buffer with a 0.5 µL trace volume of bromophenol blue to visually assist with electrophoretic tracking. Each sample was evenly dispensed between the electrodes of a 24 cm IPG strip holder (GE Healthcare) followed by placement of a 24cm Immobiline DryStrip gel pH 3-11 NL (GE Healthcare) with the acrylamide side facing down to absorb the sample. Care was taken to ensure correct alignment of the positive end of the strip with the cathode end of the IPG strip holder followed by adding mineral oil as a cover fluid to prevent evaporation of sample during the isoelectric focusing step.

Samples were then actively rehydrated at 50 V overnight using an Ettan IPGphor 3 IEF unit (GE Healthcare).

Following completion of rehydration, Immobiline strip gels were individually removed from the IPG strip holder and drained of excess mineral oil using lint-free tissues. Strip holders were cleaned using Strip Holder Cleaning Solution (GE Healthcare) followed by application of 5 mm x 5 mm wicks (that were cut from filter paper) that were wet with deionised water and placed over the strip holder electrodes. Immobiline DryStrip gels were placed back into the strip holders, followed again by covering the strips in mineral oil and placed in the Ettan IPGphor 3 IEF unit.

The Immobiline strips were focused overnight according to the following protocol:

1.	Step and hold	500 V	30 minutes
2.	Step and hold	1,000 V	30 minutes
3.	Gradient	10,000 V	30 minutes
4.	Step and hold	10,000 V	Till 65,000 Vhrs
5.	Gradient	1,000 V	30 minutes
6.	Step and hold	1,000 V	Till removed from apparatus

2.2.7.4 Second Dimension SDS-PAGE

Second dimension SDS PAGE was performed according to the manufacturer's guidelines (GE Healthcare). Immobiline strips were removed from the Ettan IPGphor 3 IEF unit and immediately incubated in 10 mL of equilibration buffer 1 for fifteen minutes at room temperature, followed by incubation in 10 mL of equilibration buffer 2 for fifteen minutes.

Specifically, the first equilibration buffer contained the anionic molecule SDS, which binds to protein in a uniform ratio proportional to a proteins mass (i.e. approximately one SDS molecule binds for every two amino acid residues). By establishing a constant negative charge per unit mass of protein, SDS makes electrophoretic separation of

protein within a polyacrylamide gel dependent on molecular weight. The reducing agent DTT was also added to equilibration buffer 1 to promote protein unfolding by reducing disulfide linkages forming with cysteine residues both within and between proteins. The second equilibration buffer contains iodoacetamide, which forms a covalent bond with the thiol group of cysteine, thereby preventing protein from forming disulfide bonds. In addition, bromophenol blue was added to visualise the dye-front during electrophoretic separation.

Casting of 24 cm 12.5% linear polyacrylamide gels was performed using an Ettan DALTsix gel caster and non-fluorescing glass plates as per manufacturer's guidelines (GE Healthcare). Each equilibrated strip was loaded and sealed at the top of each gel using 1% low melting point agarose and left to set for 5 minutes. SDS PAGE was then performed at 350 V (six gels at a time) in an Ettan DALTsix electrophoresis unit (GE Healthcare) coupled with a cooling unit set to 10^{o}C. Electrophoresis was performed for approximately 3 hours until stopping when the bromophenol blue dye-front approached 0.5 cm from the base of the gels.

2.2.7.5 DIGE Imaging and Analysis

DIGE gels were kept in their glass plates and imaged using a Typhoon 9400 Variable Mode Imager (GE Healthcare) at 200 µm. Cy2 labelled sample (pooled internal standard) was imaged using a blue 488 nm laser, 520 BP emission filter and PMT value of 550 V; the Cy3 labelled sample was imaged using a green 532 nm laser, 580 BP emission filter and PMT value of 500 V; and the Cy5 labelled sample was imaged using a red 633 nm laser, 670 BP emission filter and PMT value of 470 V. Each gel image was cropped using Image Quant Version 5.0 (GE Healthcare) to exclude non-protein related features and allow for uploading into the DIGE analysis software DeCyder Version 7.0 (GE Healthcare), under the Image Loader Module for image processing. Spot analysis was performed using DeCyder Differential In-Gel Analysis and Biological Variation Analysis modules, with each spot reviewed manually and excluded from analysis if identified as a non-protein artefact.

The average ratio of acute:follow-up abundance of each protein spot across all 18 gels (analysed within and between each of the three diagnostic groups) was calculated using the DeCyder Biological Variation Analysis module, with a Student's paired t-test also applied to identify differences in protein abundance that were of statistical significance. Protein spots with an acute:follow-up ratio of ≥ 1.1 fold, $p \leq 0.05$ were identified as proteins of interest.

2.2.7.6 Preparative 2D gels and Silver Staining

Two preparative 2D PAGE gels using depleted TIA plasma were run in order to excise protein spots of interest for mass spectrometry identification. Each gel was loaded with 450 µg of depleted unlabelled plasma protein and underwent 2D PAGE based on the methods previously described. Silver staining was performed using MS-compatible Erichrome black T (EBT) - silver staining method. This method has the sensitivity to detect proteins of low abundance within the range of 0.05 – 0.2 ng in SDS-PAGE gels (Jin, Hwang, Yoo, & Choi, 2006). Following completion of 2D-electrophoresis, gels were removed from their plates and incubated in fixative solution for 20 minutes with gentle agitation, followed by incubation in sensitiser solution for 2 minutes, destain for 2 minutes, two sets of deionised water wash for 2 minutes each, EBT silver stain for 5 minutes, two sets of deionised water washes for 20 seconds each, developer solution till spot image resolution was sufficiently achieved, and finally fixative/stop solution to cease the silver-staining reaction. Solutions were prepared fresh immediately prior to commencement of this method, with formaldehyde added to silver stain solutions immediately prior to usage.

2.2.8 Mass Spectrometry

2.2.8.1 Tryptic Digestion of 2D SDS-PAGE spots

Protein spots of interest from 2D DIGE gels were excised and underwent tryptic digestion as per the method outlined in Proteomics: A Cold Spring Harbor Laboratory

Course Manual (Link, 2009). Specifically, gel spots were manually excised from preparative silver-stained 2D gels placed on a light box, by using a 200 µL low protein-binding pipette tip attached to a OneTouch Plus depressor pipette. To avoid cross contamination, a fresh pipette tip was used for each gel spot excision. Each excised spot of approximately 1 mm^3 was placed in a 1.5 mL low protein binding microfuge tube, and any residual fixative solution was removed from the tube. Each excised gel piece was washed in 50 µL of 100mM ammonium bicarbonate, followed by vortex and incubation for 10 minutes at room temperature and then discarded. This wash step using ammonium bicarbonate was repeated a total of five times. Approximately 150 µL of acetonitrile was then added to completely cover the gel pieces, and was left for 15 minutes to dehydrate, turning gel pieces opaque. Acetonitrile was then completely removed from all tubes and gel pieces were left to dry out at 37^oC for 30 minutes to facilitate acetonitrile evaporation.

Trypsin Gold stock solution [1 µg/µL] was diluted 1:50 with 100 mM ammonium bicarbonate to create a working solution. A 20 µL aliquot of trypsin working solution was then added to each tube for 30-minute incubation/rehydration on ice to allow absorption of trypsin into gel plugs. Tubes were cap sealed and left overnight at 37^oC. The trypsin digest solution was removed and placed in a labelled mass spectrometry vial and stored at 4^oC till analysed.

2.2.8.2 In-solution Tryptic Digestion

In-solution tryptic digestion was performed on post-depleted plasma samples that had undergone ReadyPrep 2D Protein Clean-up (Section 1.2.2.5), with each protein pellet resuspended in a solution of 100 mM ammonium bicarbonate and 5% acetonitrile. The previously used resuspension buffer/2D DIGE labelling buffer was not compatible with downstream mass spectrometry analysis due to the high percentage of CHAPS detergent that contaminates columns/tubing.

In-solution digestion was based on the method described in Proteomics: A Cold Spring Harbor Laboratory Course Manual (Link, 2009). Briefly, a 1 µL aliquot of each

resuspended sample was spotted on 0-14 pH paper to ensure a pH of between 7.5 – 8.5 for optimal trypsin digestion. If samples were acidic, a pH adjustment was made by adding 1 M Tris (pH 8.0). A 1/10 volume of 50 mM DTT was added to samples and left to incubate for 5 minutes at 65^{o}C for the purpose of reducing disulfide bonds. A 1/10 volume of 100 mM iodoacetamide solution was added to samples and left to incubate for 30 minutes in the dark at 30^{o}C. The addition of iodoacetamide alkylates cysteine residues and thereby prevents disulfide bonds reforming. Sequencing-grade Trypsin Gold working solution (Section 2.2.2) was added to resuspended protein samples to give a final protein:trypsin ration of 50:1, and left to incubate overnight at 37^{o}C. Trypsin digestion reaction was stopped by acidifying the solution to a pH < 6 by adding 0.5% acetic acid and re-testing sample pH. Digested protein samples were centrifuged at 18,000 x g for 5 minutes to further clarify samples prior to mass spectrometry to prevent any solid particles blocking the micro-capillary HPLC column or the nanospray ESI tip. Digested samples were transferred into fresh microcentrifuge tubes and stored at -20oC till mass spectrometry analysis.

2.2.8.3 Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS)

Digested peptides from trypsin digested gel spots or from in-solution trypsin digested plasma were analyzed using a Thermo Orbitrap XL linear ion trap mass spectrometer (Thermo Fisher Scientific), with an attached nanospray source (Thermo Electron). Each sample was applied to a 300 µm inner diameter x 5 mm length C18 PepMap™ 100 precolumn (Dionex) at a 20 µL/minute flow rate for 3 minutes, and separated on a 75 µm x 150 mm C18 PepMap 100 column (Nikkyo Technos) using a Dionex Ultimate 3000 HPLC (Dionex) with a 55-minute gradient from 2% acetonitrile to 45% acetonitrile containing 0.1% formic acid at a flow rate of 200 nL/minute followed by a step to 77% acetonitrile for nine minutes. The mass spectrometer was operated in positive ion mode with one full scan of mass/charge 300 – 2000 at a resolution of 60,000 (FWHM). This was followed by product ion scans of the six most intense ions, with the following parameters: dynamic exclusion of 30 seconds, exclusion of 10 ppm low and high mass width relative to the reference mass, an exclusion list size of 500 proteins, and collision induced dissociation (CID) energy of 35%. Only multiple charged ions were selected for MS/MS of trypsin-digested peptides.

2.2.8.4 Protein Identification

Mass spectrometry spectra were searched with Thermo Scientific™ Proteome Discoverer™ 1.2 (Thermo Fisher Scientific) using the SEQUEST algorithm against the Swiss-Prot Human Uniprot database (May 2012 version) (Boutet, Lieberherr, Tognolli, Schneider, & Bairoch, 2007) (representing 20,244 protein entries) using trypsin specificity (KR) as the digesting protease, allowing for a maximum of two missed cleavages. Variable modifications were set for phosphorylated serine, threonine, or tyrosine (179.966 Da;(STY)), oxidated methionine (115.995 Da;(M)), acetylated N-termini or lysine (142.011 Da;(N-termini,K)), glutathionylation, and using the following filters: 1) the cross-correlation scores of matches were greater than 1.5, 2.0 and 2.5 for the charged states of 1, 2 and 3 peptide ions respectively, 2) peptide probability was greater than 0.001 and 3) each protein identified had at least two different peptides sequenced (see Appendix Figures 3-8 for peptide sequence spectra used for multiple reaction monitoring). The mass tolerance for peptide identification of precursor and product ions were 1 Da and 0.5 Da respectively.

2.2.8.5 Multiple Reaction Monitoring

Multiple reaction monitoring (MRM) assays were developed to quantify and validate six plasma proteins, with proteotypic peptides for each protein selected from the discovery 2D DIGE/LC-MS/MS experiments (Appendix Table 6-10). In-solution trypsin-digestion of immunodepleted plasma proteins (Sections 2.2.8.2 and 2.2.6 respectively) from a total of 36 acute and follow-up TIA, mimic, and healthy control volunteer samples were used for multiple reaction monitoring (MRM). Skyline 2.1 was used for method development (MacLean et al., 2010), and proteotypic peptides were checked for homogeneity using a protein BLAST search, with preference given to proteotypic peptides containing a precursor charge of +2 that did not contain cysteine residue that could otherwise interfere with mass spectral analysis. Multiple reaction monitoring was performed on a TripleTOF® 5600+ mass spectrometry system (AB Sciex) coupled to an Eksigent nanoLC 400 system (AB Sciex) fitted with a C18 spray emitter column (5 μm, 0.75 x 150 mm, Nikkyo Technos) for peptide enrichment, and peptide separation on a 3 μm, 0.3 x 10 mm ProteoCol® Polar analytical column (SGE

Analytical Science, Victoria, Australia) operating at a flow rate of 300 nL per minute, with peptides eluted using a 40 minute acetonitrile gradient from 5% to 40% followed by a step to 95% for 5 minutes. This instrument was operated in positive ion mode, using high sensitivity mode, an accumulation time of 0.1 seconds and spray voltage of 2500 V. Multiple reaction monitoring quantification was performed with Multiquant™ 2.1 software (AB Sciex) using five peptides per protein and the top 3-5 transitions per peptide (defined as transitions with the most abundant signals) selected for quantification (see Appendix Tables 11-16 for MRM data). Protein abundance was compared between acute and follow-up samples within each diagnostic group using paired t-test and inter-group comparisons performed using an unpaired t-test, where $p \leq 0.05$ was accepted as significant (Graph Pad Prism 6.05).

2.2.9 'Omic' technologies and biomarkers discovery

Biomarker discovery represents a rapidly growing field of research exploring potential links between genes (genomic), mRNA (transcriptomic), protein (proteomic) and/or metabolites (metabolomic) markers with a biological disease or condition (Kell, 2007). Unlike traditional hypothesis-driven experimental study designs, 'omic' investigations represent a holistic systems biology approach to understanding complex systems and the dynamic biochemical pathways associated with disease aetiology. In addition, 'omic' technologies are uniquely positioned to aid our understanding of disease-associated biomarkers, with significant clinical potential to screen, diagnose and prognosticate a patients' risk of disease/s. Recent innovations in 'omic' technologies have generated platforms that allow exploration of multiple analytes simultaneously with sufficient sensitivities, resolution, and dynamic range to detect and quantify entire genomic, proteomic and metabolomic material in complex biological samples in a non-targeted and non-biased workflow (Kell, 2007; Westerhoff & Palsson, 2004). This non-targeted 'holistic' experimental approach generates significantly large amounts of data to be used in hypothesis-generation for future validation and targeted explorative studies.

Furthermore, 'omic' technologies are fast becoming an integral part of the drug discovery process as well as in the assessment of drug safety and efficacy.

Pharmacogenomics explores the overlap between individual genomic variation and drug responses, and represents one of the emerging areas of personalised medical care (Evans & Relling, 2004). The holistic systems biology approach utilising 'omic' technologies gives to opportunity for a patient to provide their biological information to allow for monitoring responses to therapies, identifying optimal therapeutic dosages, tailoring pharmaceutical treatments, and screening for potential side effects (Kell, 2006).

Use of the systems biology approach to experimental design has had very limited use within the field of TIA, whilst, stroke has attracted greater representation of 'omic' research literature. The aim of this review is to provide an overview of the latest literature identifying candidate blood biomarkers associated with TIA and stroke in human studies.

Translation of biomarker research from the bench to the bedside is dependent on several established requirements in the biomarker discovery pipeline. Firstly, candidate biomarkers require identification and validation by suitable quantitative platforms. For genomic biomarker exploration, technologies such as microarrays, or more recently, next-generation sequencing (NGS) are accepted as appropriate platforms to measure differences in DNA sequences, gene expression and single nucleotide polymorphisms (Fertig, Slebos, & Chung, 2012). For proteomic biomarker discovery, two-dimensional protein separation using differential in-gel electrophoresis (DIGE) allows for unbiased quantitative analysis of thousands of proteins across different biological samples, whilst mass spectrometry-based technologies separate protein peptides according to their mass-to-charge ratio (*m/z*) which is used to create mass spectra that is used to identify proteins based on their molecular mass and structure. Mass spectrometry is the most sensitive and accurate method for detecting, identifying, and quantifying peptide molecules in complex biological samples.

2.2.10 Summary

With regards to the aims of this dissertation, genomic microarray and sequencing platforms are best suited to prognostic screening investigations of potential links between TIA/stroke risk and an individual's genetic determination. Exploration of candidate biomarkers intrinsically linked with the aetiology of TIA patients and that can also differentially diagnose acutely presenting TIA from mimic conditions is best explored using proteomics. The proteome represents all expressed proteins in the human body (over 100,000 proteins) and functions as the interface between genes and the environment. The proteome is dynamic and highly responsive to disease states making it best suited to candidate biomarker exploration in patients with acute transient symptom presentations.

3 IDENTIFICATION OF NOVEL BIOMARKERS FOR DIAGNOSING TRANSIENT ISCHAEMIC ATTACK AND DISTINGUISHING FROM MIMIC CONDITIONS – A HUMAN PROTEOMIC PILOT STUDY.

Original Contribution for Submission to:

Clinical Chemistry

Authors: Michael Djukic[1], Elaine S. Leung[1], Timothy K. Chataway[2], Alex Colella[2], Jim Jannes[1,3], Galina Gramotnev[4], Stephen J. Nicholls[5], Martin D. Lewis[1,6], Monica A. Hamilton-Bruce[1,3*], Simon A. Koblar[1,3*].

In keeping with the style of this thesis to maintain continuity, section headers, figures and tables have been re-numbered and the references have been incorporated into the thesis' reference list. The methodology for HPLC linear ion trap mass spectrometry and label-free quantitation strategy has been replicated as initially described in Chapter 2.

3.1 Statement of Authorship

Journal of Submission: *Clinical Chemistry*

Title of paper:	**Identification of Novel Biomarkers for Diagnosing Transient Ischaemic Attack and Distinguishing from Mimic Conditions - A Human Proteomic Pilot Study**
Publication status:	Unpublished and Unsubmitted work written in a manuscript style.
Publication details:	**Djukic M**, Leung ES, Chataway TK, Colella A, Jannes J, Gramotnev G, Nicholls S, Lewis MD, Hamilton-Bruce MA, Koblar SA. Identification of Novel Biomarkers for Diagnosing Transient Ischaemic Attack and Distinguishing from Mimic Conditions - A Human Proteomic Pilot Study

Author contributions

By signing the Statement of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

Name of Principal Author (PhD Candidate)	**Michael Djukic**		
Contribution to the Paper	Conceptualisation of the work, realisation of project design, documented experimental method, performed analysis of all samples, interpreted and evaluated data and wrote the manuscript.		
Overall percentage (%)	80%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	

Name of Co-Author	**Elaine S. Leung**	
Contribution to the Paper	Acquisition of the data and evaluation, and evaluate and edit manuscript.	
Signature	Date	03.09.2016

Name of Co-Author	**Timothy K. Chataway**		
Contribution to the Paper	Assisted with realisation of project design, supervised development of work, assisted with evaluation and interpretation of data, and editing of manuscript.		
Signature		Date	17.12.2016

Name of Co-Author	**Alex Colella**		
Contribution to the Paper	Assisted with data evaluation and editing of manuscript.		
Signature		Date	03.09.2016

Name of Co-Author	**Jim Jannes**		
Contribution to the Paper	Assisted with the realisation of project design, participated in evaluating and editing of manuscript.		
Signature		Date	27.08.2016

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Contribution to the Paper	Assisted with statistical analysis and data interpretation of all samples.		
Signature		Date	26.08.2016

Name of Co-Author	**Steve J. Nicholls**		
Contribution to the Paper	Assisted with evaluation and editing of manuscript.		
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Contribution to the Paper	Assisted with realisation of project design, supervised development of work, assisted with data evaluation and editing manuscript.		
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Name of Co-Senior Author	**Monica A. Hamilton-Bruce**	
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Signature	Date	24.11.2016

Name of Co-Senior Author	**Simon A. Koblar**		
Contribution to the Paper	Conceptualisation of work, realization of project design, supervised the development of work, involved in data evaluation and interpretation, contributed to evaluating and editing of manuscript, act as corresponding author.		
Signature		Date	25.11.2016

3.2 Abstract

Introduction – Transient Ischaemic Attack (TIA) is a warning sign for an imminent ischaemic stroke. Correctly distinguishing TIA from mimic conditions such as atypical migraine or focal seizures is clinically problematic. This study explored the human plasma proteome for differentially abundant plasma protein biomarkers associated with TIA diagnosis.

Methods – Acute and 90-day follow-up plasma protein samples from six well-defined TIA patients, six mimic patients and six healthy control volunteers were chromatographically depleted of abundant plasma proteins and analysed using two-dimensional difference in-gel electrophoresis (2D-DIGE). Mass spectrometry (MS) and label-free multiple reaction monitoring-MS (MRM-MS) were used to identify and quantify significant differentially abundant plasma proteins.

Results – Six plasma proteins were significantly different in abundance between TIA, mimic and healthy control groups. Apolipoprotein A-IV (APOA-IV) was exclusively elevated in TIA patients (1.61-fold, $p < 0.05$). Fibrinogen alpha-chain (FGA), Fibrinogen beta-chain (FGB) and Complement C4-A (C4A) were each elevated in mimic patients (1.22-fold, 1.17-fold, and 1.19-fold respectively, $p < 0.05$), while Apolipoprotein A-I (APOA-I) and both Gelsolin isoforms 1 and 2 (GS) were each elevated in healthy control volunteers (1.35-fold, 1.14-fold, and 1.21-fold respectively, $p < 0.05$). Apolipoprotein A-I was confirmed by MRM to be exclusively decreased in acute TIA samples (0.68-fold, $p < 0.05$), while APOA-IV was exclusively increased in follow-up TIA samples (1.80-fold, $p < 0.01$).

Conclusion – This is the first proteomic investigation to identify Apolipoprotein A-I and A-IV as differentially abundant plasma proteins associated with TIA when compared with patients presenting with mimic conditions or healthy controls. Validation in larger cohorts is needed to establish the diagnostic and prognostic utility of these candidate TIA biomarkers.

Keywords– Transient Ischaemic Attack, Plasma, 2D-DIGE, MRM-MS, Apolipoprotein, Biomarker.

3.3 Introduction

Between 15-26% of ischaemic strokes are preceded by a transient ischaemic attack (TIA) (Easton, 2011; Easton et al., 2009; Rothwell & Warlow, 2005). Evidence supports early assessment and management of TIAs, with a reported 80% risk reduction in subsequent stroke (Rothwell et al., 2007). This is a significant reduction considering the risk of stroke is 10-20% in the 90-days post-TIA onset (B. L. Cucchiara et al., 2006). A true TIA diagnosis is accepted as a medical emergency, as up to 42% of ischaemic strokes that occur within 30 days post-TIA do so within the first 24 hours (Chandratheva et al., 2009), and up to 50% within the first 48 hours (Johnston et al., 2000). This provides a very limited time-frame to both diagnose TIA and prevent an iminent stroke. Accurate diagnosis and subsequent treatment of TIA is however made difficult by mimic presentations such as atypical migraine or focal seizures (Nadarajan et al., 2014). Diagnostic uncertainty increases the difficulty of advising patients about treatments which carry potential risks, including anticoagulation therapy, thrombolysis or surgery (Jauch et al., 2013; Jensen et al., 2008). Although brain and cerebrovascular imaging using diffusion weighted magnetic resonance imaging (DWI-MRI) can diagnose ischaemic stroke with high sensitivity and specificity, or in the case of TIA, rule out the presence of an ischaemic lesion (Easton et al., 2009; Simonsen et al., 2015), these imaging modalities are time-consuming, costly, not freely accessible or available in smaller hospitals, community healthcare settings or regional areas (Burke et al., 2012; Jensen et al., 2008). An alternative test to accurately diagnose and distinguish TIA from mimic conditions is needed urgently. Blood plasma biomarkers may provide a solution for accurate, rapid and cost-effective testing.

Excluding the presence of infarction, the pathophysiology of both TIA and ischaemic stroke is underpinned by reduced cerebral blood flow causing focal neurological symptoms (Easton et al., 2009; Nadarajan et al., 2014). Promising brain-derived proteins such as S-100b (Brouns et al., 2010; Yasuda et al., 2004), brain natriuretic peptide (Kim, Kang, Kim, & Lee, 2010; Laskowitz et al., 2009), PARK7 (Allard et al., 2005) and nucleoside diphosphate kinase A (Allard et al., 2005) have been identified as potential ischaemic stroke biomarkers of diagnostic and prognostic utility, and may therefore provide the best sensitivity for diagnosing TIA and distinguishing TIA from

mimic conditions. However, the absence of cerebral infarction and lack of significant disruption to the blood brain barrier in TIA (Easton et al., 2009) means that brain-derived biomarkers in cerebrospinal fluid may not easily enter the blood stream (Brouns et al., 2010). This may delay an acute plasma concentration spike and limit their usefulness as biomarkers for rapid TIA diagnosis.

Pathological substrates that contribute to arterial occlusion may provide an althernative source TIA biomarker. Active atherosclerotic plaques at high risk of rupture and thrombus formation have been proposed as candidate TIA biomarkers for stroke risk prediction (Jensen et al., 2008). Biomarkers of atherosclerosis that have shown utility in prognosticating ischaemic stroke recurrence following TIA include high-sensitivity C-reactive protein (Arenillas et al., 2003; Di Napoli et al., 2005), and lipoprotein-associated phospholipase A2 (Delgado et al., 2012). Elevated markers of coagulation including D-dimer (Vrethem & Lindahl, 2009), fibrinogen and antithrombin-III (Meng et al., 2011) have also been implicated in TIA and ischaemic stroke aetiology and can predict a higher risk of stroke recurrance. Such biomarkers may reliably distinguish mimics from true TIA diagnoses, therefore serving as a diagnostic tool by distinguishing low-risk mimic patients from true TIA patients at high-risk of a future ischaemic stroke.

Proteomic profiling methods have identified many more promising biomarkers associated with ischaemic stroke (as reviewed in (Laborde et al., 2012)), and may provide a suitable platform for discovery of novel TIA-specific biomarkers (Ning, Lopez, Cao, Buonanno, & Lo, 2012). Two dimensional differential in-gel electrophoresis (2D-DIGE) is an accurate method for relative quantitation of human plasma proteins, enabling multiplexing within the same gel by normalisation of fluorescently labelled samples to a pooled internal reference sample, thus allowing within- and between-gel comparisons while reducing assay variability (Viswanathan, Unlu, & Minden, 2006). Use of targeted proteomic approaches involving quantitative mass spectrometry (MS) methods have recently gained popularity in the measurement of predetermined proteins discovered from previous proteomic experiments. Use of stable isotope labelling by amino acids in cell culture (SILAC) (Ong et al., 2002), isobaric tagging technology for relative and absolute quantitaition (iTRAQ) (Ross et al., 2004), or LC-MS/MS label-free multiple reaction monitoring (MRM-MS) (Megger,

Bracht, Meyer, & Sitek, 2013) can provide high accuracy and reproducibility for selected proteotryptic peptide quantification. Label-free MRM-MS quantification utilises ion intensities from extracted ion chromatograms to provide high quality measurement of protein abundance whilst eliminating the need and cost for synthesising isobaric tags or stable isotope standards for the monitoring of each transition (Chung, Colangelo, & Zhao, 2014; Zhang et al., 2011).

The objective of this discovery-phase pilot study was to compare the human plasma proteomes of TIA, mimic and healthy control volunteers, and identify differentially abundant proteins associated with a TIA diagnosis. We aimed to identify candidate proteins by comparing acute/initial with 90-day follow-up samples within each diagnostic group, as well as comparing between diagnoses using 2D-DIGE and mass spectrometry. Secondly, we aimed to measure candidate protein abundance using the quantitative label-free MRM-MS technique as a means to validate our proteomic detection method and support the establishment of an *a priori* hypothesis for a follow-up, prospective, multicentre validation study.

3.4 Methods

3.4.1 TIA Definition

Our study used the revised TIA definition endorsed by the American Heart and Stroke Associations defining TIA as: “a transient episode of neurological dysfunction caused by focal brain, spinal cord, or retinal ischaemia, without acute infarction” (Easton et al., 2009). Patients suspected of TIA were diagnosed by neurologists following clinical assessment, diffusion weighted imaging - magnetic resonance imaging (DWI-MRI) and/or computed tomography (CT) imaging to exclude the possibility of cerebral infarction and haemorrhage.

3.4.2 Study Design

To profile the plasma proteome of each participant, this study used a paired-sample design. Plasma was taken from six patients with TIA and six patients with TIA-mimicing conditions that presented within 10 days of symptom onset and compared this with a follow-up recovery sample taken 90 days later. Six healthy controls were also recruited for proteomic comparison of plasma collected initially and again at a 90 day follow-up. Clinical data, including the ABCD2 stroke-risk stratification score, lipid, and biochemical results, was collected from all 18 participants (Table 7-8), with MRI and CT scanning of the brain performed on TIA and mimic patients to confirm diagnosis. One of the six TIA patients declined to undergo an MRI scan due to claustrophobia. Written informed consent was obtained from all participants and The Queen Elizabeth Hospital Ethics Committee approved this study (HREC Approval Numbers 2009123 and 2009203).

Table 7: Demographics and clinical characteristics of TIA, Mimic and Healthy Control (HC) participants. *All quantitative data represented as mean ± standard deviation, and reflect acute/initial presentation unless otherwise stated. Clinics denoted as: COMBAT = community based rapid access TIA clinic, RAC = hospital based rapid assessment clinic. ABCD2 score = 7-point risk-stratification tool to identify patients at high risk of stroke following TIA, where scores of 1-3 = low risk, 4-5 = moderate risk, and 6-7 = high risk. Blood biochemistry tests are denoted as: TC = total cholesterol, Trig = triglycerides, HDL-C = high density lipoprotein – cholesterol, LDL-C = low density lipoprotein – cholesterol, '*' indicates significant difference by unpaired t-test when $p \leq 0.05$.*

Clinical Variables	TIA (n=6)	Mimic (n=6)	HC (n=6)	TIA/Mimic *p*-value	TIA/HC *p*-value
Age (years):	77.33 ± 11.38	68.33 ± 13.03	55.00 ± 9.01	0.23	0.0036*
Gender (male/female):	2/4	1/5	2/4	0.55	1.00
Clinic Attended (COMBAT/RAC):	3/3	3/3	-	1.00	-
Time from symptom onset to blood collection (days):	2.83 ± 1.94	4.33 ± 3.67	-	0.40	-
Cardiovascular Risk Factors					
Hypertension:	6/6	5/6	-	0.34	-
Ischaemic Heart Disease:	2/6	0/6	-	0.14	-
Hyperlipidemia:	2/6	2/6	-	1.00	-
Type 2 Diabetes Mellitus:	2/6	1/6	-	0.54	-
Smoking:	0/6	0/6	-	-	-
ABCD2 Score:	4.33 ± 1.37	-	-	-	-
Biochemistry Tests					
TC (mmol/L):	4.13 ± 0.63	4.83 ± 0.97	4.82 ± 0.86	0.17	0.15
Trig (mmol/L):	1.47 ± 0.86	1.27 ± 0.56	0.73 ± 0.22	0.64	0.07
HDL-C (mmol/L):	1.10 ± 0.17	1.50 ± 0.38	1.63 ± 0.26	0.04*	0.0017*
LDL-C (mmol/L):	2.35 ± 0.99	2.85 ± 0.96	2.83 ± 0.69	0.40	0.35
TC:HDL-C:	3.84 ± 0.79	3.42 ± 1.16	2.93 ± 0.26	0.47	0.02*
Glucose (mmol/L):	7.13 ± 2.45	6.27 ± 1.07	4.8 ± 0.46	0.45	0.04*

Table 8: Biochemistry and cardiovascular medication status of TIA, Mimic and Healthy Control (HC) participants. *All quantitative data represented as mean ± standard deviation. Ta = Acute TIA, Tf = Follow-up TIA, Ma = Acute Mimic, Mf = Follow-up Mimic, Hi = Initial Healthy Control, Hf = Follow-up Healthy Control. APOA-I = apolipoprotein A1, APOB = apolipoprotein B, hsCRP = high-sensitivity C-reactive protein.*

Clinical Variables	TIA (n=6)		Mimic (n=6)		HC (n=6)		TIA/Mimic *p*-value	TIA/HC *p*-value
	Acute	Follow-up	Acute	Follow-up	Initial	Follow-up	T*a*/M*a*/H*i*	T*f*/M*f*/H*f*
APOA-I (mmol/L):	1.21 ± 0.16	1.21 ± 0.15	1.42 ± 0.27	1.39 ± 0.17	1.39 ± 0.14	1.43 ± 0.23	0.16	0.14
APOB (mmol/L):	0.78 ± 0.15	0.68 ± 0.18	0.80 ± 0.21	0.78 ± 0.21	0.73 ± 0.11	0.77 ± 0.16	0.74	0.58
APOB:APOA-I:	0.67 ±0.18	0.57 ± 0.17	0.59 ± 0.23	0.58 ± 0.21	0.52 ± 0.08	0.55 ± 0.11	0.40	0.94
hsCRP (mg/L)	4.03 ± 3.93	3.31 ± 1.75	2.26 ± 1.98	2.06 ± 1.2	1.51 ± 0.93	2.14 ± 1.59	0.27	0.34
Cardiovascular Meds								
							T*a*/M*a*	T*f*/M*f*
Antiplatelet Therapy:	1/6	6/6	4/6	4/6	-	-	0.09	0.14
Statin:	3/6	5/6	4/6	4/6	-	-	0.60	0.55
Isosorbide Mononitrate:	1/6	1/6	0/6	0/6	-	-	0.34	0.34
ACE-Inhibitor:	3/6	3/6	1/6	1/6	-	-	0.26	0.26
Calcium Channel Blocker:	2/6	2/6	2/6	2/6	-	-	1.00	1.00
Metformin:	2/6	2/6	1/6	1/6	-	-	0.55	0.55
Glyceryl Trinitrate:	2/6	2/6	1/6	1/6	-	-	0.55	0.55
AngII receptor blocker:	2/6	2/6	3/6	3/6	-	-	0.60	0.60
Beta-1 Receptor Blocker:	1/6	1/6	2/6	2/6	-	-	0.55	0.55
Diamicron:	1/6	1/6	0/6	0/6	-	-	0.34	0.34

3.4.3 Patient Recruitment and Blood Collection

Patients with suspected TIA were assessed at either the Rapid Access Clinic at The Queen Elizabeth Hospital (RAC-TQEH) or the COMmunity-Based rapid Access TIA (COMBAT) clinic and invited to participate in this study. Each consenting patient provided two blood samples: an initial sample within 10 days of symptomatic ischaemia onset and a second sample within a 90 day follow-up period. Blood samples were also obtained from six healthy control volunteers over the age of 40 years that were recruited

from the wider Adelaide Metropolitan Community, without having any diagnosed co-morbidities and not taking prescription medications at the time of assessment.

3.4.4 Inclusion and Exclusion Criteria

Patients admitted to the RAC-TQEH or the COMBAT clinic between February 2010 and January 2011 with either a TIA (Appendix Table 3) or mimic condition were invited to participate in this study (Appendix Table 4). Participating mimic patients were diagnosed as suffering either migraine, epileptic seizure, benign positional vertigo, vasovagal syncope or transient global amnesia. Six patients from each of the two diagnosed groups (TIAs and mimics) were selected based on diagnostic certainty as determined by a GP with a special interest in stroke (ESL) and two neurologists (SAK, JJ). Mimic patients were excluded if evidence of a cerebrovascular aetiology for their ischaemia-mimicking symptoms was established. Control volunteers recruited for this study were deemed healthy by a physician's (ESL) clinical assessment, which included blood pressure, blood glucose, lipid and biochemistry measurements (Appendix Table 5), and found to have no evidence of cerebrovascular risk factors, no history of cerebrovascular events, and not presently taking any prescription medications. Haemolytic blood samples from TIA, mimic and control volunteers were excluded from proteomic investigation.

3.4.5 Sample Preparation and Depletion of Abundant Proteins

Peripheral blood samples (8.5 mL) were collected in sterile BD vacutainers® containing 1.8 mg K_2EDTA per millimeter of blood (Becton, Dickinson and Company, NJ, USA). All samples were centrifuged within 30 min of collection, and plasma was extracted and aliquoted into vials containing Halt® protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific, Illinois, USA) and stored in liquid nitrogen at -195^{o}C for later analysis.

Thawed plasma was centrifuged (5 minutes, 12,000 x g), diluted 1:4 with depletion buffer (Agilent Technologies, Santa Clara, USA), filtered by centrifugation (1 minute at 12000 x g) using 0.22 µm spin filters (Agilent Technologies) and depleted of the top six most abundant proteins using a 4.6 x 100 mm Human 6-Multiple Affinity Removal System (MARS-6, Agilent Technologies) column connected to a P-920 UPC-900 AKTA-Fast Protein Liquid Chromatography (FPLC) system (GE Healthcare, NJ, USA). The MARS-6 column (containing antibodies against albumin, transferrin, haptoglobin, immunoglobulin G, immunoglobulin A and alpha-1 antitrypsin) depleted plasma and provided a flow-through fraction devoid of the six most abundant plasma proteins, enriching lower abundant proteins and subsequently enhancing the resolution of electrophoretic separation. Five kilodalton molecular weight cut-off spin concentrators (Agilent Technologies) were used to desalinate and concentrate flow-through fractions (3000 x g), before samples were frozen and stored in liquid nitrogen. Protein concentration of depleted plasma was estimated in triplicate using an EZQ® protein quantification kit (Invitrogen, Oregon, USA) prior to CyDye fluorescent labelling.

3.4.6 Two-Dimensional DIGE Analysis and Statistics

Paired depleted plasma samples depicting acute and follow-up plasma proteomes from each of the six TIA, mimic and healthy control participants were alternately labelled with either Cy3 or Cy5 dye DIGE Fluors according to the manufacturer's protocol (GE Healthcare, Buckinghamshire, UK). Briefly, 50 µg of depleted plasma from each patient (acute and follow-up) was minimally labelled with 200 pmol of either Cy3 or Cy5 fluorochromes, whilst an internal standard was prepared by pooling 50 µg of protein from every sample and minimally labelling it with 200 pmol Cy2 DIGE Fluor. Labelling steps were performed on ice and in darkness before the reactions were stopped by adding 1 mM lysine.

Acute and follow-up samples from each of the 18 participants labeled with Cy3 and Cy5 were combined with a Cy2 labelled pooled internal standard, and diluted in rehydration buffer (7 M Urea, 2 M Thiourea, 4% CHAPS, 1% IPG Buffer, 13 mM DTT, and trace Bromophenol blue) to a final volume of 450 µL. Prior to first dimensional

isoelectric focusing, 18 x 24 cm pH 3-11 non-linear IPG strips (GE Healthcare) were re-hydrated overnight at 50 volts. Labelled samples were then added to IPG strips and isoelectric focusing was continued for approximately 70,000 total volt hours. Focused IPG strips underwent two-step equilibration including reduction and alkylation, and transferred onto 12.5% 2D SDS-polyacrylamide gels, overlayed with 1% low melting agarose and underwent SDS-PAGE at 350 volts for second dimension electrophoretic separation by molecular weight.

Eighteen gels were scanned using the Typhoon Trio variable mode imager (GE Healthcare) with a resolution of 200µm, and photomultiplier tube setting of 550V, 500V and 470V for Cy2, Cy3 and Cy5 labeled samples, respectively. Data was analysed using DeCyder^{TM} v7.0 Batch Processor software (GE Healthcare). Prior to analysis, spot filtering parameters were applied to exclude non-protein spot artefacts (i.e. slope < 1.0 and spot volume < 200,000). Candidate protein spots were assumed to be significant if present in at least 80% of the spot maps, and one-way ANOVA of standardised spot abudance were significant between groups ($p < 0.05$). The Biological Variation Analysis (BVA) module of the DeCyder^{TM} v7.0 software (GE Healthcare) applied paired t-tests for intra-cohort comparison between intial and follow-up samples, and one-way analysis of variance (one-way ANOVA) for inter-cohort comparison between the three cohorts. Significance was established when average protein spot abundance displayed a ± 1.10-fold change ($p < 0.05$) compared with the pooled internal standard. Principal component analysis was used to identify protein patterns and group samples based on relevant biological patterns.

3.4.7 Power Analysis

Establishing the random variation between samples on different gels and the extent of differential abundance or fold-change is required for sample size and power calculations. However, these variables are yet to be established in a TIA patient plasma cohort and have instead been based on power calculations from previous human plasma 2D-gel electrophoresis studies (Horgan, 2007). Assuming random biological variation of 10% between patients, to detect a 10% or ± 1.1-fold change in protein abundance, 17

participants are required to achieve a power of 80% with $p < 0.05$. We recruited 18 participants who each provided two biological replicates: initial and follow-up plasma samples.

3.4.8 Tryptic Digestion of 2D SDS-PAGE spots

Proteins of interests were visualised by mass spectrometry-compatible Eriochrome black T (EBT) silver staining of a preparative 2D-gel containing 450 ug depleted plasma, and manually excised using a depressor pipette. In-gel trypsin digestion was performed as per Schevchenko *et al.*, which briefly involved a three-step washing of excised protein spots with 100 mM ammonium bicarbonate followed by reduction (50 mM dithiothreitol), alkylation (100 mM iodoacetamide), acetonitrile dehydration and digestion overnight at 37^{o}C with 20 µL of 20 µg/ml trypsin (diluted 1:50 with 100 mM ammonium bicarbonate) (Shevchenko, Wilm, Vorm, & Mann, 1996).

3.4.9 HPLC Linear Ion Trap Mass Spectrometry

Trypsin digested peptides from gel spots were analyzed using a Thermo Orbitrap XL linear ion trap mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA), with an attached nanospray source (Thermo Electron Corporation, San Jose, CA, USA). Each sample was applied to a 300 µm inner diameter x 5 mm length C18 PepMap™ 100 precolumn (Dionex Corporation, Sunnyvale, CA, USA) at a 20 µL/minute flow rate for 3 minutes, and separated on a 75 µm x 150 mm C18 PepMap 100 column (Nikkyo Technos, Tokyo, Japan) using a Dionex Ultimate 3000 HPLC (Dionex) with a 55 minute gradient from 2-45% acetonitrile containing 0.1% formic acid at a flow rate of 200 nL/minute followed by a step to 77% acetonitrile for nine minutes. The mass spectrometer was operated in positive ion mode with one full scan of mass/charge 300 – 2000 at a resolution of 60,000 (FWHM). This was followed by product ion scans of the six most intense ions, with the following parameters: dynamic exclusion of 30 seconds, exclusion of 10 ppm low and high mass width relative to the reference mass, an

exclusion list size of 500, and collision induced dissociation (CID) energy of 35%. Only multiple charged ions were selected for MS/MS of trypsin-digested peptides.

Mass spectrometry spectra were searched with Thermo Scientific™ Proteome Discoverer™ 1.2 (Thermo Fisher Scientific) using the SEQUEST algorithm against the Swiss-Prot Human Uniprot database (May 2012 version) (Boutet et al., 2007) (representing 20,244 protein entries) using trypsin specificity (KR) as the digesting protease, allowing for a maximum of two missed cleavages. Variable modifications were set for phosphorylated serine, threonine, or tyrosine (179.966 Da; (STY)), oxidated methionine (115.995 Da; (M)), acetylated N-termini or lysine (142.011 Da; (N-termini, K)), glutathionylation, and using the following filters: 1) the cross-correlation scores of matches were greater than 1.5, 2.0 and 2.5 for the charged states of 1, 2 and 3 peptide ions respectively, 2) peptide probability was greater than 0.001 and 3) each protein identified had at least two different peptides sequenced. The mass tolerance for peptide identification of precursor and product ions was 1 Da and 0.5 Da respectively.

3.4.10 Label-Free Quantitation Strategy

Multiple reaction monitoring (MRM) assays were developed to quantify and validate six plasma proteins, with proteotypic peptides for each protein selected from the discovery 2D DIGE/LC-MS/MS experiments (Appendix Table 6-10). In-solution trypsin-digestion of immunodepleted plasma protein from a total of 36 acute and follow-up TIA, mimic and healthy control volunteer samples was used for multiple reaction monitoring (MRM). Skyline 2.1 was used for method development (MacLean et al., 2010), and proteotypic peptides were checked for homogeneity using a protein BLAST search, with preference given to proteotypic peptides containing a precursor charge of +2 that did not contain cysteine residue that could otherwise interfere with mass spectral analysis. Multiple reaction monitoring was performed on a TripleTOF® 5600+ mass spectrometry system (AB Sciex, Concord, Ontario, Canada) coupled to an Eksigent nanoLC 400 system (AB Sciex) fitted with a C18 spray emitter column (5 μm, 0.75 x 150 mm, Nikkyo Technos) for peptide enrichment, and peptide separation on a 3 μm, 0.3 x 10 mm ProteoCol® Polar analytical column (SGE Analytical Science,

Victoria, Australia) operating at a flow rate of 300 nL per minute, with peptides eluted using a 40 minute acetonitrile gradient from 5% to 40% followed by a step to 95% for 5 minutes. This instrument was operated in positive ion mode, using high sensitivity mode, an accumulation time of 0.1 seconds and spray voltage of 2500 V. Multiple reaction monitoring quantification was performed with Multiquant™ 2.1 software (AB Sciex) using 4-5 peptides per protein and the top 3-5 transitions per peptide (defined as transitions with the most abundant signals) selected for quantification. Protein abundance was compared between acute and follow-up samples within each diagnostic group using paired t-test and inter-group comparisons performed using an unpaired t-test, where $p \leq 0.05$ was accepted as significant (Graph Pad Prism 6.05).

3.5 Results

3.5.1 Patient Characteristics

Study participant demographics and clinical characteristics of TIA, mimic and healthy control groups are provided (Table 7 and Appendix Table 3-5). Apart from TIA patients being significantly older than healthy controls (77.33 ± 11.38 years vs. 55.00 ± 9.01 years, $p < 0.01$), the three groups were not differentiable by any demographic characteristic, while TIA and mimic groups were not differentiable by any cardiovascular risk factors or medication usage. Within the TIA group, fewer patients were receiving antiplatelet therapy (1/6 patients) and statins (3/6 patients) at acute presentation, however both classes of medications were mostly being taken at 90-day follow-up (6/6 and 5/6 patients receiving the medications respectively) (Table 8). Acute TIA patients presented with significantly lower levels of the protective high-density lipoprotein cholesterol when compared with both mimic and healthy controls (TIA = 1.10 ± 0.17 mmol/L vs. mimic = 1.50 ± 0.38 mmol/L and healthy controls = 1.63 ± 0.26, $p < 0.05$) (Table 8). Both the ratio of total cholesterol:HDL-C and mean blood glucose levels were significantly elevated in TIA patients compared with healthy controls (3.84 ± 0.79 vs. 2.93 ± 0.26, and 7.13 ± 2.45 mmol/L vs. 4.80 ± 0.46 mmol/L respectively, $p < 0.05$).

3.5.2 Two-Dimensional DIGE Proteomic Analysis

To minimise technical variation when comparing initial and follow-up plasma proteomes of individual patients, both samples were multiplexed and analysed on the same gel. A representative DIGE gel image (Figure 16) detected approximately 391 protein features following spot filtering. Protein spots were matched across 18 gels that represented the plasma proteome of the 18 patients and control volunteers (Appendix Figure 1). Seven protein spots that were found to be different in abundance (± 1.1-fold change with $p < 0.05$) were selected for further analysis (Table 9). Mass spectrometry successfully identified peptide sequences of seven candidate protein spots, which corresponded with known proteins in the Swiss-Prot Human UniProt database. Proteins identified with ≥ 2 unique peptides were considered to have sufficient coverage of amino acid sequence to provide conclusive identification. Log standardised abundance of Apolipoprotein A-IV was exclusively increased in TIA patients both at initial presentation and at 90-day follow-up when compared with identically sampled mimic patients and healthy controls (Figure 17). Complement C4-A, Fibrinogen alpha-chain and beta-chain proteins were significantly elevated in mimic patients, while both Apolipoprotein A-I, Gelsolin and Fibrinogen beta-chain was significantly elevated in healthy control volunteers (Figure 17). No statistically significant difference was found in any of the seven measured proteins when comparing their concentration between initial and follow-up samples from the same patient/volunteer.

Figure 16: Analysis of plasma proteome by 2D-DIGE. *A representative 2D-DIGE image showing the plasma protein profile of a TIA patient. Seven differentially abundant protein spots were detected by DeCyder™ analysis and identified by mass spectrometry. Labelled plasma proteins were separated in the first dimension across a pH range of 3-11 (isoelectric focusing) and in the second dimension by molecular weight (from 250-10 kDa).*

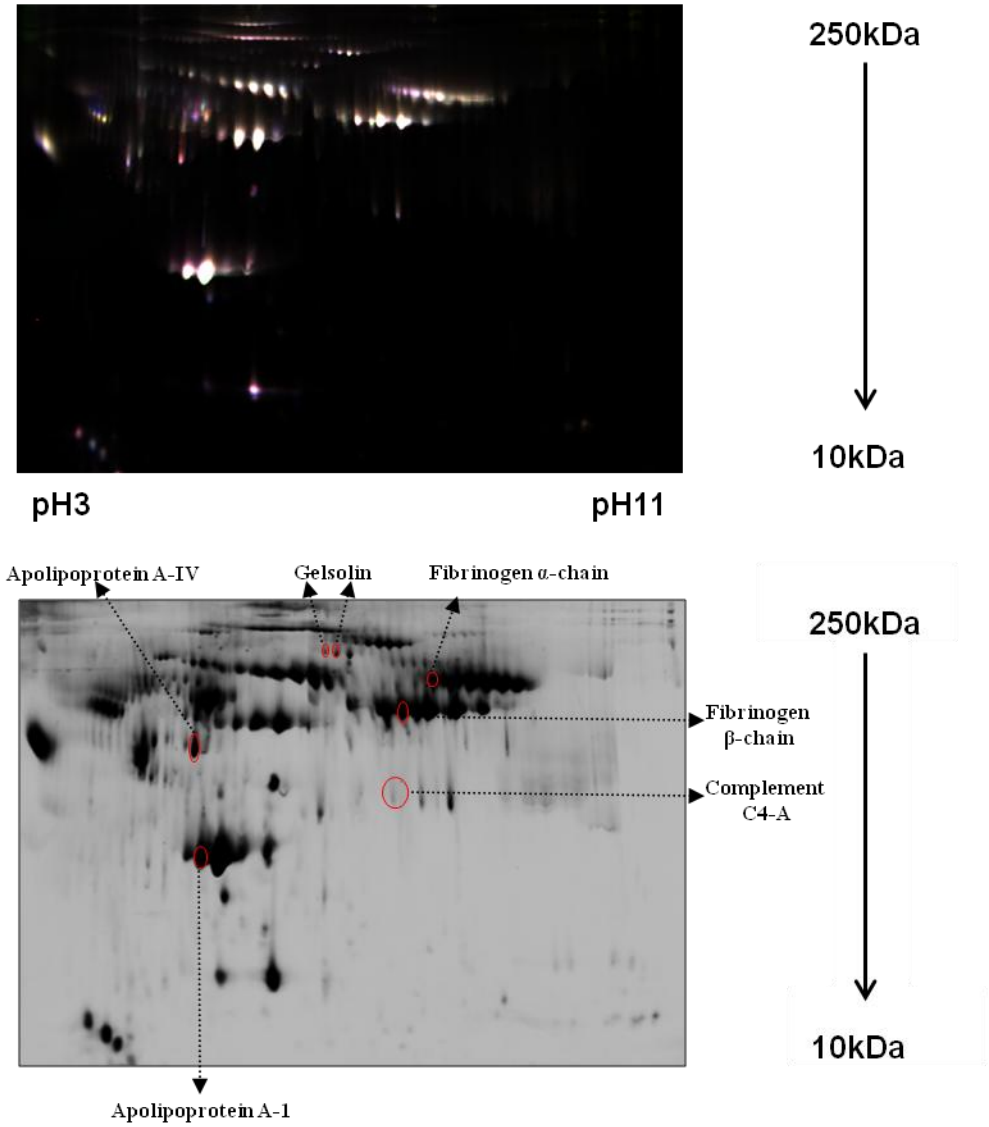
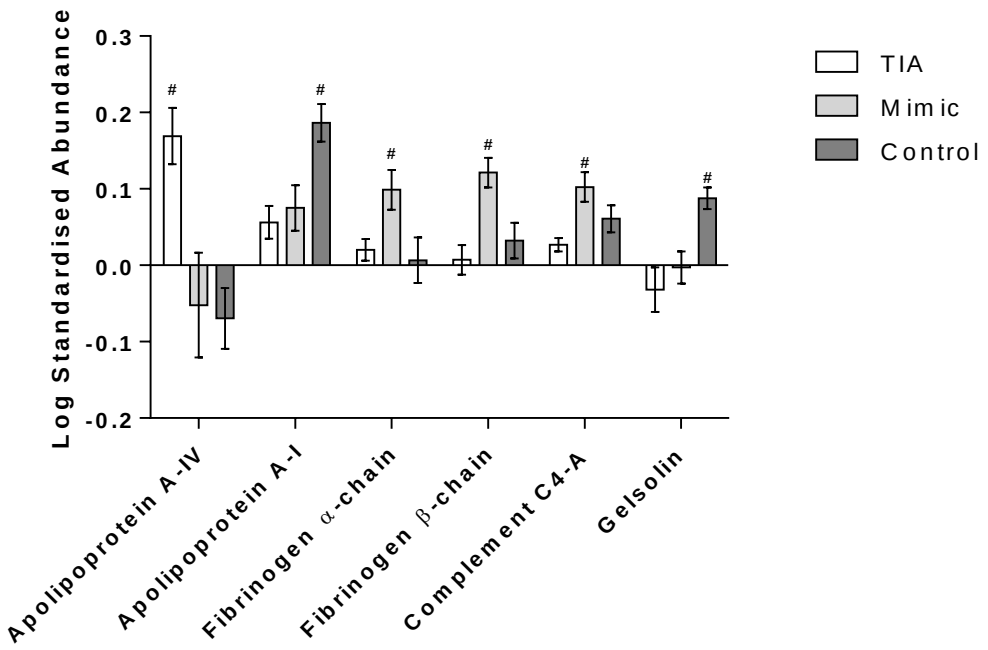


Table 9: Summary of differentially abundant plasma proteins identified by nanospray LTQ Orbitrap XL-MS/MS. *Fold Δ = significant fold change determined by increased cohort vs. pooled internal standard, where p<0.05, MW= molecular weight, kDa= kilodaltons, Calc. pI = calculated isoelectric point, TIA = transient ischaemic attack, M = mimic, HC = healthy control, '*' = non-unique peptide sequence for each Gelsolin isoform.*

Protein Identified	UniProtKB Accession Number	Unique Peptide matches	Fold Δ ± SD (p-value)	Increased cohort	% Sequence coverage MS/MS	Calc. pI/MW [kDa]	Biological Function	Unique Peptide Sequence
Apolipoprotein A-IV	P06727	22	1.61 ± 0.20 (0.02)	TIA	60.86	5.38/45.4	Lipid transport.	-ALVQQMEQLR -IDQTVEELR -ISASAEELR -LAPLAEDVR -SLAPYAQDTQEK -TQVNTQAEQLR
Apolipoprotein A-I	P02647	7	1.35 ± 0.10 (0.03)	HC	29.59	5.76/30.8	Anti-atherogenic.	-DLATVYVDVLK -DYVSQFEGSALGK -LLDNWDSVTSTFSK -LSPLGEEMR -THLAPYSDELR -VSFLSALEEYTK
Fibrinogen α- chain	P02671	8	1.22 ± 0.09 (0.04)	M	16.86	6.01/94.9	Blood coagulation.	-QFTSSTSYNR -TVIGPDGHK -VQHIQLLQK -GDFSSANNR -GDSTFESK -GGSTSYGTGSETESPR
Fibrinogen β- chain	P02675	16	1.17 ± 0.08 (0.03)	M	53.97	8.27/55.9	Blood coagulation.	-DNDGWLTSDPR -EDGGGWWYNR -LIQPDSSVKPYR -TMTIHNGMF -TPCTVSCNIPVVSGK
Complement C4-A	P0C0L4	5	1.19 ± 0.06 (0.03)	M	44.04	7.08/192.7	Immune response, inflammation, complement activation.	-ANSFLGEK -APVDLLGVAHNNLMAM -DHAVDLIQK -FGLLDEDGK
Gelsolin (Isoform 1)	P06396	10	1.14 ± 0.08 (0.03)	HC	22.89	6.28/85.6	Signalling, motility, apoptosis.	-GASQAGAPQGR -EVQGFESATFLGYFK* -GGVASGFK* -TGAQELLR*
Gelsolin (Isoform 2)	P06396-2	7	1.21 ± 0.10 (0.02)	HC	13.82	5.85/80.6	Signalling, motility, apoptosis.	-EVQGFESATFLGYFK* -GGVASGFK* -TGAQELLR*

Figure 17: 2D-DIGE data representing mean log standardised abundance values for six differentially abundant proteins. *Bars represent the mean log standardised abundance of combined acute and follow-up samples from six TIA, mimic and healthy control volunteers (n=18), with error bars representing the standard error of the means. Patient/volunteer samples representing ± 1.1-fold change in abundance ($p<0.05$) when compared with pooled internal standard are denoted by (#). The zero value for the log standardised abundance corresponds to the pooled internal standard.*

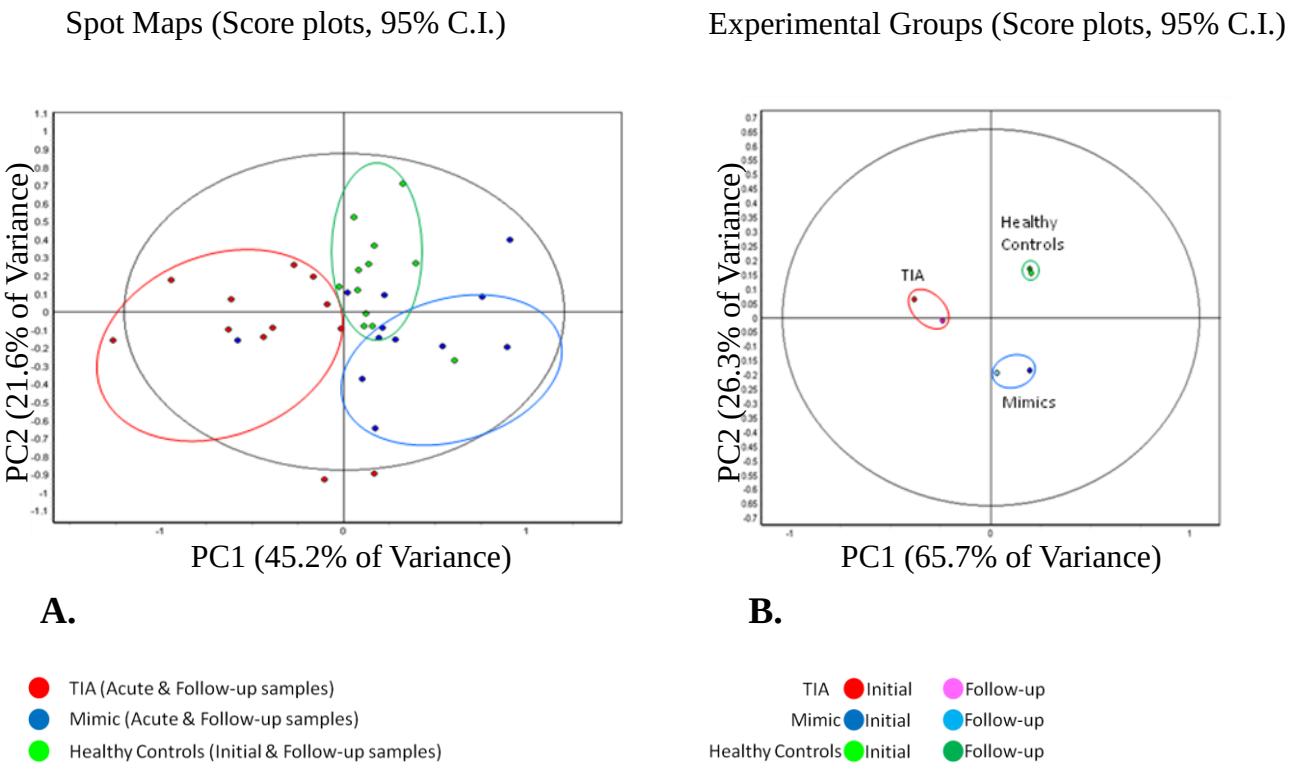


3.5.3 Principal Component Analysis

To reduce the complexity of multidimensional data sets, and to clearly highlight trends within data, principal component analysis was applied (Figure 18). The protein profiles of seven candidate protein spots were matched on > 80% of the spot maps, whereby the first principal component (PC1) represented 45.2% of the total variance in the data set, while the second principal component (PC2) displayed an additional 21.6% of the variance. We observed a distinct clustering of spot maps into three main regions according to the three diagnostic groups (Figure 18.A.). When examining initial and follow-up sample categories from each of the three experimental groups, a tighter cluster of samples from healthy control volunteers and to a lesser extent TIA and

mimics was observed (Figure 18.B.). This may indicate a small magnitude of experimental variability when analysing biological replicates (initial vs. follow-up) of the same human sample from a particular diagnostic group. The first principal component suggests that the diagnosis of TIAs, mimics, and healthy controls represents the largest form of variation in this study, and implies that the expression profile of seven differentially abundant proteins in this study might allow us to distinguish between TIA and non-TIA patients. Of the seven candidate protein spots, all were successfully identified by mass spectrometry, with gelsolin appearing twice with both isoforms.

Figure 18: Principal Component Analysis (PCA) of significant TIA, mimic and healthy control plasma proteins. *PCA of six candidate proteins identified in 2D-DIGE that are separated according to the two largest sources of variance in the data set (PC1 and PC2). Score plots of the first two principal components generated three distinct clusters that discriminate between TIA, mimic and healthy control groups. (A) Distribution of 36 individual spot maps with PC1 representing 45.2% of variance, PC2 representing 21/6% of variance, and cumulative variance of 66.8%. (B) Distribution of each of the three experimental groups with PC1 representing 65.7% of variance, PC2 representing 26.3% of variance, and cumulative variance of 92.0%. Ellipses surrounding related samples are displayed only to emphasise the group distribution in the plot.*



3.5.4 Multiple Reaction Monitoring (MRM) Quantification

Six candidate proteins – APOA-IV, APOA-I, Fibrinogen α-chain, Fibrinogen β-chain, Gelsolin, and Complement C4-A were quantified using MRM (Table 10). The peptide abundance profiles of APOA-IV, APOA-I and Gelsolin matched the expression profiles depicted in the previous 2D-DIGE work (Figure 19.), while Complement C4-A and Fibrinogen α and β-chains displayed greater peptide abundance in TIA samples

compared to previous over-representation in mimic samples analysed by 2D-DIGE. Apolipoprotein A-IV abundance was significantly higher in TIA compared to mimic groups (Figure 19), with an exclusive increase in follow-up TIA samples compared with their paired acute samples (1.74-fold increase, $p < 0.05$) (Figure 20). Apolipoprotein A-I abundance was significantly lower in acute TIA samples compared to paired follow-up TIA samples (0.71-fold, $p < 0.05$) and compared to both mimic and control groups (0.68-fold, $p < 0.05$) (Figure 20). Complement C4A, Fibrinogen α and β-chain abundance was not significantly different between groups, however, each displayed a trend favouring an increased abundance in acute TIA samples when compared to mimic and control groups (Appendix Figure 2). Gelsolin abundance was lower in acute TIA and mimic patients in comparison to the control group, however, this trend was not statistically significant either (Appendix Figure 2).

Table 10: Summary of differentially abundant candidate TIA plasma protein biomarkers quantified by multiple reaction monitoring (MRM). *TIA = transient ischaemic attack, '*' = significance following paired t-test, p<0.05, '#' = significance following unpaired t-test, p<0.05.*

Protein	Sub-group displaying unique abundance profile	Direction of abundance	Fold change (p-value) vs. paired sub-group	Fold change (p-value) vs. all other samples
Apolipoprotein A-IV	Follow-up TIA	Increased	1.74 (0.029) vs. Acute TIA*	1.80 (0.001)#
Apolipoprotein A-I	Acute TIA	Decreased	0.71 (0.029) vs. Follow-up TIA*	0.68 (0.038)#
Fibrinogen α-chain	Acute TIA	Increased	1.08 (0.294) vs. Follow-up TIA	1.06 (0.356)
Fibrinogen β-chain	Acute TIA	Increased	1.01 (0.457) vs. Follow-up TIA	1.18 (0.211)
Complement C4-A	Acute TIA	Increased	1.06 (0.293) vs. Follow-up TIA	1.21 (0.185)
Gelsolin	Acute TIA	Decreased	0.81 (0.062) vs. Follow-up TIA	0.87 (0.303)

Figure 19: Multiple reaction monitoring quantification of candidate TIA plasma protein biomarkers. *Statistically significant changes in MRM abundance profiles of Apolipoprotein A-IV and Apolipoprotein A-I within and between cohorts, with unpaired t-test significance denoted by '*' when $p \leq 0.05$.*

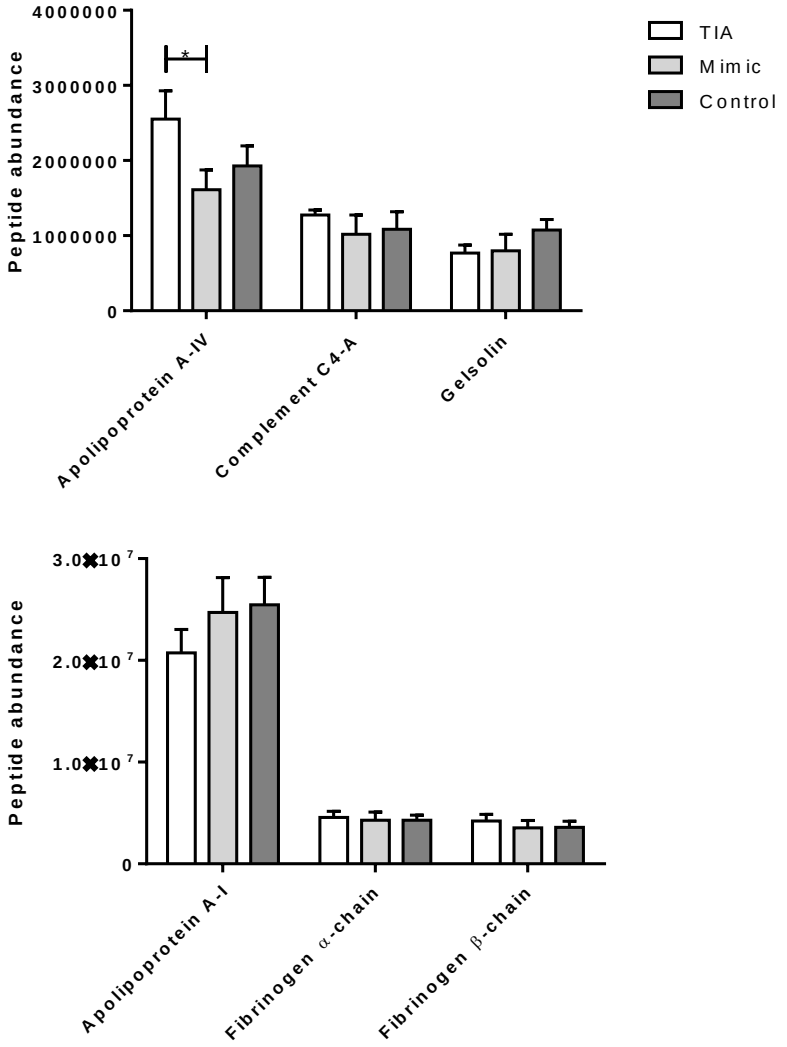
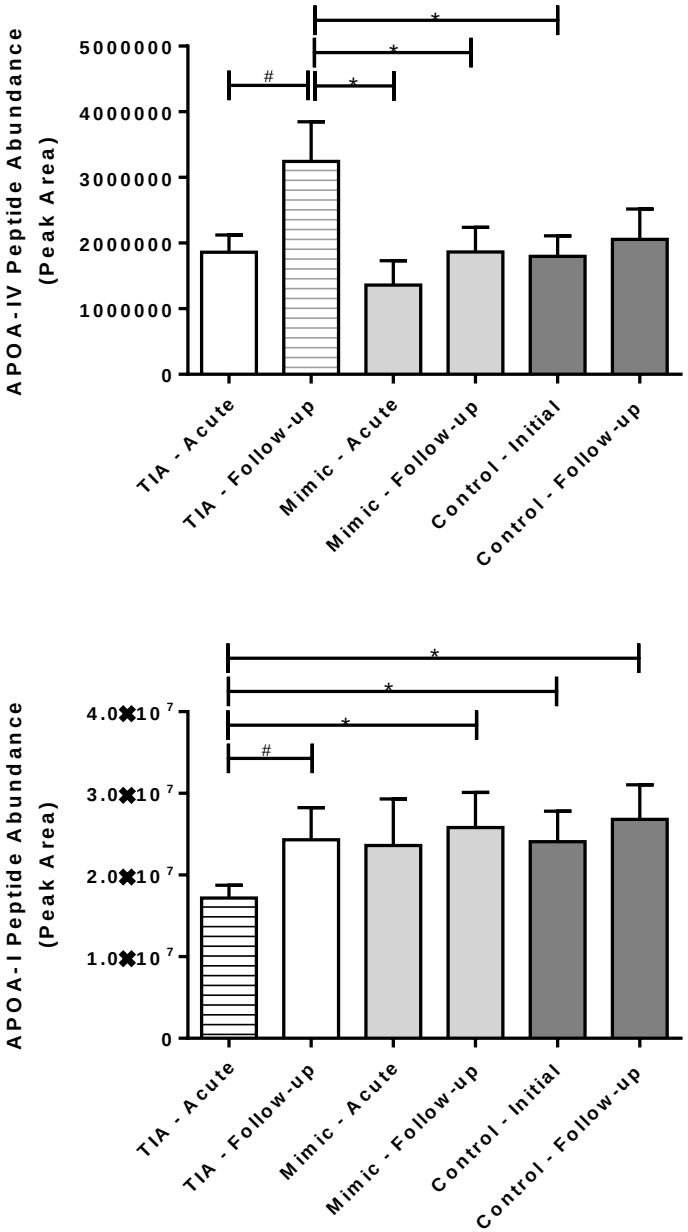


Figure 20: Multiple reaction monitoring quantification of Apolipoprotein A-I and A-IV in acute and follow-up volunteers. *Bar graph representation of mean peak area ± SEM of peptides measured by MRM (n=18). Combined acute and follow-up samples from TIA, Mimic and Control cohorts displaying peptide abundance of six candidate proteins, with paired t-test significance denoted by '#' and unpaired t-test significance denoted by '*' when $p \leq 0.05$.*



3.6 Discussion

This is the first study of its kind to apply a discovery-based plasma proteomic method utilising 2D-DIGE, LC-MS/MS, and MRM quantification in patients with TIA. We identified six candidate plasma protein biomarkers that are differentially abundant between TIA, mimic and healthy control volunteers. This study has shown for the first time that the anti-atherogenic protein APOA-I was decreased exclusively in the acute presentation of TIA, while the lipid transport protein APOA-IV was increased exclusively in TIA patients at follow-up. These findings warrant future mechanistic studies to investigate lipid regulatory pathways in the pathogenesis of TIA, and validate their potential use as diagnostic or prognostic biomarkers.

3.6.1 Apolipoproteins and TIA

Apolipoproteins are lipid-binding proteins that transport lipid through the circulatory system. Apolipoprotein A-I is the major plasma protein component in high-density lipoprotein (HDL) that displays anti-atherogenic properties by transporting cholesterol from the tissues to the liver (Zannis, Chroni, & Krieger, 2006). The protective effects of APOA-I against atherosclerosis, coronary heart disease and stroke have been well documented (Contois et al., 1996; Walldius & Jungner, 2007), whereby reduced APOA-I has specifically been shown to predict ischaemic stroke in patients with previous TIA (Bhatia et al., 2006). We have shown for the first time that both HDL-C and APOA-I concentrations are significantly reduced in acute TIA patients compared with mimic or healthy controls, and can distinguish acute TIA from acute mimic diagnoses. Furthermore, a study by Hyka *et al.* has shown that HDL-associated APOA-I has anti-inflammatory properties through direct inhibition of the inflammatory cytokines tumour necrosis factor (TNF)-α and interleukin-1β (Hyka et al., 2001). HDL-associated APOA-I has been shown to decrease in acute inflammation, allowing for upregulation of these pro-inflammatory cytokines (Hyka et al., 2001). Hence, this HDL-associated APOA-I deficiency may reflect a pro-inflammatory mechanism unique to the pathogenesis of TIA and the inherently high risk of stroke, which conversely is not present in mimics or healthy controls.

Apolipoprotein A-IV is a glycoprotein predominantly synthesised in the small intestine and secreted into plasma where it functions in the assembly of chylomicrons that facilitate high-density lipoprotein transport and metabolism (Steinmetz & Utermann, 1985). However, a study by Shen *et al.* has also linked APOA-IV with satiety regulation, and characterised APOA-IV synthesis and distribution within the brain (Shen et al., 2008). Of significance, APOA-IV detected in cerebral spinal fluid (CSF) was not derived from systemic circulation as it was unable to cross the blood-brain-barrier, but rather produced within hypothalamic neuronal cells (Shen et al., 2008). In a transient embolic or thrombotic cerebrovascular occlusion, a biochemical cascade of neurotoxicity is initiated, compromising the structural integrity of the blood-brain-barrier by increasing permeability and in effect damaging neurons, glia and microvascular endothelium (Dambinova et al., 2003). When considering the significant increase of APOA-IV in TIA patients at follow-up compared to mimics, and given that co-morbidities have been standardised between both groups (making diagnosis the major variable), delayed release of brain-derived APOA-IV in response to TIA may be a source for this increased plasma concentration, and may explain the absence of an increased APOA-IV concentration at acute presentation.

3.6.2 Gelsolin and TIA

In a healthy human, plasma gelsolin functions as an actin scavenging protein that removes actin filaments shed from the cytoskeleton of damaged or dead cells into the systemic circulation. Circulating actin is directly toxic to endothelial cells (Erukhimov et al., 2000), and is associated with secondary tissue injury (Bucki, Levental, Kulakowska, & Janmey, 2008). Deficiency of plasma gelsolin compromises the sequestration of circulating actin as well as proinflammatory mediators, increasing the risk of further injury as seen in chronic inflammatory disorders such as rheumatoid arthritis (Osborn, Verdrengh, Stossel, Tarkowski, & Bokarewa, 2008). Our 2D-DIGE study identified a significantly lower plasma gelsolin concentration in both TIA and mimic groups compared with healthy controls. This reduction signifies gelsolin's association as a biomarker of a healthy physiological state. Despite lacking statistical

significance when measured using the more sensitive mass spectrometry-based MRM method, gelsolin quantification depicted the same trend as 2D-DIGE. This is the first study to identify reduced plasma gelsolin concentration in TIA patients. However, given the lack of fluctuation of gelsolin abundance in each patient over a 90-day period, its deficiency in our mimic participants may be a reflection of chronic systemic morbidities rather than transient ischaemia. Nevertheless, Guo *et. al.* established that a reduced plasma gelsolin concentration below 52 µg/mL was an independent predictor for 1-year mortality after a first-ever ischemic stroke compared with healthy controls (73.0% sensitivity and 65.2% specificity) (Guo et al., 2011). This trend warrants further investigations to validate plasma gelsolin in TIA patients, to assess whether levels are comparable to ischaemic stroke patients, and if TIA and stroke patients share the same risk of mortality based on decreased plasma gelsolin.

3.6.3 Complement and Coagulation in TIA

Studies of human populations have shown Complement factor C4 (C4A) to be associated with increased levels of cardiovascular risk factors including diabetes, hypertension and obesity (Nilsson et al., 2014). The acute-phase C-reactive protein (CRP) is a key activator of the classical complement pathway, and has been shown to be elevated in combination with C4A in atherosclerotic plaque tissue (Yasojima, Schwab, McGeer, & McGeer, 2001). A longitudinal study observed an increased incidence of cardiovascular disease in patients with a plasma C4A concentration in the top 10% of the population distribution, demonstrating a potential link between elevated C4A and atherogenesis (Engstrom, Hedblad, Janzon, & Lindgarde, 2007). Activation of the complement cascade has been implicated in the inhibition of fibrinolysis through promotion of platelet activation, fibrin formation and thrombosis (Carter, 2012). In addition, the coagulation factor fibrinogen increases in the presence of atherosclerotic plaque, and functions as an acute phase protein that increases following stroke (Swarowska et al., 2014). This link between inflammation, complement activation, and thrombosis supports our quantitative findings of significantly increased high-sensitivity CRP and the trend (non-significant) indicating an increased abundance of C4A, Fibrinogen α-chain and β-chain in acutely presenting TIA patients. Given the vascular aetiology of TIA and the lack of significant difference in cardiovascular comorbidities

between TIA and mimic patients, our findings have shown these elevated mediators of inflammation, complement activation and coagulation may be implicated in the pathogenesis of TIA.

3.6.4 Study Limitations

This pilot study has identified differentially abundant proteins that can distinguish TIA from mimic and healthy control volunteers. However, the expression profile of six candidate biomarkers identified by 2D-DIGE did not change significantly between acute/initial and 90-day follow-up assessments in TIA and mimic patients, so it remains to be determined whether the expression profile was the effect of a transient ischaemic attack or of a chronic condition stemming from underlying co-morbidities. Every effort was made to standardise TIA and mimic patients with regards to co-morbidities, however each of these proteins has been previously implicated in either neurological (Huang et al., 2011), cardiovascular (Liu, Yin, & Chen, 2011; Manpuya, Guo, & Zhao, 2001), infectious (Albuquerque et al., 2009) or inflammatory conditions (T. W. Li et al., 2010; Osborn et al., 2008), which should not be overlooked when considering their suitability in distinguishing TIA from non-TIA patients. Furthermore, it is unclear whether the arbitrarily-defined 1.1-fold cut-off value based on power calculations from previous human plasma 2D electrophoresis studies was suitable to detect biologically relevant changes in our patient population. Given that the dynamic range of plasma protein abundance varies widely on a day-to-day basis, this may further add to the risk of false-positive discoveries (Type 1 error) attributed to spontaneous variation.

3.7 Conclusion

This is the first proteomic investigation using 2D-DIGE, LC-MS/MS, and MRM to identify and quantify six plasma proteins that are differentially abundant between TIA, mimic and healthy control volunteers. Apolipoproteins A-I and A-IV were exclusively associated with TIA and require validation in larger cohorts to establish their suitability

as biomarkers for diagnosing and distinguishing TIA from mimic conditions, and exploring their prognostic implications regarding the development of ischaemic stroke.

List of abbreviations

TIA: transient ischaemic attack; 2D-DIGE: two dimensional difference in-gel electrophoresis; MRM: multiple reaction monitoring; APOA-IV: apolipoprotein A4; FGA: fibrinogen alpha-chain; FGB: fibrinogen beta-chain; C4A: complement component C4A; APOA-I: apolipoprotein A-1; GS: gelsolin; DWI-MRI: diffusion weighted magnetic resonance imaging; CT: computed tomography; RAC: rapid access clinic; COMBAT: community-based rapid access TIA clinic; K_2EDTA: potassium ethylene diamine tetraacetic acid; FPLC: fast protein liquid chromatography; MARS-6: human 6 multiple affinity removal system; PCA: principal component analysis; LC: liquid chromatography; MS: mass spectrometry.

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Competing interests **–** The authors declare that they have no competing interests.

Authors' contributions **–** MD contributed to the conception of the study design, carried out sample collection, pre-analytical processing, proteomic studies, statistical analyses and drafting of the manuscript. EL contributed to the clinical study design, patient recruitment and review of the manuscript. TC contributed to conception of study design, interpretation of proteomic studies and mass spectrometry, and reviewed of the manuscript. AC performed multiple-reaction-monitoring mass spectrometry and analysis. JJ contributed to clinical study design and patient recruitment. GG contributed to review of statistical analyses. SN contributed to review of manuscript. ML involved in the conception of the study design and review of the manuscript. MB contributed to the clinical study design, drafting the manuscript and critical review. SK involved in the conception of the study design, participated in drafting and critical review of the manuscript.

4 A 2D-DIGE Based Proteomic Analysis Excludes a Low-Dose Aspirin Effect on Candidate Plasma Biomarkers for Transient Ischaemic Attack: A Case Study.

Original Short Report Contribution for Submission to:

Stroke: A Journal of Cerebral Circulation

Authors: Michael Djukic[1], Timothy K. Chataway[2], Martin D. Lewis[1,4], Monica A. Hamilton-Bruce[1,3*], Simon A. Koblar[1,3*].

In keeping with the style of this thesis to maintain continuity, section headers, figures and tables have been re-numbered and the references have been incorporated into the thesis' reference list. The methodology for HPLC linear ion trap mass spectrometry and label-free quantitation strategy has been replicated as initially described in Chapter 2.

24th November, 2016

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4.1 Statement of Authorship

Journal of Submission: *Stroke: A Journal of Cerebral Circulation*

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Author contributions

By signing the Statement of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

Name of Principal Author (PhD Candidate)	**Michael Djukic**		
Contribution to the Paper	Conceptualisation of the work, realisation of project design, documented experimental method, performed analysis of all samples, interpreted and evaluated data and wrote the manuscript.		
Overall percentage (%)	80%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	

Name of Co-Author	**Timothy K. Chataway**		
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Contribution to the Paper	Assisted with realisation of project design, supervised development of work, assisted with manuscript editing.		
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Contribution to the Paper	Assisted with realisation of project design, supervised development of work, participated in critical review and editing of manuscript.		
Signature		Date	24.11.2016

Name of Co-Senior Author	**Simon A. Koblar**		
Contribution to the Paper	Conceptualisation of work, realization of project design, supervised the development of work, involved in data evaluation and interpretation, contributed to evaluation and editing of manuscript, act as corresponding author.		
Signature		Date	25.11.2016

4.2 Abstract

Background and Purpose – A test to accurately diagnose and distinguish TIA from mimic conditions is urgently needed. Recent exploration of the human plasma proteome has identified differentially abundant plasma protein biomarkers associated with TIA diagnosis. However, these findings have been limited by the potential variation induced by aspirin intake following acute TIA presentation. This study explored the effect of aspirin on the human plasma proteome to rule out an aspirin-induced bias towards previously proposed candidate TIA biomarkers.

Methods –A baseline 'pre-aspirin' blood sample was collected from a single healthy control volunteer prior to receiving a daily 100 mg dose of aspirin. A 'post-aspirin' follow-up blood sample was collected after a 7-day treatment period. Pre- and post-aspirin plasma was immunodepleted before fluorescent labelling and protein separation using difference in-gel electrophoresis (2D-DIGE). Mass spectrometry (MS) was used to identify differentially abundant plasma proteins associated with aspirin dosage, and directly compared with candidate TIA plasma proteins previously published.

Results – Fifteen protein spots were identified as significantly different in abundance between pre- and post-aspirin intake. Seven unique proteins appeared in multiple spots, with hemopexin (HEMO), zinc-alpha-2-glycoprotein (ZA2G), Apolipoprotein E (APOE), and fibrinogen gamma chain (FIBG) increasing following 7-days of aspirin intake. C-reactive protein (CRP) precursor, Apolipoprotein A-I (APOA-I) and serum amyloid P-component (SAMP) decreased following aspirin-intake. Plasma APOA-I was differentially abundant following aspirin usage as well as between acutely presenting and follow-up TIA patients. However, APOA-I is reduced following aspirin intake, whilst TIA patients express an increased APOA-I at follow-up.

Conclusion –This case study does not suggest an aspirin-associated effect on increasing plasma APOA-I and APOA-IV abundance, supporting further validation as candidate plasma biomarkers associated with TIA.

Key Words: Transient Ischaemic Attack, Aspirin, Plasma, Proteomics, Biomarker, Apolipoprotein.

4.3 Introduction

Rapid assessment and immediate treatment of transient ischaemic attack (TIA) has been associated with an 80% risk reduction in 90-day stroke risk (Lavallee et al., 2007; Luengo-Fernandez, Gray, & Rothwell, 2009). Aspirin is the most readily available and most widely used anti-platelet agent in this setting, with an associated 13% relative risk reduction of stroke in previous ischaemic stroke or TIA patients (Algra & van Gijn, 1996). When clinically indicated to receive antiplatelet therapy, current clinical guidelines for stroke management recommend aspirin prescription for stroke risk reduction in TIA patients (National Stroke Foundation, 2010; Sacco et al., 2008). Low-dose aspirin (75 – 150 mg) has shown to be as clinically effective at preventing stroke as higher doses (300 – 1300 mg), and with lower associated risks of gastrointestinal side effects (Antithrombotic Trialists' Collaboration, 2002).

Recent application of proteomic technologies to explore the plasma proteome of TIA, mimic and healthy control volunteers at acute and 90-day follow-up time points has identified several candidate TIA-associated biomarkers linked with lipid transport, coagulation, and inflammation (Chapter 3, Djukic et al.). However, it remains unclear what potential impact low-dose aspirin intake has had on the relative abundance of these candidate biomarkers. Hence, we investigated the plasma proteome of a healthy volunteer subjected to a 7-day low-dose aspirin trial to determine which plasma proteins are altered, and whether previously proposed candidate TIA biomarkers were biased by the effects of aspirin following acute presentation.

4.4 Methods

4.4.1 Volunteer Recruitment and Study Design

This pilot study analysed blood plasma from a healthy volunteer presenting from the wider Adelaide Metropolitaln Community to The Queen Elizabeth Hospital in October 2010 to participate in this study. The inclusion criteria was to be over the age of 40

years, not having any clinically diagnosed medical conditions, and not taking any prescription or complementary medicines at the time of assessment. Exclusion criteria were any known allergy to aspirin or a nonsteroidal anti-inflammatory drug (NSAID), conditions that increases the risk of bleeding, pregnancy or breastfeeding, asthma or other breathing problems, or having had a stomach or intestinal ulcer in the past. Written informed consent was obtained from the volunteer and The Queen Elizabeth Hospital Ethics Committee approved this study (HREC Approval Number 2009123).

The consenting volunteer was screened for evidence of cerebrovascular risk factors and deemed to be healthy and safe to participate in this study by a physician's (ESL) clinical assessment. Two blood samples collected in 1.8 mg/mL K_2EDTA BD vacutainers® (Becton, Dickinson and Company, NJ, USA) were provided: an initial 'baseline' sample, and a second sample that followed a daily oral intake of Cartia® 100 mg low dose aspirin (Aspen Pharmacare, NSW, Australia) over a 7-day period.

4.4.2 Sample preparation, depletion and CyDye labelling

Blood plasma sample preparation and depletion methods have previously been described (Chapter 2). Briefly, both blood vacutainers were centrifuged within 30 min of collection, with plasma extracted and mixed with an aliquot of Halt® protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific, Illinois, USA) and stored in liquid nitrogen for future analysis. Prior to analysis, plasma samples were depleted of albumin, transferrin, haptoglobin, immunoglobulin G, immunoglobulin A and alpha-1 antitrypsin using a 4.6 x 100 mm Human 6-Multiple Affinity Removal System (MARS-6) (Agilent Technologies, Santa Clara, USA), quantified using EZQ® protein quantification kit (Invitrogen, Oregon, USA), and fluorescently labelled using 400 pmol of Cy3 and Cy5 CyDye DIGE Fluors per 50 µg of depleted plasma from pre- and post-aspirin samples respectively (GE Healthcare, Buckinghamshire, UK).

4.4.3 2D-DIGE

CyDye labelled pre- and post-aspirin samples were separated by first dimensional isoelectric focusing using a 24 cm pH 3-11 non-linear IPG strip (GE Healthcare) followed by a reduction and alkylation equilibration process before transfering the IPG strip onto a 12.5% 2D SDS-polyacrylamide gel for second dimension electrophoretic separation by molecular weight. Scanning was performed using the Typhoon Trio variable mode imager (GE Healthcare) with a resolution of 200µm, and photomultiplier tube setting of 500V and 470V for Cy3 and Cy5 labeled samples respectively. Differential-in-gel analysis (DIA) of pre- and post-aspirin plasma was performed in DeCyder™ v7.0 (GE Healthcare) with the difference between pre- and post-aspirin plasma samples reported as a volume ratio (Figure 21.A-B.). A threshold of two-times the standard deviation of the distribution of the log volume ratio from this pre- and post-aspirin comparision was used to provide a measure of experimental variation that encompass 95% of the spot changes atributed to experimental variation, with the assumption that the data follows a normal distribution (Figure 21.C.). Therefore, protein spots with a fold-change greater than this threshold are considered significant changes with a 95% confidence that the change is a difference between pre- and post-aspirin plasma is not due to random chance. Spots from non-protein artefacts with a slope < 1.0 and spot volume < 200,000 were filtered out while spots having a ± 1.50-fold change in abundance were included for analysis.

4.4.4 Mass Spectrometry

Proteins of interests from the analytical 2D-DIGE gel were visualised using mass spectrometry-compatible Eriochrome black T (EBT) silver staining, manually excised and digested using trypsin. Digested gel spots were analysed using a Thermo Orbitrap XL linear ion trap mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA), with an attached nanospray source (Thermo Electron Corporation, San Jose, CA, USA). Mass spectrometry spectra were searched with Thermo Scientific™ Proteome Discoverer™ 1.2 (Thermo Fisher Scientific) using the SEQUEST algorithm against the Swiss-Prot Human Uniprot database (May 2012 version) (Boutet et al., 2007) (representing 20,244 protein entries).

4.5 Results

The 2D-DIGE gel image represents the depleted plasma protein profile of a clinically healthy 50 year old male volunteer, with pre- and post-aspirin plasma depleted of the top six most abundant proteins (Figure 21). Of the 1,364 gel spots detected, 427 were excluded and 937 spots included in DIA, with 14 (1.5%) increased, 67 (7.2%) decreased, and 856 (91.4%) unchanged between pre- and post-aspirin plasma samples. Following the aforementioned spot filtering parameters for DIA, fifteen spots were selected for further analysis (Figure 21). Candidate TIA biomarkers identified by our previous study are listed in Table 12.

Of the fifteen selected gel spots, seven unique proteins were identified whilst four spots were unidentified. Fibrinogen gamma chain (FIBG), hemopexin (HEMO), zinc-alpha-2-glycoprotein (ZA2G), and apolipoprotein E (APOE) increased following 7-days of aspirin intake, with fold-change spanning 2.14 - 6.44 fold increases, $p < 0.05$ (Table 11). Conversely, apolipoprotein A-I (APOA-I), C-reactive protein (CRP) precursor, and serum amyloid P-component (SAMP) decrease following aspirin intake, with fold-change spanning 1.84 – 2.27 fold decreases, $p < 0.05$. Both fibrinogen gamma chain and apolipoprotein A-I proteins appeared in multiple spots.

In comparison to our previous study, APOA-I is the only proposed candidate TIA biomarker that is differentially abundant in both studies. However, in the presence of aspirin, APOA-I is reduced, whilst TIA patients have increased plasma APOA-I abundance at follow-up (Table 12).

Figure 21: 2D-DIGE analysis of pre- and post-aspirin plasma from a healthy volunteer. *Proteins were labelled with corresponding Cydyes (see Methods section) and separated using isoelectric focusing (pH range 3-11, 24 cm) and a 12.5% SDS-polyacrylamide gel. (A) 2D-DIGE analysis where the fluorescence emission from Cy3 and Cy5 dyes are superimposed. (B) Representative image of the analysis in a gray scale with differentially regulated spots highlighted (green indicating increased abundance, and red indicating decreased abundance) when comparing pre-and post-aspirin plasma. Further information about protein identifications can be found in Table 11. (C) Histogram plot for log volume ratios in the DIA module. (D) 3D relative abundance image of two differentially abundant protein spots circled in purple (spot number 887 and 690) after DIA analysis by DeCyder 7.0 software.*

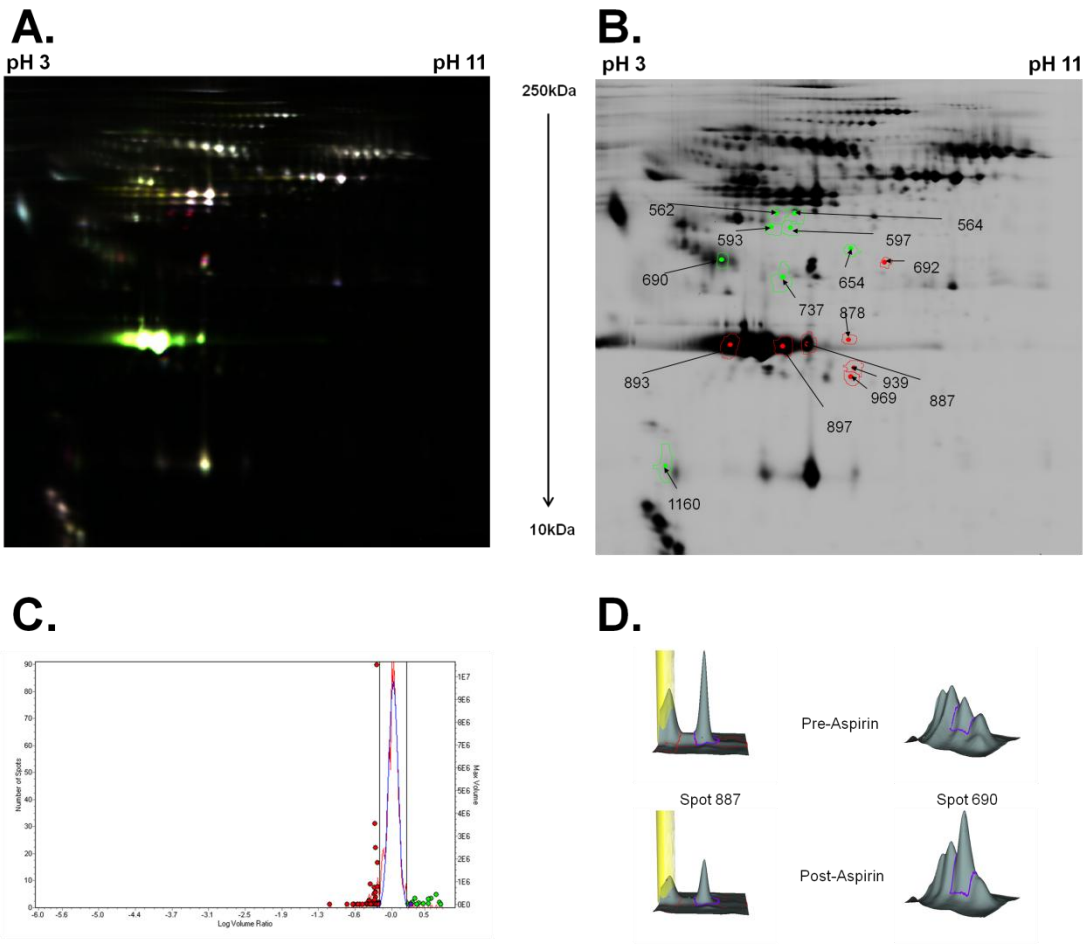


Table 11: List of identified differentially abundant plasma proteins when comparing pre- and post-aspirin intake in a healthy volunteer. *Differentially expressed protein spots were identified by nanospray LTQ Orbitrap XL-MS/MS. Mass spectrometry spectra were searched with Thermo Scientific™ Proteome Discoverer™ 1.2 using the SEQUEST algorithm against the Swiss-Prot Human UniProt database (May 2012 version) with associated codes listed. All listed protein spots have a fold change cut-off ≥ 1.5 with increased/decreased abundance determined from DIA analysis. Fold change = spot volume ratio when comparing labelled pre- and post-aspirin samples, MW = molecular weight, kDa = kilodaltons, Calc. pI = calculated isoelectric point.*

Identified Protein	UniProt Code	Spot#	Post-Aspirin	Function	Fold change	MW (kDa)	Calc. pI
Not identified	-	654	Increased	-	6.44	-	-
Fibrinogen gamma chain	P02679	597	Increased	Platelet activation, coagulation	6.05	49.57	5.34
Fibrinogen gamma chain	P02679	564	Increased	Platelet activation, coagulation	5.41	49.57	5.34
Fibrinogen gamma chain	P02679	562	Increased	Platelet activation, coagulation	4.41	49.57	5.24
Hemopexin	P02790	1160	Increased	Heme transporter	4.00	18.29	4.56
Fibrinogen gamma chain	P02679	593	Increased	Platelet activation, coagulation	2.73	49.57	5.24
Zinc-alpha-2-glycoprotein	P25311	690	Increased	Lipolysis stimulation	2.34	40.26	4.97
Apolipoprotein E	P02649	737	Increased	Lipoprotein transporter.	2.14	34.97	5.35
Serum amyloid P-component	P02743	878	Decreased	Acute phase response	-1.81	26.28	5.55
Not identified	-	969	Decreased	-	-1.84	-	-
C-reactive protein precursor	P02741	893	Decreased	Acute phase response	-1.86	23.76	5.12
Apolipoprotein A-I	P02647	887	Decreased	Cholesterol transport	-1.98	23.45	5.48
Apolipoprotein A-I	P02647	897	Decreased	Cholesterol transport	-2.04	23.00	5.22
Not identified	-	692	Decreased	-	-2.16	-	-
Not identified	-	939	Decreased	-	-2.27	-	-

4.6 Discussion

Aspirin inhibits platelet function by irreversibly acetylating a serine residue in the cycloxygenase-1 (COX-1) enzyme used for prostaglandin and thromboxane synthesis, thereby significantly reducing the production of the platelet aggregating and vasoconstrictive thromboxane A2 compound and exerting a protective cardiovascular effect (Roth & Majerus, 1975). Aspirin's irreversible inactivation of COX-1 exerts an antithrombotic and anticoagulant effect through the suppression of thrombin generation and fibrin network formation (Undas, Brummel-Ziedins, & Mann, 2007). Aspirin also exerts an analgesic and anti-inflammatory effect by suppression of pro-inflammatory prostaglandin production (Botting, 2010). Due to the wide-ranging mechanisms of action, the aim of this study was to identify aspirin's potential impact on the broader scope of plasma proteins so as to exclude a potential aspirin bias on previously proposed candidate TIA biomarkers (Chapter 3, Djukic et al.).

To better understand the effect of aspirin on plasma proteins, we adopted a proteomic approach. In the initial processing step of this study, we removed the top six highest abundant plasma proteins as they can mask lower abundant 'disease-specific' proteins in the 2D-DIGE image. As a result, fifteen gel spots with ≥ 1.5-fold differences in relative abundance between pre- and post-aspirin plasma were detected, with seven unique proteins identified as differentially abundant. Among them, APOA-I was the only protein identified from our previous TIA biomarker pilot study to have a significantly altered level of abundance as a result of aspirin intake.

Apolipoprotein A-I is a major protein component of high-density lipoprotein in plasma. Cycloxygenase inhibition by aspirin has previously been shown to down-regulate protein expression of APOA-I (Horani, Gobal, Haas, Wong, & Mooradian, 2004). Aspirin has been shown to reduce serum APOA-I concentration by reducing the transcriptional activity of the APOA-I gene and suppressing APOA-I mRNA expression (Akaike et al., 2002). This is of clinical significance given the genetic inheritance of lipoprotein A (an independent risk factor for atherosclerosis) to express itself from APOA-I gene sequences. Hence, aspirin has been shown to lower serum lipoprotein A and thereby exert a lipid-lowering anti-arthrogenic effect by suppressing APOA-1

transcription and expression. Our study supports earlier research showing aspirin lowers APOA-I by suppression of APOA-I gene transcription. We previously identified APOA-I as significantly reduced in abundance in acute TIA patients when compared with mimic or healthy controls. However, following aspirin administration, TIA patients presented with higher (normalised) APOA-I levels. This suggests aspirin may not significantly affect APOA-I expression, supporting the proposed APOA-I association with TIA diagnosis.

This case study has shown the utility of the DIGE/MS method to quantitatively analyse over 1,300 resolved plasma proteins when comparing pre- and post-aspirin treatment in a well-defined healthy asymptomatic volunteer. However, analysis of differentially abundant proteins in a single volunteer study design lacks validity and replicability to confirm differences between pre- and post-aspirin treatment. Furthermore, this case study design does not explore the effects of polypharmacy and cardiovascular comorbidities commonly in patients presenting with TIA, thereby limiting the translation of these findings to a TIA biomarker model. Hence, within the specific context of a single healthy volunteer, administration of low-dose (100 mg) aspirin over 7-days was not shown to significantly increase the abundance of plasma APOA-I and APOA-IV proteins previously proposed to be associated with TIA.

Table 12: Summary of differentially abundant candidate TIA-associated proteins identified by 2D-DIGE and nanospray LTQ Orbitrap XL-MS/MS. *Adapted from Chapter 3 (Djukic et al.). Fold Δ = significant fold change determined by increased cohort vs. pooled internal standard, where p<0.05, MW= molecular weight, kDa= kilodaltons, Calc. pI = calculated isoelectric point, TIA = transient ischaemic attack, M = mimic, HC = healthy control.*

Protein Identified	UniProtKB Accession Number	Fold Δ ± SD (p-value)	Increased cohort	Calc. pI/MW [kDa]	Biological Function
Apolipoprotein A-IV	P06727	1.61 ± 0.20 (0.02)	TIA	5.38/45.4	Lipid transport.
Apolipoprotein A-I	P02647	1.35 ± 0.10 (0.03)	HC	5.76/30.8	Anti-atherogenic
Fibrinogen α- chain	P02671	1.22 ± 0.09 (0.04)	M	6.01/94.9	Blood coagulation.
Fibrinogen β- chain	P02675	1.17 ± 0.08 (0.03)	M	8.27/55.9	Blood coagulation.
Complement C4-A	P0C0L4	1.19 ± 0.06 (0.03)	M	7.08/192.7	Immune response, inflammation, complement activation.
Gelsolin (Isoform 1)	P06396	1.14 ± 0.08 (0.03)	HC	6.28/85.6	Signalling, motility, apoptosis.
Gelsolin (Isoform 2)	P06396-2	1.21 ± 0.10 (0.02)	HC	5.85/80.6	Signalling, motility, apoptosis.

5 SIGNIFICANCE AND FUTURE DIRECTIONS

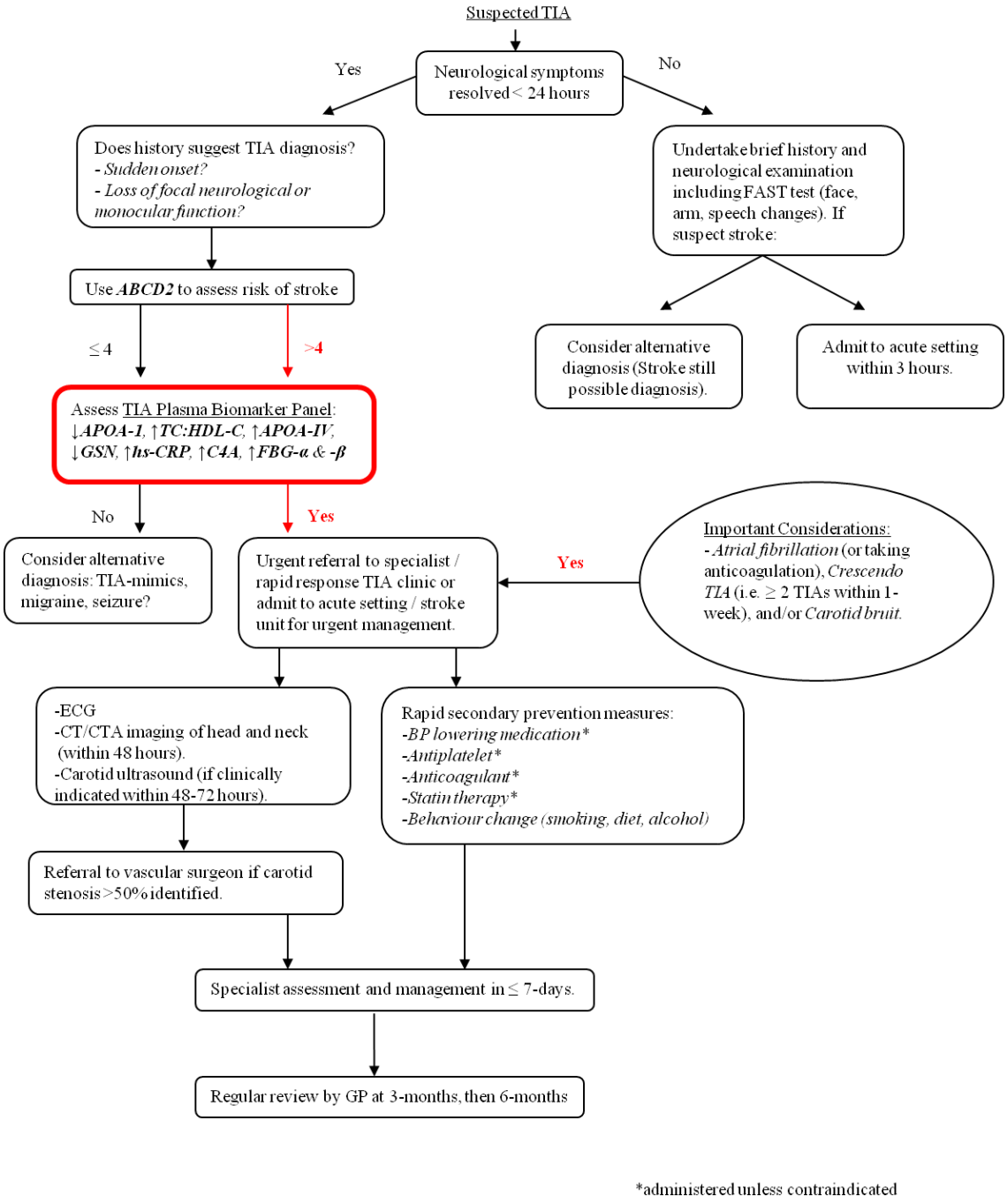
In 2016, stroke will occur in around 50,000 Australians, making it the second single greatest cause of death and the leading cause of disability that presently costs the Australian health system an estimated $2.14 billion per year (National Stroke Foundation, 2014; National Stroke Foundation, 2015). It is anticipated that up to 10,000 Australians that develop a stroke annually will have presented first with a TIA (Coull et al., 2004; Giles & Rothwell, 2007; Johnston et al., 2000; Rothwell & Warlow, 2005). Early and accurate diagnosis of stroke or its common precursor, TIA, may allow for rapid theraputic intervention and complete reversal of stroke symptoms without permanent brain damage.

Early assessment and treatment of TIA using rapid assessment clinics reduce 3-month stroke risk from 5.96% (based on ABCD2 risk stratification score) to an actual risk of 1.24% (Lavallee et al., 2007; Luengo-Fernandez et al., 2009; Rothwell et al., 2007). There is a reliance on neuroimaging modalities and clinical features to differentially diagnose high risk TIA from mimic conditions. Evidence shows that acute DWI-MRI findings in combination with clinical features of transient ischaemic attack as determined by the ABCD2 risk stratification score increases the accuracy (sensitivity 80%, specificity 73%) of predicting 7-day stroke risk following TIA (Ay et al., 2009). However, DWI-MRI remains a stroke-exclusion tool rather than an imaging modality that can diagnose TIA. Furthermore, limited DWI-MRI availability in smaller hospitals

or remote community health settings, stringent patient contraindications, and high running costs limit neuroimaging from becoming a gold standard diagnostic tool. There is a need for an accurate, time-efficient and cost-effective test such as a blood plasma assay for TIA-specific biomarkers to assist in both early and accurate diagnosis of TIA and prognosis of stroke risk.

Improved diagnostic and prognostic certainty will allow for selective triage and implementation of appropriate preventative therapies for high-risk individuals such as blood pressure control, appropriate pharmaceutical application of statins, antiplatelet, antithrombotic agents, and in the presence of significant internal carotid artery disease, the option for early carotid revascularisation (Figure 22). This will allow for more efficient and effective use of hospital resources for TIA patients at high risk of future stroke, whilst allowing appropriate referral pathways for low risk patients to receive ongoing management in a primary healthcare setting. Identification and triage of high-risk patients to appropriate hospital monitoring and treatment facilities could be of significant benefit to one-in-five future stroke sufferers.

Figure 22: Hypothesised TIA management pathway for community GP or hospital based referral. *Given the risk of imminent stroke post-TIA, the implementation of a biomarker panel to diagnose and distinguish TIAs from mimic conditions could assist in the hyper-acute management/triage of high risk patients to an acute stroke unit, whilst low risk patients are triaged to a rapid access clinic for stroke prevention and vascular risk reduction therapy.*



This thesis outlines the design and implementation of a human plasma proteomic pilot study involving small sample cohorts of TIA, mimic and healthy control volunteers. A 2D-DIGE/MS method has detected and identified six medium and high abundant plasma proteins; apolipoprotein A-I, apolipoprotein A-IV, gelsolin, fibrinogen alpha-chain, fibrinogen beta-chain, and complement C4-A, that are differentially abundant between TIA, mimic and control volunteers. This study developed label-free MRM assays to quantify six plasma proteins using a TripleTOF 5600+ mass spectrometer. Although MRM-MS could detect significant differences in the plasma level of both apolipoproteins A-I and A-IV between acute and follow-up TIA patients, these findings are hypothesis-generating and will require future study designs of adequate sample size and statistical power to hypothesis-test and validate these candidate biomarkers in both TIA and mimic groups.

Two-dimensional DIGE coupled with mass spectrometry is a labour intensive and costly method, with a low throughput of patient samples that can be analysed by this combined method. However, the advantages of this proteomic study design over genomic-based biomarker discovery methods is that exploration of the proteome identifies functional bio-active macromolecules, making it easier to transfer protein-based study results into clinically utilised tools in comparison to results of genomic-based studies. Two-dimensional DIGE allows for the separation of thousands of proteins which include protein isoforms and disease-associated post-translational modifications (Lilley, Razzaq, & Dupree, 2002). However, several technical caveats exist with use of the 2D-DIGE platform, including exclusion of very large (>250 kDa) or small molecular weight proteins (< 5 kDa), highly acidic (pH < 3), highly alkaline (pH >11), or hydrophobic proteins. In addition, inter-gel variation requires further biological replicates to achieve sufficient statistical power to identify differentially abundant proteins. Label-free proteomic methods like MRM-MS allows for higher patient sample throughput with greater dynamic range detection sensitivity, whilst not suffering from these same technical limitations that plague gel-based methods. Nevertheless, different proteins can be detected by 2D-DIGE and label-free MRM methods, thereby making their combined use a recommended standard for a ‘discovery-based’ proteomic pilot study. This is the first study of its kind to conduct a multi-platform proteomic analysis of differential plasma protein (biomarker) abundance in TIA and mimic pateints at acute presentation,

and followed-up for the assessment of a stroke outcome within a 3-month period. The impact of these results and ideas for further studies are discussed below.

Identification of moderate to high abundant plasma proteins as described above have been identified as biomarkers in other diseases. This suggests an inherrent limitation that these abundant plasma proteins may not distinguish people with TIA's from mimic or healthy individuals. Any set of biomarkers identified in a discovery-phase pilot study need to first be validated in that target population (i.e. patients with clearly confirmed TIA and mimic diagnoses) before expanding testings into the wider population. Hence, the future direction of this thesis is the validation of plasma proteins in an independent and larger prospectively recruited group of acute and follow-up TIA plasma samples.

The goal of the Stroke Research Programme, of which this thesis research is a part, is the development of diagnostic and prognostic screening tests for TIA. The plasma proteomic work described in this thesis is the initial phase of a TIA biomarker discovery process to identify candidate TIA biomarkers that subsequently require validation in a larger population using less refined and undepleted blood plasma. Collaboration with the mulit-state and multi-centre Australian Stroke Biomarker Collaboration (ASBC) is currently underway, with the aim to complete a prospective *a priori* validation study comparing TIA and mimic patient plasma samples.

In addition, a sub-study to explore the sensitivity and efficacy of detecting candidate TIA biomarkers in the form of a non-invasive saliva test will be undertaken. Briefly, we plan to obtain human bio-samples including whole blood, plasma, DNA, and saliva prospectively using four established clinical bio-repository centres across Australia (The Neurosciences Department at Gosford Hospital, Gosford; the Neurology Department at John Hunter Hospital, Newcastle; The Queen Elizabeth Hospital, Adelaide; and the Royal Perth Hospital, Perth). Recruited participants will be referred to an outpatient TIA rapid assessment clinic within 72 hours of symptomatic ischaemic onset after referral from GP or emergency department triage. Participants will be consenting patients suspected of having a TIA, with diagnostic confirmation provided by neurologists in accordance with the definitions from the scientific statement of the American Heart

Association ‘Definition and evaluation of TIA’ (Easton et al., 2009). Clinical and biological material will include:

1. Medical history (including signs and symptoms as reported by patient) and physical examination findings of GP and/or referring medical officer (to be obtained retrospectively once patients are included into this study).
2. Medical assessment by neurologist at TIA rapid assessment clinic, including blood testing, electrocardiogram, carotid ultrasound scanning and brain CT and/or MRI (see 2.1.1. for full battery of clinical tests).
3. Venous blood sampling (20 mL), buffy coat (for DNA extraction), and unstimulated whole saliva sampling (1 mL) for pre-analytical processing and long-term storage (2.1.4.1. for pre-analytical considerations protocol).
4. Three month follow-up assessment by neurologist at TIA rapid assessment clinic including venous blood collection and saliva sampling for later biomarker.

Genetic and protein samples will be deidentified to researchers in order to maintain blinding to diagnosis and collection order. A set of 11 proposed TIA-associated genetic and protein diagnostic biomarkers (see Table 1) including apolipoprotein A-I and A-IV will be measured. At the end of the recruitment phase, participant samples will be genotyped in one batch using polymerase chain reaction procedure for two proposed single nucleotide polymorphism biomarkers: PAI-1 -675 4G/5G and 5,10-MTHFR C677T. Protein biomarker measurements will be performed at the end of the follow-up phase in one batch of testing using sandwich enzyme-linked immunosorbent assay’s (ELISA) for: S-100B, GFAP, NSE, MBP, PBP, CP, C8-gamma, APOA-I and APOA-IV.

The aim of this prospective TIA biomarker validation study is to assess the potential for any of the listed or identified candidate biomarkers to contribute to diagnostic utility beyond the current clinical assessments and testings. Harrell’s rule has been applied to power calculations in diagnostic studies, whereby for each determinant used in multivariate logistic regression analysis, at least ten participants are required in the smallest category of the outcome variable (Harrell et al., 1984). Therefore, given that a TIA rapid assessment clinic can expect approximately 70% of referred patients having a

confirmed TIA diagnosis, approximately 30% will be mimic conditions and hence form the smallest outcome category (Lavallee et al., 2007). As we propose to evaluate 11 potential diagnostic biomarker determinants, 110 patients with mimic conditions will be required. This means that a total of (110 x (1/0.3)) = 367 patients with TIA diagnosis will be required. To allow for loss of participants due to drop-out, missing data points, or exclusion, we aim to recruit 150 patients with mimic conditions and 400 patients with TIA diagnoses.

Historically, the risk of recurrent ischaemic stroke in the first 3-months following a TIA or minor stroke was between 12 to 20% (Johnston, Gress, Browner, & Sidney, 2000; Lovett et al., 2003). However, changes in definition and managment of TIA including use of specialised rapid assessment TIA units that allow for immediate initiation of antiplatelet therapies, anticoagulation (in the presence of atrial fibrillation), revascularisation (in patients with severe carotid stenosis) and other secondary stroke-prevention strategies (i.e. use of statins and/or blood-pressure lowering medications) has mitigated the incidence of a recurrent ischaemic stroke event (Amarenco et al., 2016; Easton et al., 2009; Lavallee et al., 2007; Rothwell et al., 2007). Findings from a recent multicentre TIA registry project identified the current risk of stroke and other vascular events is 3.7% at 90 days (Amarenco et al., 2016). We can therefore estimate that our prospective study will yeild approximately 15 patients with TIA that will develop an ischaemic stroke within 3-months. This sample size will be insufficiently powered to allow multivariable regression analysis to explore the prognostic utility of the proposed candidate biomarkers to predict ischaemic stroke events, and therefore can only provide anecdotal/hypothesis-generating results.

This thesis has demonstrated that 2D-DIGE combined with MS and MRM-MS forms a powerful proteomic study design to identify differentially expressed plasma proteins. When comparing depleted human plasma from TIA, mimic and healthy control volunteers at acute presentation and 90-day follow-up, this thesis has identified for the first time that apolipoprotein's A-I and A-IV may be potential diagnostic biomarker candidates for TIA. MRM-MS was found to be a suitable platform to quantify and verify the significant decrease in APOA-I abundance in acute TIA presentation, while confirmed the exclusive increase in APOA-IV abundance in follow-up TIA patients.

Although APOA-I and APOA-IV are potential plasma TIA biomarker candidates, larger-scale validation studies involving sufficiently powered groups of TIA and mimic patients is required.

In conclusion, transient ischaemic attack is a common precursor to an ischaemic stroke, making it an important but difficult condition to diagnose. The research carried out in this thesis is a step towards a blood-based diagnostic screening test, which could identify and distinguish a true TIA condition from mimic presentations, and help improve the acute management and prevention of ischaemic stroke.

6 APPENDICES

APPENDIX TABLE 1: CLINICAL INFORMATION SHEET FOR PROSPECTIVE PATIENT VOLUNTEERS.

Patient Detail	Date of Test: Time of Test: Name: DOB: ID no:
Diagnosis	
Time of onset of symptoms: or 3-month follow-up ☐	
Ethnicity	☐ Caucasian ☐ Aboriginal ☐ South Asian ☐ Oriental ☐ Afro-Caribbean
Sex	☐M ☐ F
Age	
Menopausal status/menstrual cycle	☐ Post-menopausal ☐ Cycle LMP: Day:
Fasting	
Exercise	
Smoking	
Alcohol	
Caffeine	
Co-morbidities	
Medications	

APPENDIX TABLE 2: PROTEASE AND PHOSPHATASE INHIBITOR COCKTAIL TARGETED ENZYME CLASSES.

Inhibitor	**Targeted Enzyme Class**
Sodium Fluoride	Ser/Thr and Acidic Phosphatases
Sodium Orthovanadate	Tyr and Alkaline Phosphatases
B-glycerophosphate	Ser/Thr Phosphatases
Sodium Pyrophosphate	Ser/Thr Phosphatases
Aprotinin	Ser Proteases
Bestatin	Amino-peptidases
E64	Cysteine Proteases
Leupeptin	Ser/Cys Proteases
EDTA (vacutainer)	Metalloproteases

APPENDIX TABLE 3. DEMOGRAPHICS AND CLINICAL CHARACTERISTICS OF INDIVIDUAL TIA PATIENTS.

	TIA 1	TIA 2	TIA 3	TIA 4	TIA 5	TIA 6
Age	77	86	82	80	84	55
Gender	F	M	M	F	F	F
Cardiovascular Risk Factors	HTN, IHD, HLD, T2DM	HTN	HTN	HTN, IHD, HLD	HTN	HTN, T2DM
Smoking	No	No	No	No	No	No
Clinical Cause (TIA)	Atrial Fibrillation	Left Internal Carotid Stenosis	Unknown	Unknown	Right Middle Cerebral Artery Stenosis	Patent Foramen Ovalae
Clinic Attended	COMBAT	RAC	COMBAT	RAC	COMBAT	RAC
Time from symptom onset to blood collection (days)	1	3	4	1	6	2
ACUTE PRESENTATION						
ABCD2 Score	6	3	4	3	4	6
Cardiovascular Meds	AP, Stat, IMDUR, ACE-I, CCB, MF, GCTN	ACE-I	ARB	CCB, ARB, Stat, BB, GCTN	Stat	ACE-I, MF, DM
TC (mmol/L)	3.10	5.00	3.90	4.40	4.30	4.1
Trig (mmol/L)	3.00	0.90	0.70	1.30	1.00	1.9
HDL-C (mmol/L)	1.20	1.1	0.90	1.30	1.20	0.9
LDL-C (mmol/L)	0.50	3.5	2.70	2.50	2.60	2.3
TC:HDL-C	2.60	4.55	4.30	3.4	3.60	4.6
APOA-I (mmol/L)	1.48	1.13	1.01	1.25	1.26	1.10
APOB (mmol/L)	0.60	0.92	0.72	0.81	0.66	0.98
APOB:APOA-I	0.41	0.81	0.71	0.65	0.52	0.89
hsCRP (mg/L)	0.81	7.80	9.80	0.98	3.90	0.88
Glucose (mmol/L)	11.10	4.70	5.3	8.6	5.50	7.6
3 MONTH FOLLOW–UP						
Cardiovascular Meds	AP, Stat, IMDUR, ACE-I, CCB, MF, GCTN	AP, Stat, ACE-I, CCB	AP, ARB	AP, ARB, Stat, BB, GCTN	AP, Stat, INHH	AP, Stat, MF, ACE-I, DM.
APOA-I (mmol/L)	1.45	1.07	1.09	1.21	1.33	1.12
APOB (mmol/L)	0.73	0.43	0.78	0.69	0.52	0.92
APOB:APOA-I	0.50	0.40	0.72	0.57	0.39	0.82
hsCRP (mg/L)	0.83	4.80	1.60	3.90	5.20	3.50

Cardiovascular Risk Factors denoted as: HTN = hypertension, IHD = Ischemic Heart Disease, HLD = Hyperlipidemia, T2DM = diabetes mellitus type 2. Clinics denoted as: COMBAT = community based rapid access TIA clinic, RAC = hospital based rapid assessment clinic. Medications are denoted as: ACE-I = angiotensin converting enzyme inhibitor, ARB = angiotensin II receptor blocker, AP = antiplatelet therapy, BB = beta-1-receptor blocker, CCB = calcium channel blocker, DM = Diamicron, GCTN = Glyceryl Trinitrate, IMDUR = Isosorbide Mononitrate, MF = Metformin, Stat = Statin. Blood biochemistry tests are denoted as: TC = total cholesterol, Trig = triglycerides, HDL-C = high density lipoprotein – cholesterol, LDL-C = low density lipoprotein – cholesterol, APOA-I = apolipoprotein A1, APOB = apolipoprotein B, hsCRP = high-sensitivity C-reactive protein.

APPENDIX TABLE 4. DEMOGRAPHICS AND CLINICAL CHARACTERISTICS OF INDIVIDUAL MIMIC PATIENTS.

	Mimic 1	Mimic 2	Mimic 3	Mimic 4	Mimic 5	Mimic 6
Age	52	68	83	82	55	70
Gender	F	M	F	F	F	F
Cardiovascular Risk Factors	HTN	HTN	HTN, HLD	HTN, HLD	HTN, HLD, T2DM	Nil
Smoking	No	No	No	No	No	No
Diagnosis (Mimic)	Migraine	Benign positional vertigo	Vasovagal Syncope	Epileptic Seizure	Migraine	Transient Global Amnesia
Clinic Attended	RAC	RAC	RAC	COMBAT	COMBAT	COMBAT
Time from symptom onset to blood collection (days)	1	1	10	2	5	7
ACUTE PRESENTATION						
Cardiovascular Meds	ARB	AP, Stat, BB, ACE-I	AP, Stat, BB	AP, GCTN, Stat, ARB	AP, MF, Stat, CCB, ARB	CCB
TC (mmol/L)	5.70	4.90	3.70	3.80	4.80	6.10
Trig (mmol/L)	1.80	2.0	0.80	1.40	0.60	1.00
HDL-C (mmol/L)	1.20	1.00	1.60	1.60	1.50	2.10
LDL-C (mmol/L)	3.70	3.60	1.70	1.60	3.00	3.50
TC:HDL-C	4.8	4.90	2.30	2.40	3.20	2.90
APOA-I (mmol/L)	1.22	1.02	1.40	1.61	1.51	1.76
APOB (mmol/L)	1.12	0.84	0.58	0.54	0.86	0.85
APOB:APOA-I	0.92	0.82	0.41	0.34	0.57	0.48
hsCRP (mg/L)	4.80	1.00	0.22	3.90	1.40	15.0*
Glucose (mmol/L)	5.40	6.20	5.60	8.2	6.70	5.50
3 MONTH FOLLOW–UP						
Cardiovascular Meds	ARB	AP, Stat, BB, ACE-I	AP, Stat, BB	AP, GCTN, Stat, ARB	AP, MF, Stat, CCB, ARB	CCB
APOA-I (mmol/L)	1.26	1.16	1.32	1.54	1.44	1.62
APOB (mmol/L)	1.09	0.94	0.69	0.60	0.53	0.84
APOB:APOA-I	0.86	0.81	0.52	0.39	0.37	0.52
hsCRP (mg/L)	2.30	1.30	0.50	3.60	2.6	190*

Risk Factors denoted as: HTN = hypertension, HLD = Hyperlipidemia, T2DM = diabetes mellitus type 2. Clinics denoted as: COMBAT = community based rapid access TIA clinic, RAC = hospital based rapid assessment clinic. ACE-I = angiotensin converting enzyme inhibitor, ARB = angiotensin II receptor blocker, AP = antiplatelet therapy, BB = beta-1-receptor blocker, CCB = calcium channel blocker, GCTN = Glyceryl Trinitrate, MF = Metformin, Stat = Statin. Blood biochemistry tests are denoted as: TC = total cholesterol, Trig = triglycerides, HDL-C = high density lipoprotein – cholesterol, LDL-C = low density lipoprotein – cholesterol, APOA-I = apolipoprotein A1, APOB = apolipoprotein B, hsCRP = high-sensitivity C-reactive protein, (*) = standard CRP assay measurement excluded from final analysis.

APPENDIX TABLE 5. DEMOGRAPHICS AND CLINICAL CHARACTERISTICS OF INDIVIDUAL HEALTHY CONTROL VOLUNTEERS.

	Healthy Control 1	Healthy Control 2	Healthy Control 3	Healthy Control 4	Healthy Control 5	Healthy Control 6
Age	61	50	69	43	53	54
Gender	M	F	M	F	F	F
Smoking	No	No	No	No	No	No
INITIAL PRESENTATION						
TC (mmol/L)	3.50	4.60	4.50	5.00	5.20	6.10
Trig (mmol/L)	0.90	1.10	0.60	0.60	0.60	0.60
HDL-C (mmol/L)	1.40	1.60	1.40	1.70	1.6	2.10
LDL-C (mmol/L)	1.70	2.50	2.80	3.00	3.3	3.70
TC:HDL-C	2.50	2.90	3.20	2.90	3.2	2.90
APOA-I (mmol/L)	1.34	1.47	1.21	1.32	1.41	1.61
APOB (mmol/L)	0.52	0.74	0.74	0.73	0.80	0.84
APOB:APOA-I	0.39	0.50	0.61	0.55	0.57	0.52
hsCRP (mg/L)	3.10	0.91	1.90	0.45	1.60	1.10
Glucose (mmol/L)	4.80	4.60	4.80	4.20	5.6	4.8
3 MONTH FOLLOW–UP						
APOA-I (mmol/L)	1.25	1.58	1.09	1.51	1.42	1.73
APOB (mmol/L)	0.46	0.81	0.79	0.84	0.81	0.93
APOB:APOA-I	0.37	0.51	0.72	0.56	0.57	0.54
hsCRP (mg/L)	3.40	1.30	4.60	0.77	2.20	0.59

Blood biochemistry tests are denoted as: TC = total cholesterol, Trig = triglycerides, HDL-C = high density lipoprotein – cholesterol, LDL-C = low density lipoprotein – cholesterol, APOA-I = apolipoprotein A1, APOB = apolipoprotein B, hsCRP = high-sensitivity C-reactive protein.

APPENDIX TABLE 6: PROTEOTYPIC PEPTIDE SEQUENCES AND SELECTED MRM TRANSITIONS FOR APOLIPOPROTEIN A-I.

Protein (Swiss-Prot Primary Accession Number)	Peptide Sequence	Retention Time (min)	Q1	Q3
Apolipoprotein A-I (P02647)	DLATVYVDVLK	19.117	618.3464 (2+)	**736.42 ($y6^{1+}$)** 835.49 ($y7^{1+}$) 936.55 ($y8^{1+}$) 1007.58 ($y9^{1+}$) 573.36 ($y5^{1+}$)
	DYVSQFEGSALGK	17.8	700.8392 (2+)	**1023.51 ($y10^{1+}$)** 808.42 ($y8^{1+}$) 661.35 ($y7^{1+}$) 532.31 ($y6^{1+}$) 378.17 ($y4^{1+}$)
	LLDNWDSVTSTFSK	18.683	806.8962 (2+)	**971.45 ($y9^{1+}$)** 670.34 ($y6^{1+}$) 856.45 ($y8^{1+}$) 1386.60 ($y12^{1+}$) 1157.54 ($y10^{1+}$)
	LSPLGEEMR	15.567	516.2629 (2+)	**621.27 ($y5^{1+}$)** 831.40 ($y7^{1+}$) 734.35 ($y6^{1+}$) 918.43 ($y8^{1+}$)
	THLAPYSDELR	14.367	434.5548 (3+)	**619.31 ($y5^{1+}$)** 532.27($y4^{1+}$) 417.25 ($y3^{1+}$)

Candidate proteins and unique signal peptide sequences with 3-5 of the best transitions used for quantification selected using Skyline 2.1 software. **BOLD** = Transition with the most abundant signal.

APPENDIX TABLE 7: PROTEOTYPIC PEPTIDE SEQUENCES AND SELECTED MRM TRANSITIONS FOR APOLIPOPROTEIN A-IV.

Protein (Swiss-Prot Primary Accession Number)	Peptide Sequence	Retention Time (min)	Q1	Q3
Apolipoprotein A-IV (P06727)	ALVQQMEQLR	16.733	608.3266 (2+)	**932.46 ($y7^{1+}$)** 804.40 ($y6^{1+}$) 676.34 ($y5^{1+}$) 545.31 ($y4^{1+}$) 1031.54 ($y8^{1+}$)
	IDQTVEELR	14.467	551.7905 (2+)	**746.41 ($y6^{1+}$)** 645.36 ($y5^{1+}$) 546.29 ($y4^{1+}$) 989.49 ($y8^{1+}$) 874.47 ($y7^{1+}$)
	ISASAEELR	13.35	488.2587 (2+)	**862.43 ($y8^{1+}$)** 775.40 ($y7^{1+}$) 704.35 ($y6^{1+}$) 617.32 ($y5^{1+}$) 546.29 ($y4^{1+}$)
	LAPLAEDVR	15.333	492.2786 (2+)	**589.29 ($y5^{1+}$)** 274.19 ($y2^{1+}$) 799.44 ($y7^{1+}$) 702.38 ($y6^{1+}$)
	SLAPYAQDTQEK	13.617	675.8308 (2+)	**1079.50 ($y9^{1+}$)** 819.38 ($y7^{1+}$) 982.45 ($y8^{1+}$) 1150.52 ($y10^{1+}$) 748.34 ($y6^{1+}$)

Candidate proteins and unique signal peptide sequences with 3-5 of the best transitions used for quantification selected using Skyline 2.1 software. **BOLD** = Transition with the most abundant signal.

APPENDIX TABLE 8: PROTEOTYPIC PEPTIDE SEQUENCES AND SELECTED MRM TRANSITIONS FOR FIBRINOGEN ALPHA- AND BETA-CHAIN.

Protein (Swiss-Prot Primary Accession Number)	Peptide Sequence	Retention Time (min)	Q1	Q3
Fibrinogen alpha-chain (P02671)	QFTSSTSYNR	12.383	595.7749 (2+)	**915.41 ($y8^{1+}$)** 727.34 ($y6^{1+}$) 814.37 ($y7^{1+}$) 640.30 ($y5^{1+}$) 539.26 ($y4^{1+}$)
	TVIGPDGHK	10.75	462.251 (2+)	**723.38 ($y7^{1+}$)** 610.29 ($y6^{1+}$) 553.26 ($y5^{1+}$)
	VQHIQLLQK	14.333	553.8378 (2+)	**879.54 ($y7^{1+}$)** 742.48 ($y6^{1+}$) 629.39 ($y5^{1+}$)
	GGSTSYGTGSETESPR	11.483	786.8423 (2+)	**1020.47 ($y10^{1+}$)** 862.37 ($y8^{1+}$) 1183.51 ($y11^{1+}$) 1270.55 ($y12^{1+}$)
Fibrinogen beta-chain (P02675)	DNDGWLTSDPR	16.417	638.2833 (2+)	**272.17 ($y2^{1+}$)** 688.36 ($y6^{1+}$) 575.28 ($y5^{1+}$)
	EDGGGWWYNR	17.4	620.2619 (2+)	**638.30 ($y4^{1+}$)** 452.22 ($y3^{1+}$) 824.38 ($y5^{1+}$) 881.40 ($y6^{1+}$) 995.46 ($y8^{1+}$)
	LIQPDSSVKPYR	13.75	468.2616 (3+)	**435.24 ($y3^{1+}$)** 563.32 ($y4^{1+}$) 662.41 ($y5^{1+}$)
	TMTIHNGMF	17.817	526.7296 (2+)	**820.35 ($y7^{1+}$)** 606.23 ($y5^{1+}$) 719.32 ($y6^{1+}$)
	TPCTVSCNIPVVSGK	15.55	810.3965 (2+)	586.35 ($y6^{1+}$) 291.17 ($y3^{1+}$) 390.24 ($y4^{1+}$)

Candidate proteins and unique signal peptide sequences with 3-5 of the best transitions used for quantification selected using Skyline 2.1 software. **BOLD** = Transition with the most abundant signal.

APPENDIX TABLE 9: PROTEOTYPIC PEPTIDE SEQUENCES AND SELECTED MRM TRANSITIONS FOR GELSOLIN.

Protein (Swiss-Prot Primary Accession Number)	Peptide Sequence	Retention Time (min)	Q1	Q3
Gelsolin (P06396)	GASQAGAPQGR	8.983	500.2516 (2+)	**656.35 ($y7^{1+}$)** 585.31 ($y6^{1+}$) 457.25 ($y4^{1+}$)
	EVQGFESATFLGYFK	19.433	861.9222 (2+)	**294.18 ($y2^{1+}$)** 514.27 ($y4^{1+}$) 627.36 ($y5^{1+}$) 774.42 ($y6^{1+}$) 875.51 ($y7^{1+}$)
	GGVASGFK	12.567	361.6962 (+2)	**656.35 ($y7^{1+}$)** 585.31 ($y6^{1+}$) 457.25 ($y4^{1+}$)
	TGAQELLR	14.483	444.2509 (2+)	**509.27 ($y5^{1+}$)** 438.23 ($y4^{1+}$) 608.33 ($y6^{1+}$)

Candidate proteins and unique signal peptide sequences with 3-5 of the best transitions used for quantification selected using Skyline 2.1 software. **BOLD** = Transition with the most abundant signal.

APPENDIX TABLE 10: PROTEOTYPIC PEPTIDE SEQUENCES AND SELECTED MRM TRANSITIONS FOR COMPLEMENT C4-A.

Protein (Swiss-Prot Primary Accession Number)	Peptide Sequence	Retention Time (min)	Q1	Q3
Complement C4-A (P0C0L4)	AEMADQAAAWLTR	18.167	717.346 (2+)	**717.44 ($y6^{1+}$)** 788.44 ($y7^{1+}$) 1102.57 ($y10^{1+}$) 916.50 ($y8^{1+}$) 1031.53 ($y9^{1+}$)
	ANSFLGEK	14.3	433.2234 (3+)	**680.36 ($y6^{1+}$)** 446.26 ($y4^{1+}$) 333.17 ($y3^{1+}$)
	DHAVDLIQK	13.867	519.7818 (2+)	**786.47 ($y7^{1+}$)** 715.42 ($y6^{1+}$) 616.37 ($y5^{1+}$)
	FGLLDEDGK	17.217	497.2477 (0)	**846.42 ($y8^{1+}$)** 789.39 ($y7^{1+}$) 676.31 ($y6^{1+}$) 563.23 ($y5^{1+}$)

Candidate proteins and unique signal peptide sequences with 3-5 of the best transitions used for quantification selected using Skyline 2.1 software. **BOLD** = Transition with the most abundant signal.

APPENDIX TABLE 11: APOLIPOPROTEIN A-I MRM DATA

	Peptide 1	Peptide 2	Peptide 3	Peptide 4	Peptide 5	Protein Abundance	Mean Abundance (SEM)
TIA Initial 1	2,088,676	2,499,715	7,808,259	1,239,421	1,337,894	14,973,966	17,183,926 (1,565,085)
TIA Initial 2	1,438,086	2,176,066	6,646,723	1,060,641	1,803,007	13,124,523	
TIA Initial 3	1,842,580	2,741,097	7,042,995	972,043	1,178,061	13,776,776	
TIA Initial 4	2,395,769	3,559,003	9,079,282	1,677,808	1,857,766	18,569,627	
TIA Initial 5	2,837,520	4,469,829	10,611,247	2,210,006	2,654,072	22,782,675	
TIA Initial 6	2,171,252	3,868,301	10,054,209	1,637,599	2,144,630	19,875,992	
TIA Follow-up 1	2,775,430	3,968,679	12,688,461	2,105,435	2,634,049	24,172,055	24,300,541 (3,949,435)
TIA Follow-up 2	2,039,527	2,894,448	7,352,669	1,099,635	977,765	14,364,045	
TIA Follow-up 3	1,603,793	2,831,186	6,664,586	1,036,088	1,322,694	13,458,345	
TIA Follow-up 4	4,992,985	7,995,908	16,817,934	3,874,838	3,326,672	37,008,337	
TIA Follow-up 5	3,652,805	6,497,675	16,353,316	3,317,638	3,895,486	33,716,919	
TIA Follow-up 6	2,819,307	5,084,002	10,032,066	2,805,246	2,342,926	23,083,546	
Mimic Initial 1	928,194	1,458,556	4,391,675	692,754	921,643	8,392,822	23,603,034 (5,715,105)
Mimic Initial 2	808,727	1,337,567	3,591,089	516,224	564,052	6,817,659	
Mimic Initial 3	2,569,311	3,658,990	9,842,062	1,540,362	1,686,416	19,297,141	
Mimic Initial 4	5,282,494	7,284,287	16,295,571	1,407,172	3,008,995	33,278,519	
Mimic Initial 5	3,862,285	6,700,811	18,665,115	3,961,097	3,456,759	36,646,067	
Mimic Initial 6	4,118,409	6,939,154	19,008,745	3,548,154	3,571,534	37,185,995	
Mimic Follow-up 1	639,738	1,296,397	4,006,583	600,245	1,076,720	7,619,683	25,803,036 (4,327,725)
Mimic Follow-up 2	1,963,613	3,692,078	10,485,930	1,677,487	1,519,901	19,339,010	
Mimic Follow-up 3	2,805,352	5,222,447	14,942,310	3,055,367	2,798,755	28,824,231	
Mimic Follow-up 4	3,345,685	6,125,758	15,681,109	1,547,827	2,974,617	29,674,996	
Mimic Follow-up 5	4,054,482	6,247,133	15,896,084	2,990,844	3,525,366	32,713,910	
Mimic Follow-up 6	5,014,453	7,622,837	18,276,073	3,018,381	2,714,643	36,646,388	
Control Initial 1	1,387,174	1,878,584	5,288,619	854,632	1,007,403	10,416,412	24,098,520 (3,730,266)
Control Initial 2	1,732,048	3,022,147	9,091,265	1,317,344	2,037,496	17,200,299	
Control Initial 3	2,400,841	4,327,718	11,620,161	1,982,856	2,398,747	22,730,322	
Control Initial 4	4,544,542	6,873,043	14,941,741	2,443,794	2,718,383	31,521,503	
Control Initial 5	2,700,823	5,177,544	14,861,573	2,696,360	2,747,518	28,183,818	
Control Initial 6	4,018,574	7,450,038	17,564,521	2,699,551	2,806,079	34,538,763	
Control Follow-up 1	1,232,977	2,403,061	6,234,366	913,650	1,717,101	12,501,156	26,827,390 (4,207,335)
Control Follow-up 2	2,661,309	4,216,943	12,143,157	1,954,162	2,506,693	23,482,265	
Control Follow-up 3	3,032,727	5,538,009	12,990,195	3,673,604	2,858,274	28,092,808	
Control Follow-up 4	4,772,739	8,164,193	19,977,269	4,068,324	4,014,977	40,997,502	
Control Follow-up 5	2,577,775	4,387,163	9,948,619	1,547,794	2,076,985	20,538,335	
Control Follow-up 6	4,016,709	7,080,366	17,103,209	3,559,850	3,592,144	35,352,277	

APPENDIX TABLE 12: APOLIPOPROTEIN A-IV MRM DATA

	Peptide 1	Peptide 2	Peptide 3	Peptide 4	Peptide 5	Protein Abundance	Mean Abundance (SEM)
TIA Initial 1	571,855	395,588	781,402	831,281	197,028	2,777,154	
TIA Initial 2	214,280	134,722	303,018	338,183	75,337	1,065,539	
TIA Initial 3	296,864	173,378	348,838	411,834	142,286	1,373,199	1,862,457
TIA Initial 4	317,968	231,773	425,895	445,777	196,233	1,617,647	(259,231)
TIA Initial 5	361,281	290,845	549,781	535,527	267,257	2,004,691	
TIA Initial 6	449,854	327,953	632,088	650,613	276,006	2,336,514	
TIA Follow-up 1	740,797	489,327	991,877	1,004,778	244,079	3,470,857	
TIA Follow-up 2	445,090	246,229	552,014	546,796	131,105	1,921,235	
TIA Follow-up 3	312,177	186,705	384,231	417,634	182,862	1,483,609	3,243,666
TIA Follow-up 4	1,102,759	822,667	1,528,735	1,512,851	702,359	5,669,371	(604,117)
TIA Follow-up 5	586,725	481,142	899,036	897,030	401,962	3,265,895	
TIA Follow-up 6	700,753	509,820	1,007,245	921,343	511,866	3,651,026	
Mimic Initial 1	187,131	120,189	260,463	282,955	103,837	954,576	
Mimic Initial 2	61,672	48,477	95,337	102,339	21,572	329,397	
Mimic Initial 3	197,426	151,201	297,824	328,371	134,434	1,109,256	1,928,354
Mimic Initial 4	418,028	293,568	610,154	670,358	138,318	2,130,426	(642,066)
Mimic Initial 5	811,120	706,656	1,299,790	1,329,700	613,612	4,760,878	
Mimic Initial 6	415,411	330,283	636,018	599,858	304,023	2,285,593	
Mimic Follow-up 1	176,060	99,825	239,878	249,932	107,782	873,477	
Mimic Follow-up 2	282,593	167,268	350,081	339,075	85,136	1,224,154	
Mimic Follow-up 3	488,040	383,714	733,033	740,093	361,746	2,706,626	2,598,043
Mimic Follow-up 4	553,736	395,857	733,371	818,079	197,656	2,698,698	(795,265)
Mimic Follow-up 5	1,141,618	900,106	1,738,885	1,669,744	818,951	6,269,305	
Mimic Follow-up 6	377,415	232,108	480,848	520,867	204,763	1,816,001	
Control Initial 1	138,767	88,350	171,932	200,530	79,829	679,408	
Control Initial 2	267,906	202,464	397,607	421,847	97,832	1,387,657	
Control Initial 3	386,465	274,087	569,611	589,405	244,891	2,064,458	1,797,927
Control Initial 4	413,363	259,417	520,031	518,251	227,973	1,939,035	(314,329)
Control Initial 5	324,764	237,868	466,801	480,528	206,188	1,716,149	
Control Initial 6	578,977	423,296	789,427	877,350	331,804	3,000,855	
Control Follow-up 1	180,677	104,515	236,091	258,872	99,509	879,663	
Control Follow-up 2	298,369	214,079	419,452	461,866	105,942	1,499,710	
Control Follow-up 3	628,846	542,852	958,755	1,004,898	422,211	3,557,562	2,056,174
Control Follow-up 4	577,429	402,823	790,023	768,229	416,335	2,954,839	(460,860)
Control Follow-up 5	173,907	126,161	242,962	248,175	96,667	887,872	
Control Follow-up 6	433,749	382,747	725,165	689,690	326,045	2,557,397	

APPENDIX TABLE 13: FIBRINOGEN ALPHA CHAIN MRM DATA

	Peptide 1	Peptide 2	Peptide 3	Peptide 4	Protein Abundance	Mean Abundance (SEM)
TIA Initial 1	1,764,484	898,300	928,821	542,979	4,134,584	
TIA Initial 2	1,813,007	995,493	999,222	725,365	4,533,088	
TIA Initial 3	1,481,705	738,625	616,043	413,592	3,249,965	4,568,856
TIA Initial 4	2,193,335	1,206,624	1,019,960	715,842	5,135,761	(600,233)
TIA Initial 5	3,285,817	1,628,374	1,356,741	885,172	7,156,104	
TIA Initial 6	1,425,203	732,870	643,192	402,369	3,203,634	
TIA Follow-up 1	1,627,581	826,579	918,868	555,222	3,928,250	
TIA Follow-up 2	2,018,691	1,077,803	1,183,005	684,697	4,964,196	
TIA Follow-up 3	1,672,442	957,941	767,488	482,428	3,880,298	4,971,082
TIA Follow-up 4	3,367,563	1,934,176	1,701,147	980,929	7,983,814	(628,963)
TIA Follow-up 5	2,217,048	1,088,019	837,327	650,940	4,793,335	
TIA Follow-up 6	1,813,933	890,785	934,445	637,431	4,276,595	
Mimic Initial 1	1,600,835	860,340	868,444	503,749	3,833,367	
Mimic Initial 2	973,458	621,849	539,215	337,110	2,471,632	
Mimic Initial 3	1,280,761	631,142	549,606	500,733	2,962,242	4,285,021
Mimic Initial 4	1,303,555	756,556	551,466	463,533	3,075,111	(791,793)
Mimic Initial 5	2,739,876	1,302,818	1,147,684	949,004	6,139,382	
Mimic Initial 6	3,146,060	1,613,730	1,417,322	1,051,280	7,228,393	
Mimic Follow-up 1	2,223,650	1,022,476	1,014,589	741,393	5,002,108	
Mimic Follow-up 2	3,517,884	2,009,828	1,949,160	1,203,715	8,680,587	
Mimic Follow-up 3	2,388,314	1,404,328	1,376,661	1,030,448	6,199,752	6,331,871
Mimic Follow-up 4	4,005,379	2,043,501	1,924,894	1,276,624	9,250,398	(934,988)
Mimic Follow-up 5	2,633,284	1,286,679	1,068,439	698,907	5,687,309	
Mimic Follow-up 6	1,420,621	801,171	513,601	435,678	3,171,072	
Control Initial 1	2,302,834	1,225,680	1,342,867	827,232	5,698,614	
Control Initial 2	2,336,533	1,223,709	1,064,801	726,662	5,351,705	
Control Initial 3	1,594,506	848,467	760,323	555,721	3,759,017	4,282,455
Control Initial 4	1,764,429	987,429	836,246	442,888	4,030,992	(495,575)
Control Initial 5	1,921,303	985,306	893,523	726,817	4,526,949	
Control Initial 6	1,035,466	543,984	463,100	284,902	2,327,452	
Control Follow-up 1	1,683,484	867,447	953,711	565,047	4,069,688	
Control Follow-up 2	2,160,824	1,183,134	1,070,028	700,060	5,114,045	
Control Follow-up 3	5,686,163	2,925,106	3,322,749	2,872,755	14,806,773	6,191,542
Control Follow-up 4	2,823,518	1,527,665	1,448,196	799,337	6,598,716	(1,806,530)
Control Follow-up 5	1,118,375	674,566	482,574	333,209	2,608,725	
Control Follow-up 6	1,786,984	866,519	794,893	502,911	3,951,306	

APPENDIX TABLE 14: FIBRINOGEN BETA CHAIN MRM DATA

	Peptide 1	Peptide 2	Peptide 3	Peptide 4	Peptide 5	Protein Abundance	Mean Abundance (SEM)
TIA Initial 1	2269246	1315328	71194	376468	7119	4,039,356	
TIA Initial 2	2444742	1207748	66449	358788	6679	4,084,406	
TIA Initial 3	1718948	847441	72103	189691	4947	2,833,131	4,209,423
TIA Initial 4	3458487	1988012	118881	451434	9473	6,026,287	(649,871)
TIA Initial 5	3582257	1988763	119416	361180	11154	6,062,770	
TIA Initial 6	1390893	646228	86167	83355	3945	2,210,587	
TIA Follow-up 1	2372852	1281562	58252	186558	5553	3,904,778	
TIA Follow-up 2	2805039	1433608	84575	234750	6858	4,564,831	
TIA Follow-up 3	1688859	915969	78067	177807	3985	2,864,686	4,147,734
TIA Follow-up 4	4028581	2639951	134847	200208	10390	7,013,977	(624,643)
TIA Follow-up 5	2167819	1092690	73129	113390	4637	3,451,664	
TIA Follow-up 6	1755196	1044836	77867	200187	8379	3,086,466	
Mimic Initial 1	2250225	1261805	59300	388082	6151	3,965,562	
Mimic Initial 2	1210370	562582	38350	180399	2293	1,993,995	
Mimic Initial 3	1386770	738403	59048	123027	3792	2,311,040	3,533,460
Mimic Initial 4	1163404	671447	37475	37946	1070	1,911,343	(738,402)
Mimic Initial 5	2819182	1455853	144317	114641	9459	4,543,451	
Mimic Initial 6	4001121	2026510	189104	242567	16066	6,475,368	
Mimic Follow-up 1	3630695	1985586	101926	408379	5913	6,132,498	
Mimic Follow-up 2	5166307	2529347	134472	561345	9313	8,400,784	
Mimic Follow-up 3	2830375	1485259	102506	247172	9438	4,674,751	5,705,393
Mimic Follow-up 4	5250676	2912294	200230	220852	16577	8,600,628	(1,016,785)
Mimic Follow-up 5	2632905	1261248	118071	110471	7809	4,130,504	
Mimic Follow-up 6	1485372	689574	50062	67380	807	2,293,194	
Control Initial 1	3379359	1728797	108320	526027	8558	5,751,061	
Control Initial 2	2519612	1362960	93227	368188	5607	4,349,595	
Control Initial 3	1875824	999532	65089	119624	6060	3,066,129	3,586,327
Control Initial 4	2305719	1225853	83705	128919	3887	3,748,084	(594,232)
Control Initial 5	2113052	969342	86384	53540	8372	3,230,691	
Control Initial 6	876270	423343	39778	29264	3751	1,372,406	
Control Follow-up 1	2095284	1050990	66541	153235	4280	3,370,330	
Control Follow-up 2	2541258	1382612	89318	268089	5745	4,287,021	
Control Follow-up 3	6935802	3977859	248619	1137545	26795	12,326,620	4,945,590
Control Follow-up 4	3117356	1781354	118660	297829	7442	5,322,641	(1,563,759)
Control Follow-up 5	1028467	516725	46607	59258	1039	1,652,096	
Control Follow-up 6	1762674	840294	70715	33063	8089	2,714,835	

APPENDIX TABLE 15: GELSOLIN MRM DATA

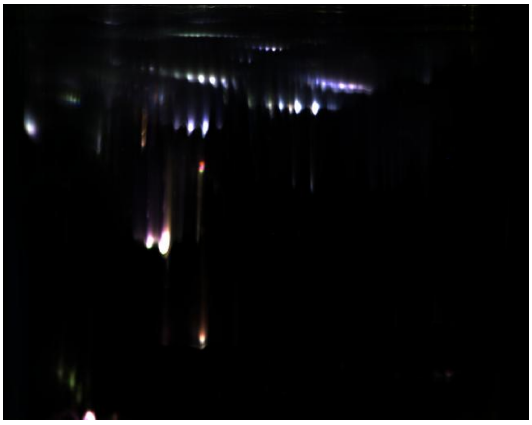
	Peptide 1	Peptide 2	Peptide 3	Peptide 4	Protein Abundance	Mean Abundance (SEM)
TIA Initial 1	33403	76907	214206	394128	718,644	
TIA Initial 2	53294	34970	177021	363306	628,591	
TIA Initial 3	51956	47826	149254	313405	562,442	768,321
TIA Initial 4	46796	46984	404407	677424	1,175,610	(105,378)
TIA Initial 5	30662	30738	335452	592186	989,038	
TIA Initial 6	68650	25018	151384	290551	535,603	
TIA Follow-up 1	25067	65068	214926	408232	713,293	
TIA Follow-up 2	39726	39097	197222	333735	609,780	
TIA Follow-up 3	45405	32564	220983	408984	707,936	942,434
TIA Follow-up 4	65068	348919	277472	553446	1,244,905	(120,939)
TIA Follow-up 5	39097	612336	181606	416366	1,249,405	
TIA Follow-up 6	32564	537818	209637	349266	1,129,286	
Mimic Initial 1	45414	19291	167210	234726	466,641	
Mimic Initial 2	15384	56079	68586	137927	277,976	
Mimic Initial 3	50571	13297	190790	421548	676,205	799,870
Mimic Initial 4	15558	399659	57569	133425	606,211	(217,391)
Mimic Initial 5	56079	785878	308978	618718	1,769,652	
Mimic Initial 6	37742	258687	247953	458154	1,002,537	
Mimic Follow-up 1	53294	34970	181709	258459	528,433	
Mimic Follow-up 2	46796	46984	276280	490371	860,430	
Mimic Follow-up 3	68650	25018	421547	809809	1,325,024	961,361
Mimic Follow-up 4	34970	374243	232956	409974	1,052,143	(144,641)
Mimic Follow-up 5	46984	681879	228840	421766	1,379,469	
Mimic Follow-up 6	29382	330175	82221	180891	622,669	
Control Initial 1	28140	36437	231169	222813	518,559	
Control Initial 2	98526	44264	544936	929104	1,616,829	
Control Initial 3	32211	37742	207189	420051	697,193	952,475
Control Initial 4	43833	344865	215803	378881	983,381	(170,036)
Control Initial 5	44264	290525	304026	603524	1,242,339	
Control Initial 6	13297	396568	75691	170992	656,548	
Control Follow-up 1	66975	66514	191815	200552	525,855	
Control Follow-up 2	87455	35993	330881	605519	1,059,848	
Control Follow-up 3	72533	29382	578689	1016572	1,697,177	1,199,424
Control Follow-up 4	66514	875817	403471	686691	2,032,493	(227,239)
Control Follow-up 5	35993	619273	92542	183001	930,809	
Control Follow-up 6	25018	361021	182996	381328	950,364	

APPENDIX TABLE 16: COMPLEMENT C4-A MRM DATA

	Peptide 1	Peptide 2	Peptide 3	Peptide 4	Protein Abundance	Mean Abundance (SEM)
TIA Initial 1	404604	292342	320100	475392	1,492,439	
TIA Initial 2	196273	188329	239950	415186	1,039,738	
TIA Initial 3	256395	191974	436742	374750	1,259,861	1,275,056
TIA Initial 4	148860	322448	372833	457535	1,301,676	(66,353)
TIA Initial 5	211864	362500	127977	695324	1,397,665	
TIA Initial 6	281074	158169	319651	400064	1,158,958	
TIA Follow-up 1	446289	274333	191569	479012	1,391,202	
TIA Follow-up 2	180640	240953	302097	471542	1,195,232	
TIA Follow-up 3	257507	199055	313559	351381	1,121,501	1,199,728
TIA Follow-up 4	90802	182800	323540	349792	946,934	(100,260)
TIA Follow-up 5	117873	190032	188287	471846	968,038	
TIA Follow-up 6	373195	344078	388056	470130	1,575,459	
Mimic Initial 1	98746	190052	133447	310165	732,410	
Mimic Initial 2	115650	76846	182837	208814	584,147	
Mimic Initial 3	139709	155134	142375	311462	748,680	1,018,892
Mimic Initial 4	57913	51133	288578	92723	490,348	(254,880)
Mimic Initial 5	704060	390901	85333	890392	2,070,686	
Mimic Initial 6	221945	219949	471542	573644	1,487,080	
Mimic Follow-up 1	106416	203085	200237	319651	829,389	
Mimic Follow-up 2	630129	537255	184502	821929	2,173,815	
Mimic Follow-up 3	395815	468673	169006	787566	1,821,059	1,532,738
Mimic Follow-up 4	409306	349396	314255	583367	1,656,325	(282,789)
Mimic Follow-up 5	596485	342961	475392	759743	2,174,582	
Mimic Follow-up 6	59590	65646	208814	207206	541,256	
Control Initial 1	172943	148627	204936	388056	914,563	
Control Initial 2	214558	266827	159628	685813	1,326,827	
Control Initial 3	180185	191289	165544	333122	870,140	1,085,290
Control Initial 4	104270	179554	123616	354685	762,126	(233,711)
Control Initial 5	485876	329444	479012	828181	2,122,513	
Control Initial 6	29936	53678	256048	175912	515,575	
Control Follow-up 1	156075	121752	178801	256048	712,676	
Control Follow-up 2	130172	181488	369716	472037	1,153,413	
Control Follow-up 3	634404	747214	270107	1255291	2,907,016	1,396,844
Control Follow-up 4	318483	401114	193905	679875	1,593,377	(333,232)
Control Follow-up 5	97312	83578	310165	215220	706,274	
Control Follow-up 6	235275	190290	415186	467557	1,308,309	

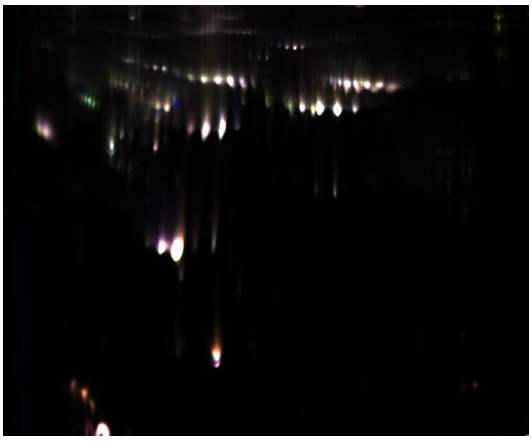
APPENDIX FIGURE 1: 2D-DIGE COMPARISON OF IMMUNODEPLETED PLASMA PROTEIN SAMPLES FROM SIX TIA, SIX MIMIC AND SIX HEALTHY CONTROL PARTICIPANTS.

TIA Patient 1

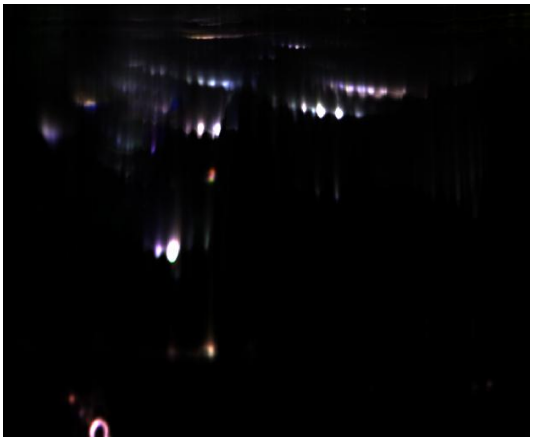


TIA Patient 2

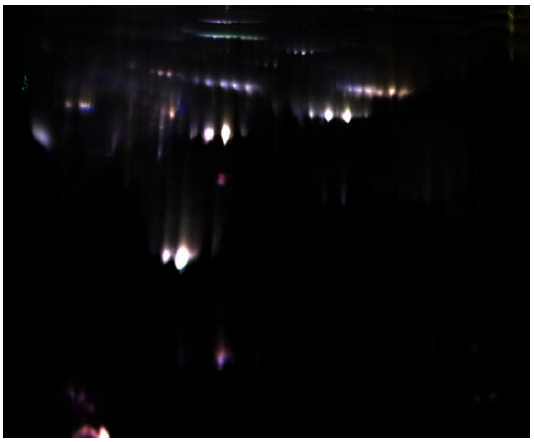
Mimic Patient 1



Mimic Patient 2

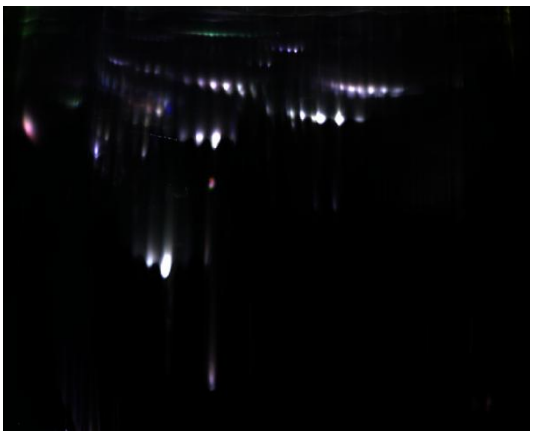


Healthy Control Volunteer 1



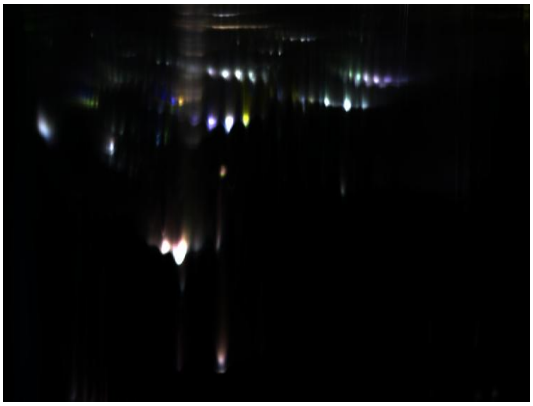
Healthy Control Volunteer 2

TIA Patient 3

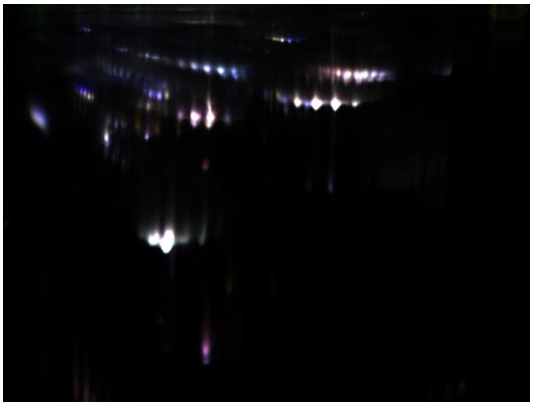


TIA Patient 4

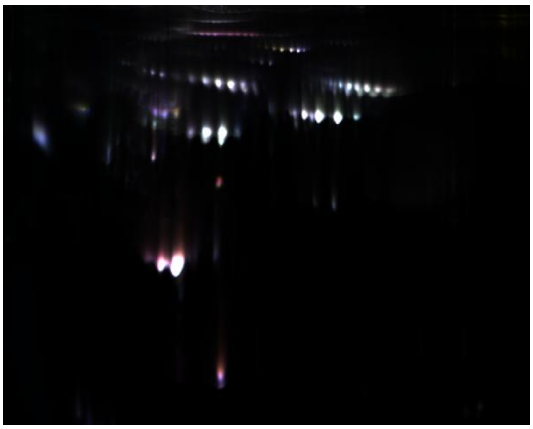
Mimic Patient 3



Mimic Patient 4



Healthy Control Volunteer 3



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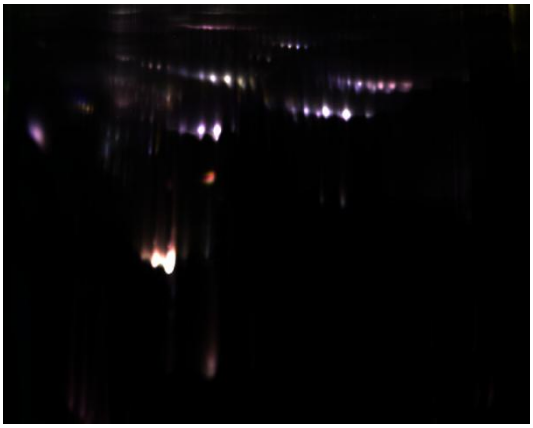
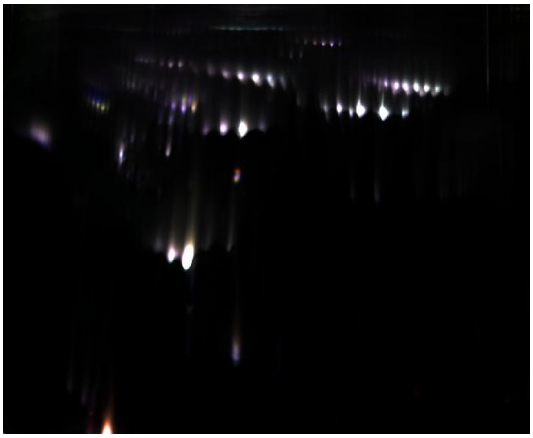
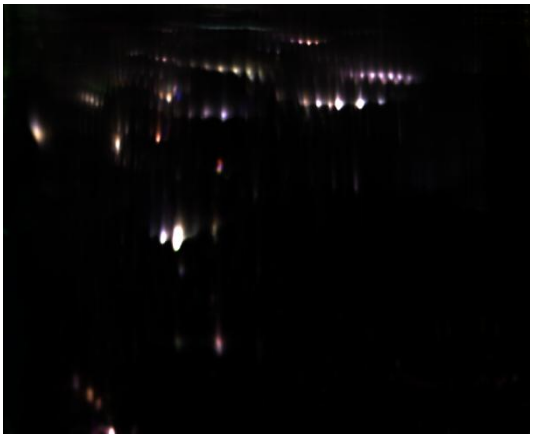


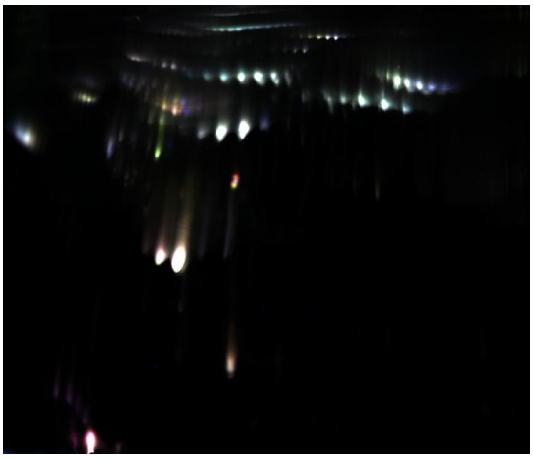
TIA Patient 5



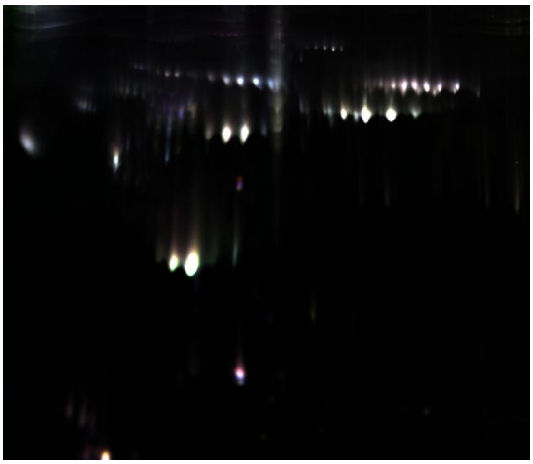
TIA Patient 6



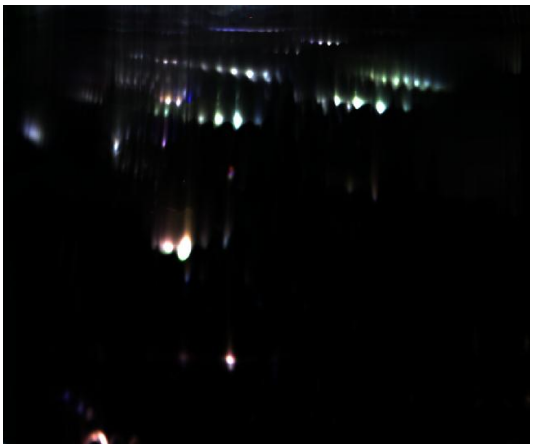
Mimic Patient 5



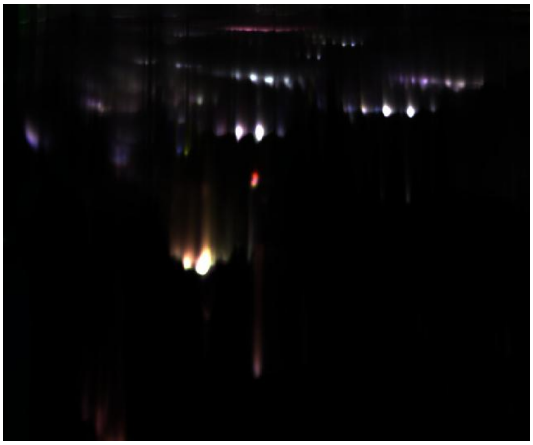
Mimic Patient 6



Healthy Control Volunteer 5



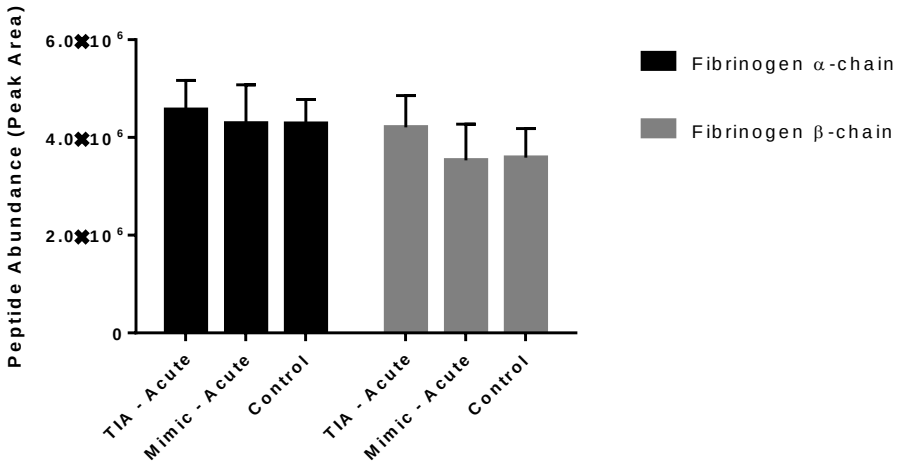
Healthy Control Volunteer 6



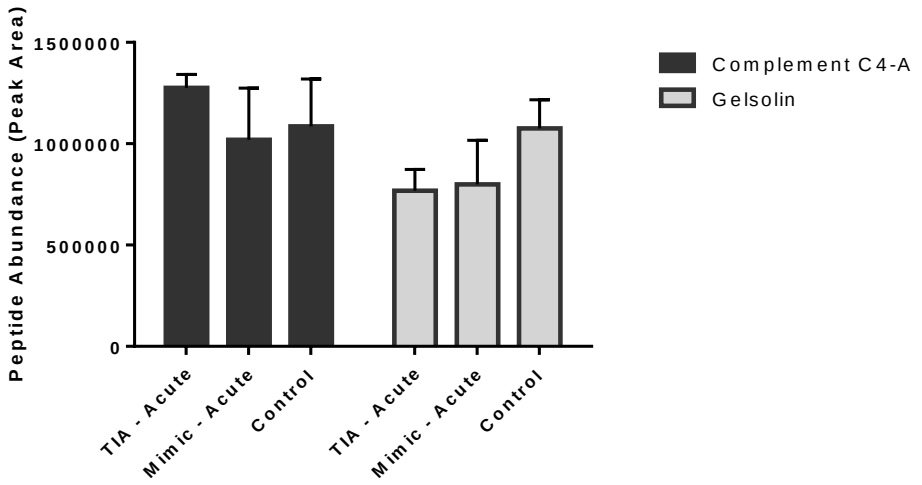
Eighteen gels representative of each participant and depicting three CyDye labelled samples: 1) acute, 2) 3-month follow-up, 3) pooled internal standard. Fluorescently labelled samples were combined and separated in the first dimension across a pH range of 3-11 (isoelectric focusing) and in the second dimension by molecular weight (from 250-10 kDa).

APPENDIX FIGURE 2: MULTIPLE REACTION MONITORING (MRM) QUANTIFICATION OF CANDIDATE PLASMA PROTEINS.

A.



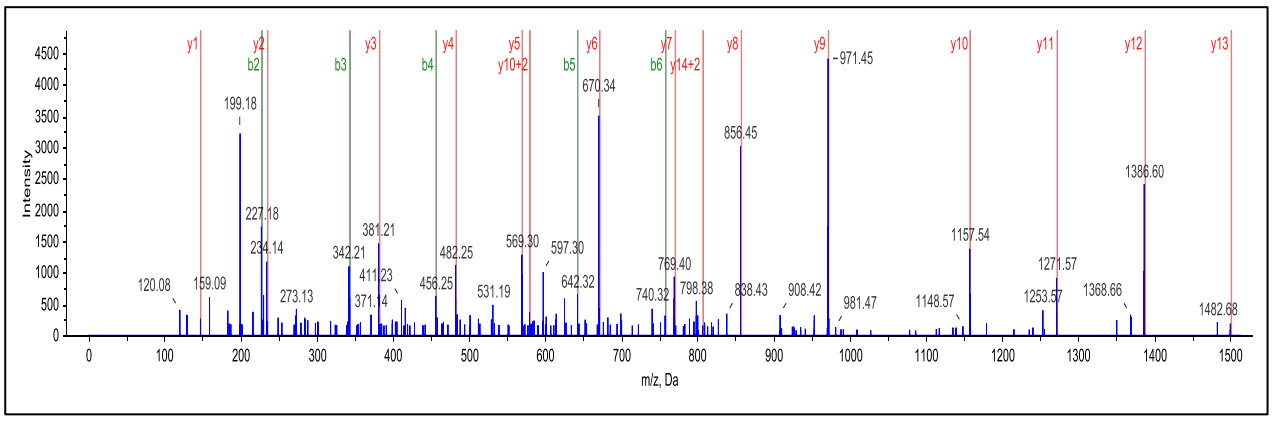
B.



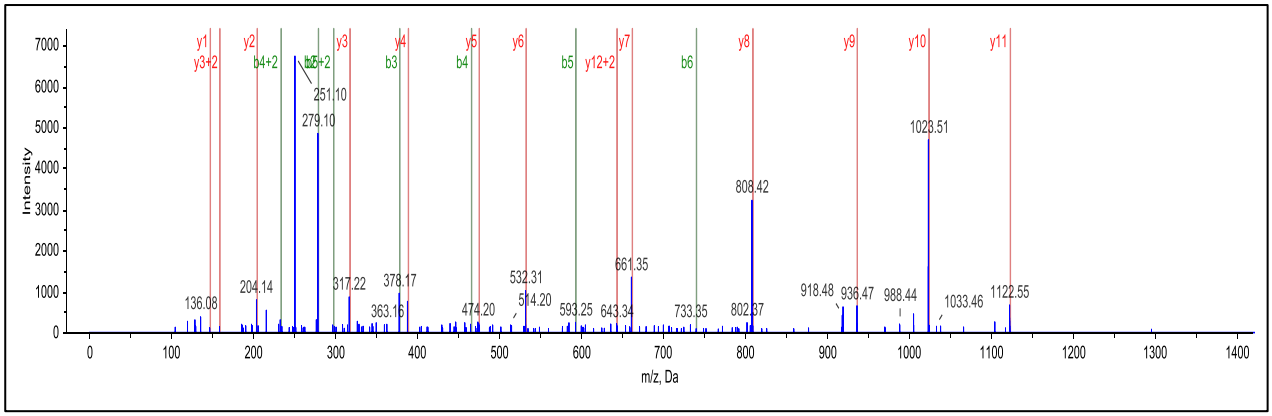
Statistically non-significant trends in the MRM abundance profiles of **(A)** Fibrinogen α-chain, Fibrinogen β-chain, and **(B)** Complement C4-A, and Gelsolin between acute-only samples from each cohort.

APPENDIX FIGURE 3:APOLIPOPROTEIN A1 PEPTIDE SEQUENCE SPECTRA

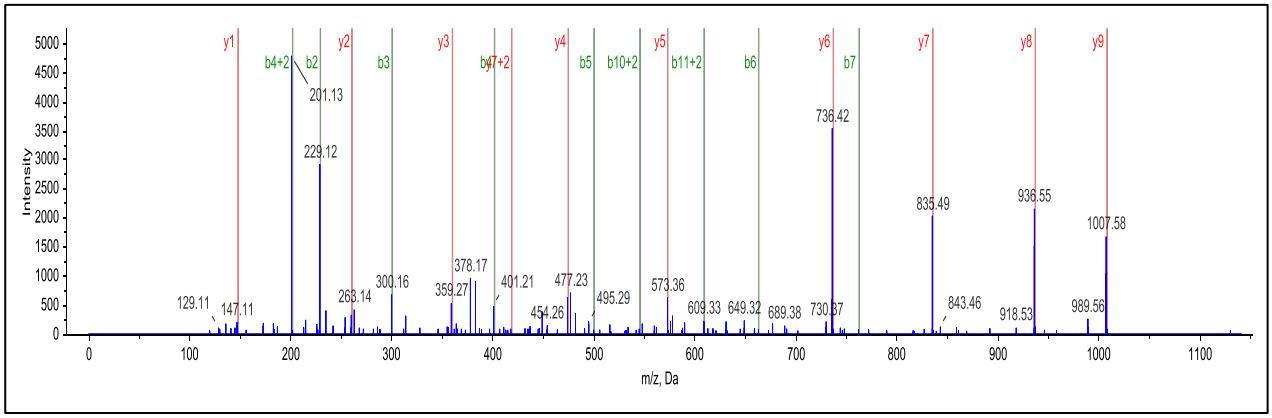
DLATVYVDVLK



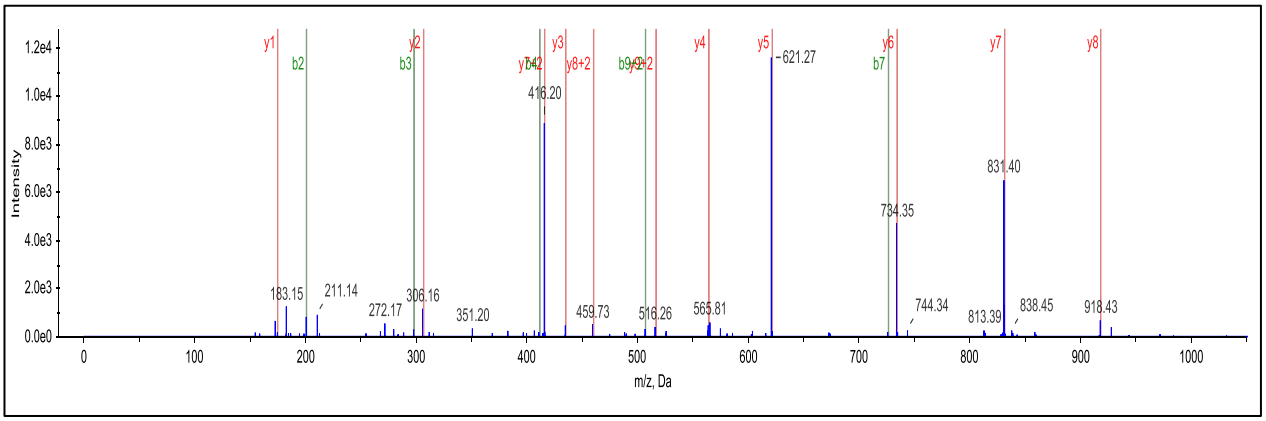
DYVSQFEGSALGK



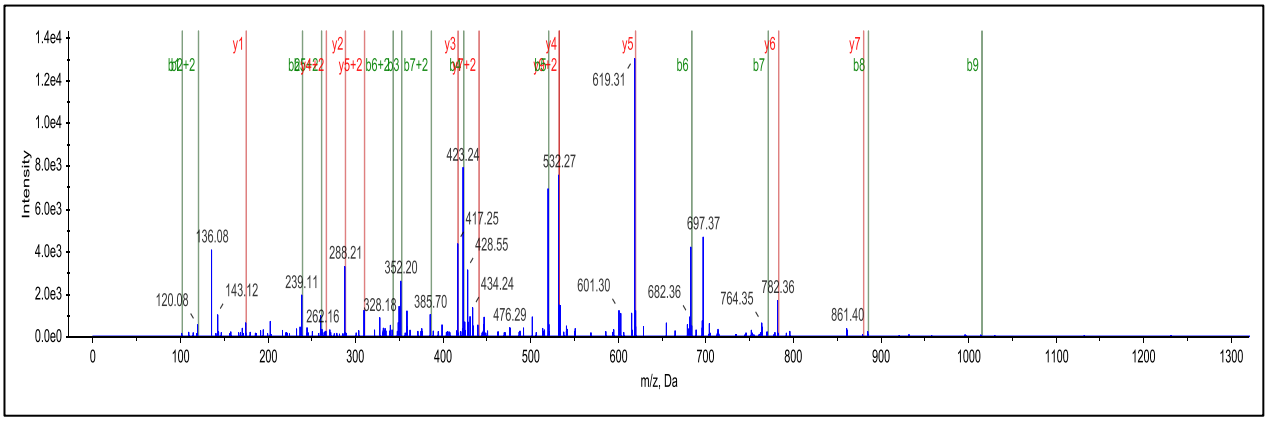
LLDNWDSVTSTFSK



LSPLGEEMR

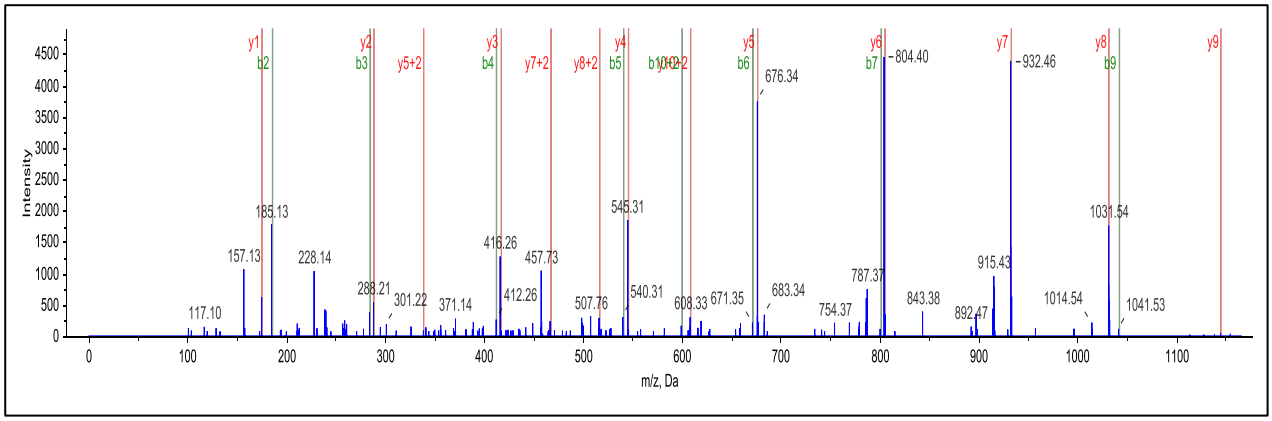


THLAPYSDELR

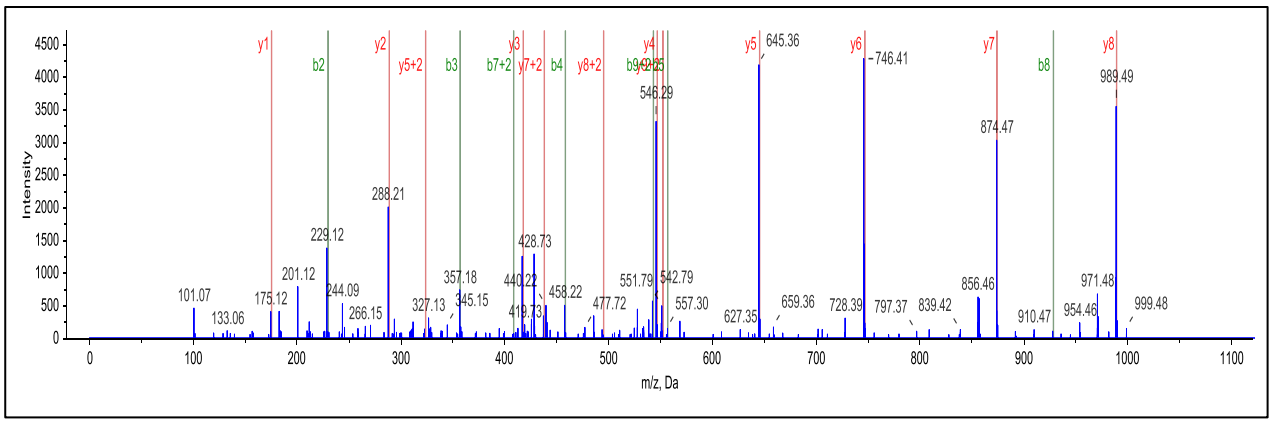


APPENDIX FIGURE 4: APOLIPOPROTEIN A-IV PEPTIDE SEQUENCE SPECTRA

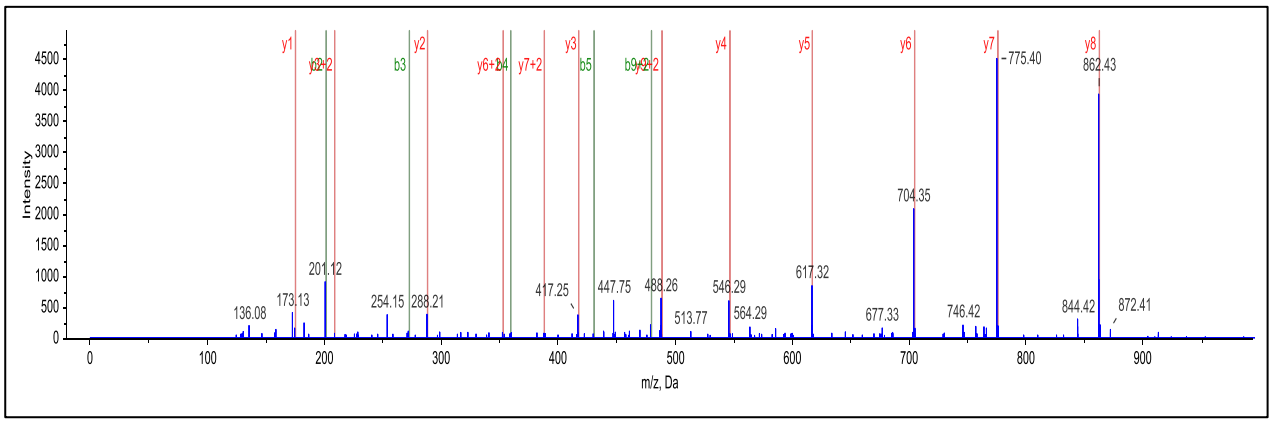
ALVQQMEQLR



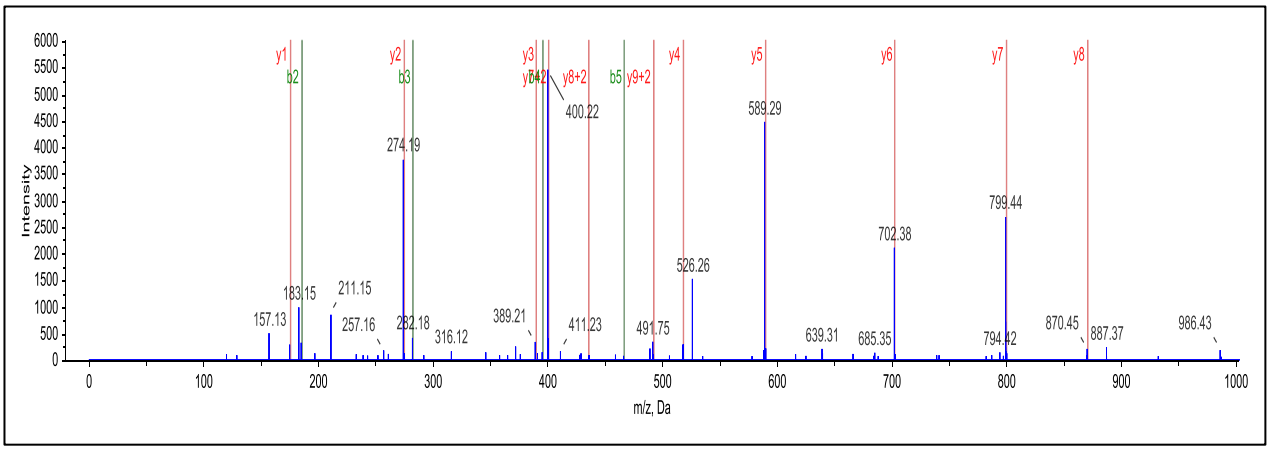
IDQTVEELR



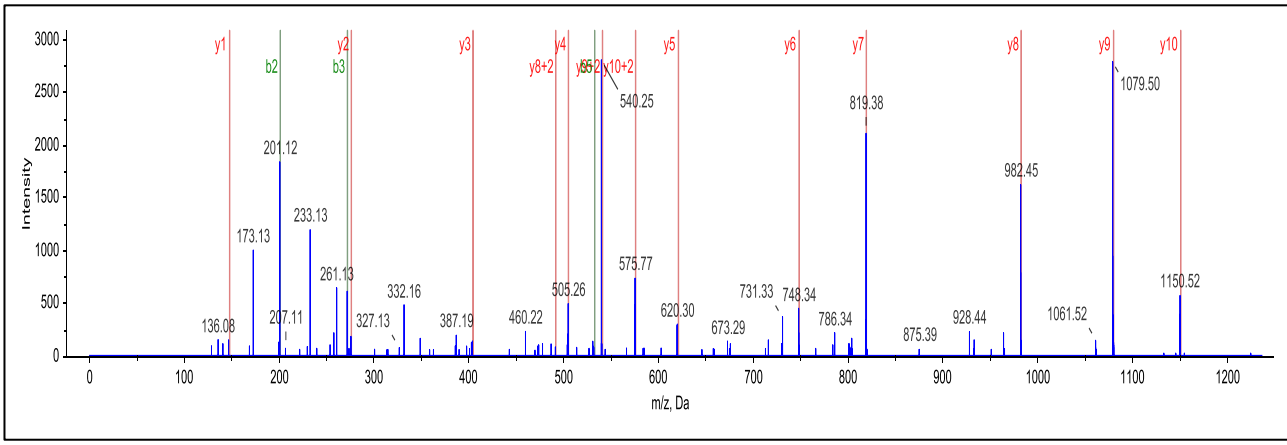
ISASAEELR



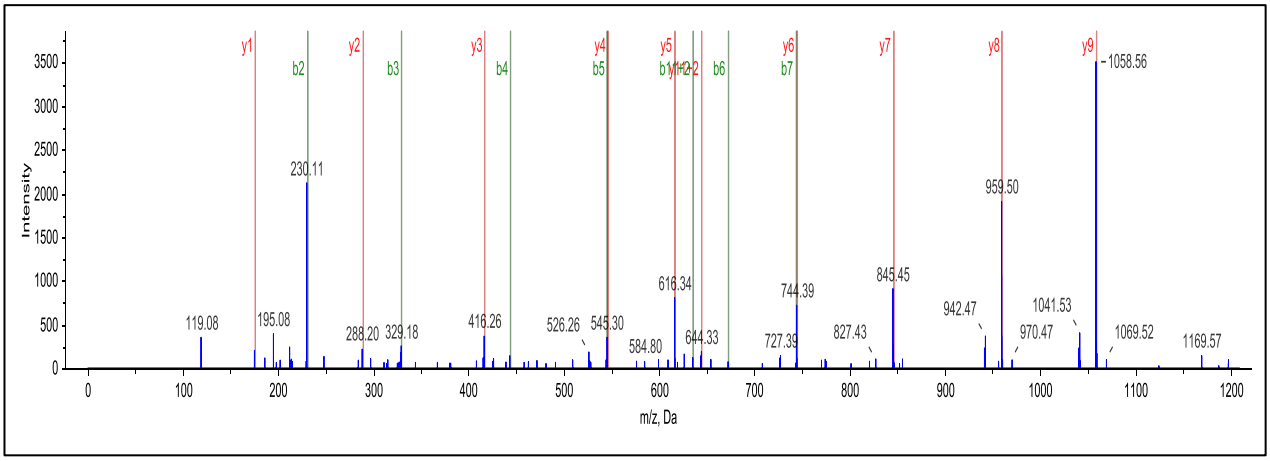
LAPLAEDVR



SLAPYAQDTQEK

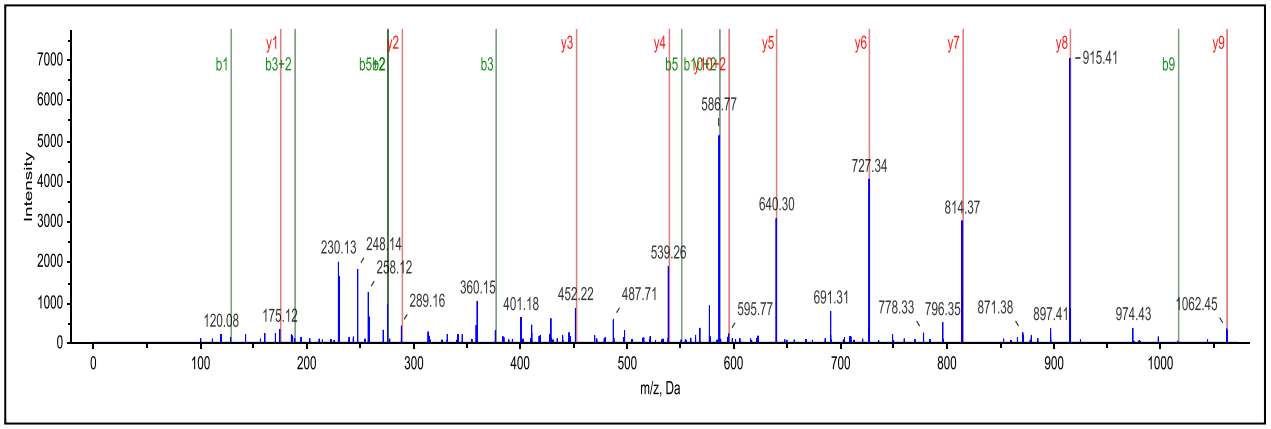


TQVNTQAEQLR

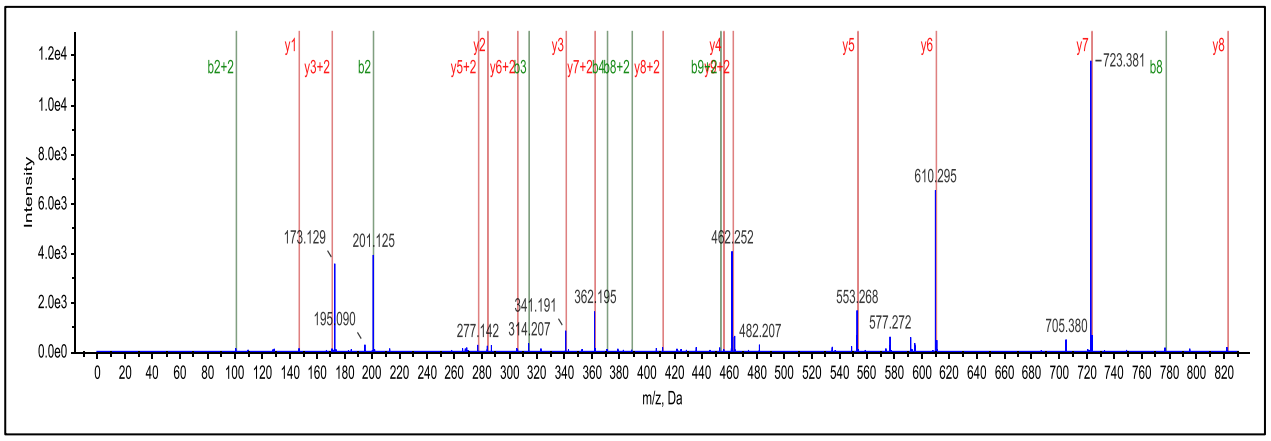


APPENDIX FIGURE 5: FIBRINOGEN ALPHA-CHAIN PEPTIDE SEQUENCE SPECTRA

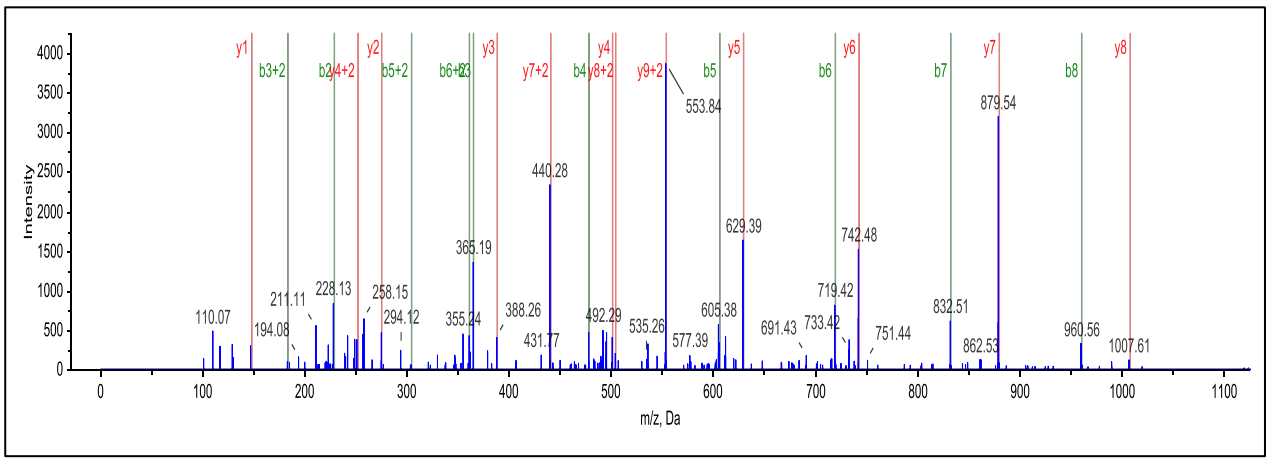
QFTSSTSYNR



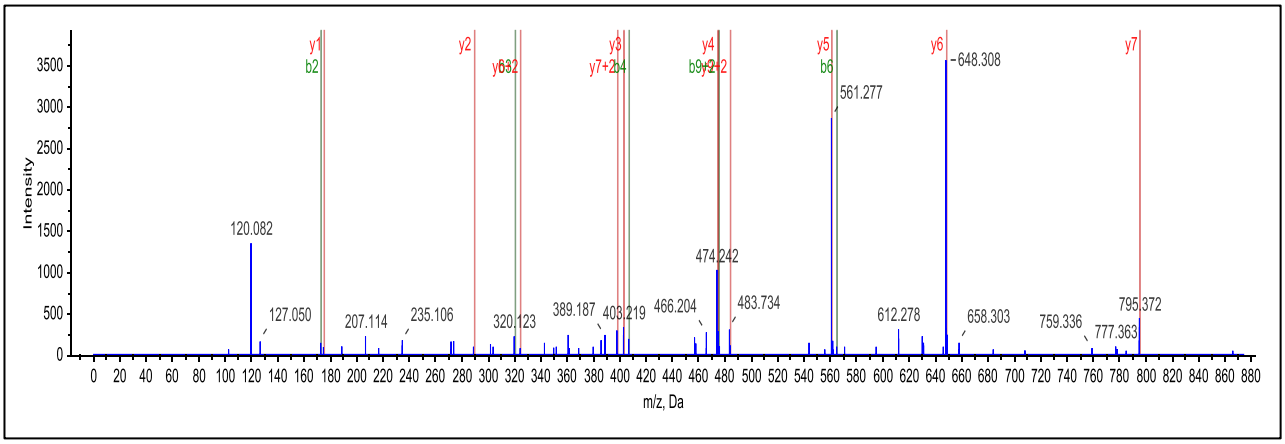
TVIGPDGHK



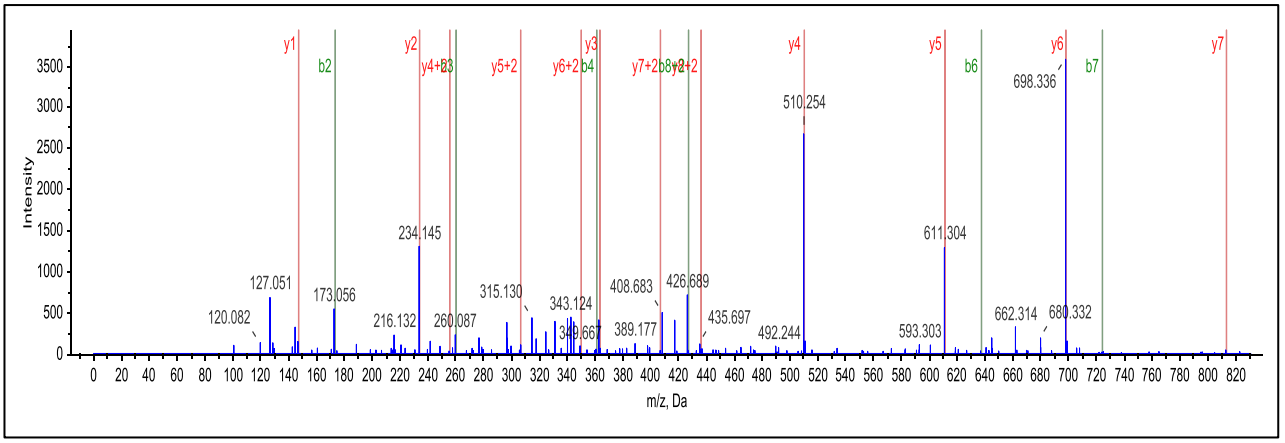
VQHIQLLQK



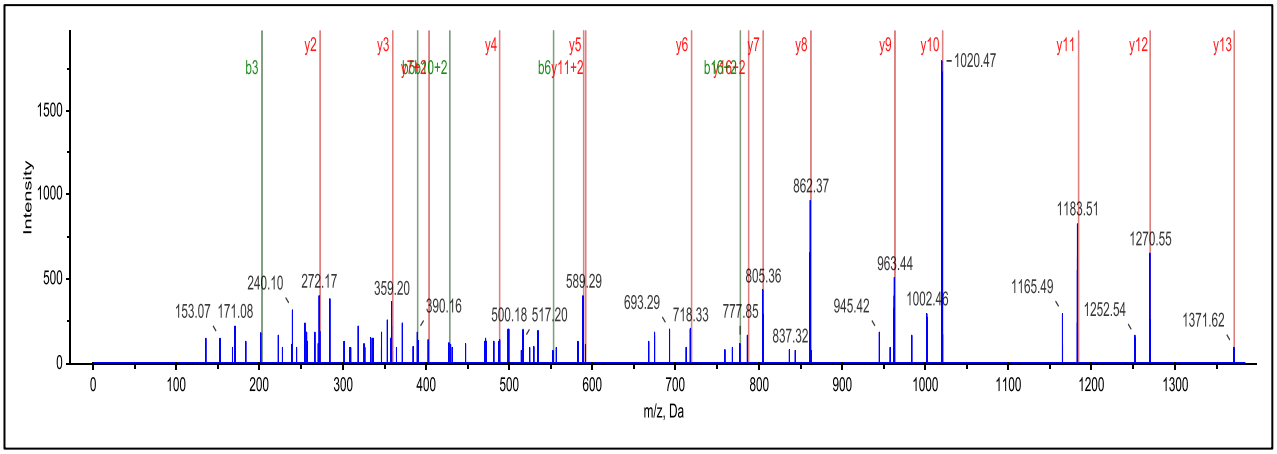
GDFSSANNR



GDSTFESK

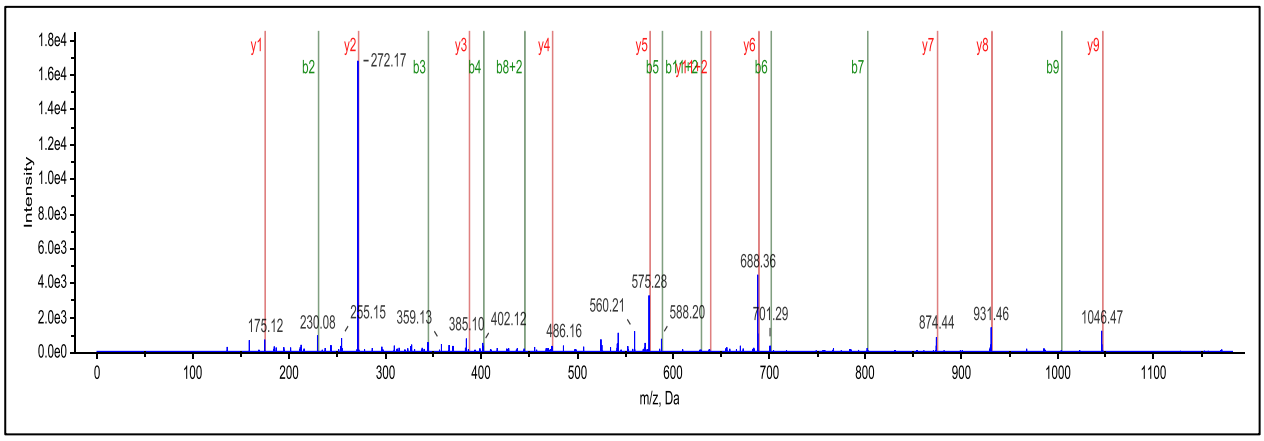


GGSTSYGTGSETESPR



APPENDIX FIGURE 6: FIBRINOGEN BETA-CHAIN PEPTIDE SEQUENCE SPECTRA

DNDGWLTSDPR



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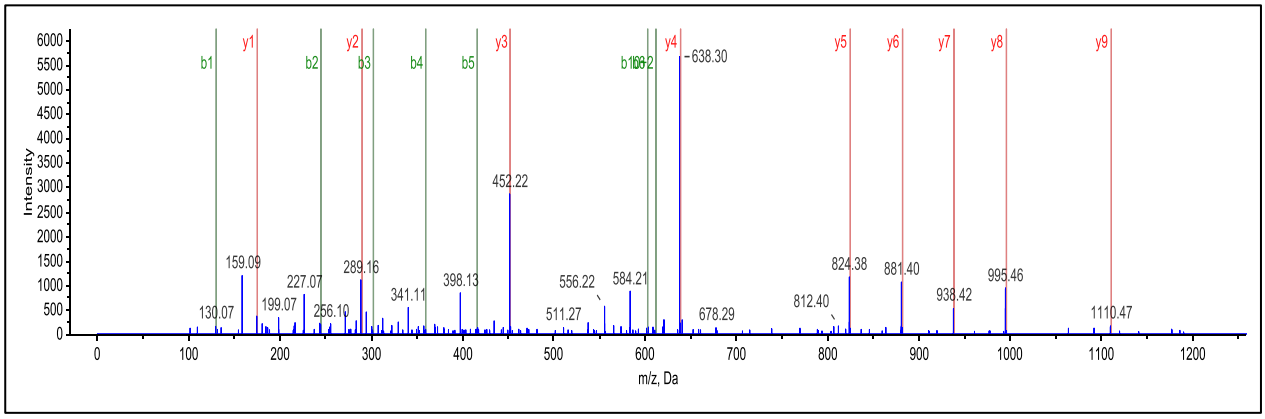
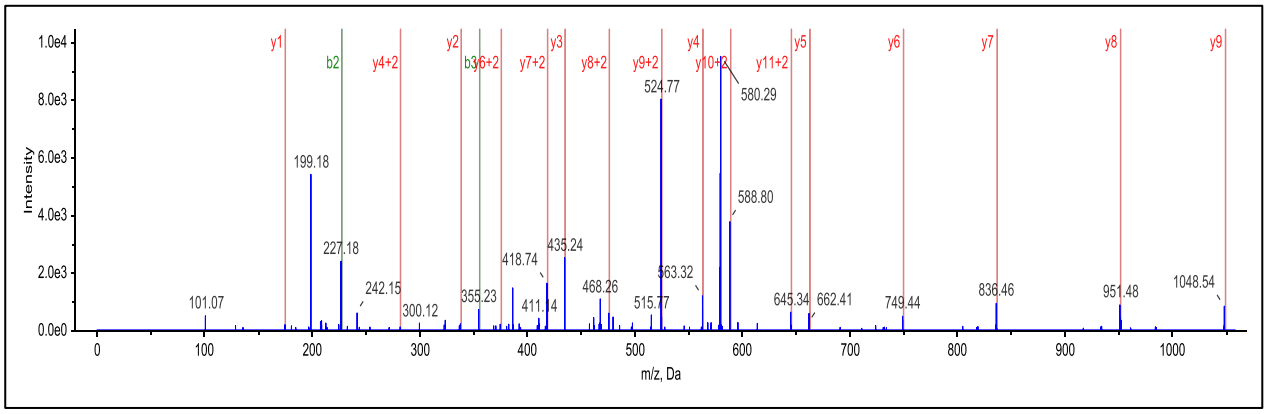
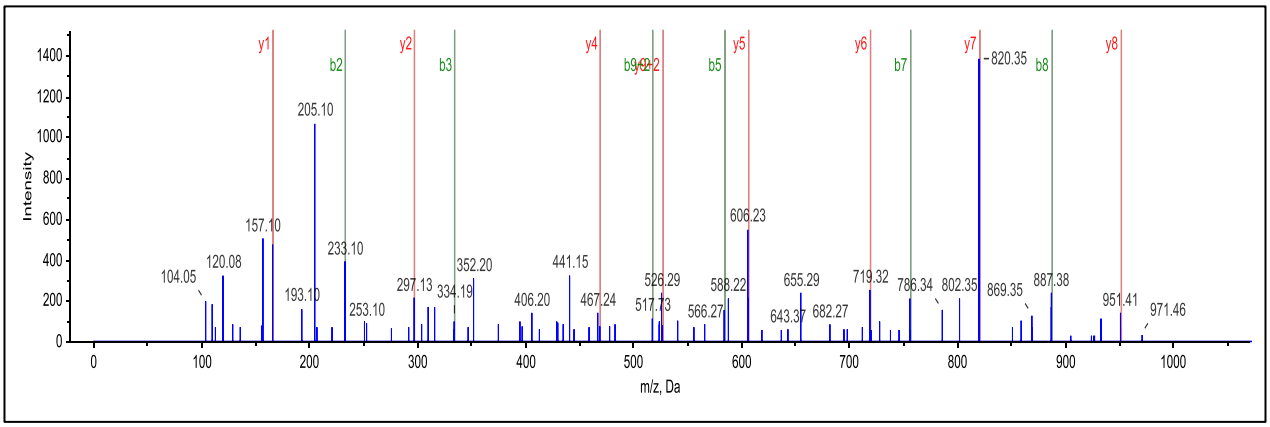


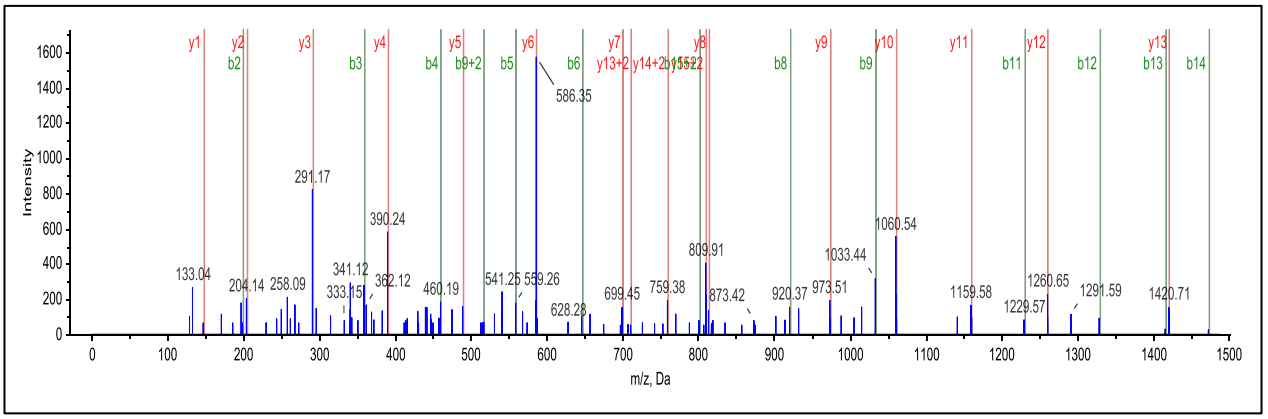
LIQPDSSVKPYR



TMTIHNGMF

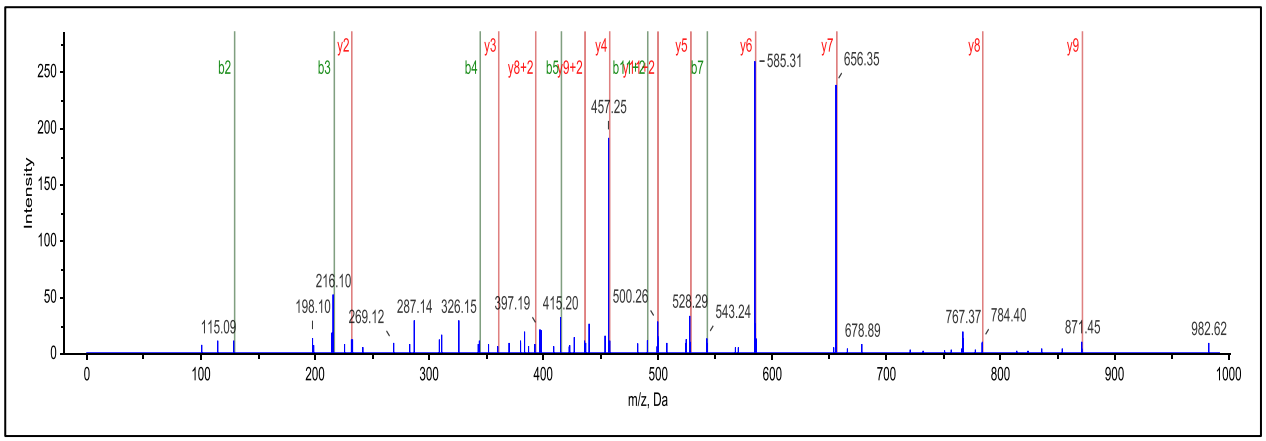


TPCTVSCNIPVVSGK



APPENDIX FIGURE 7: GELSOLIN PEPTIDE SEQUENCE SPECTRA

GASQAGAPQGR



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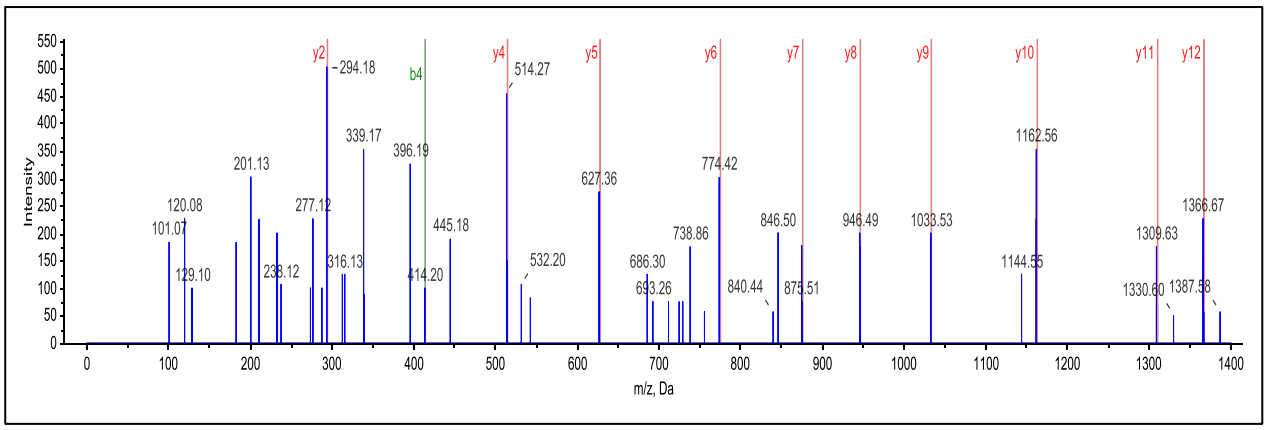

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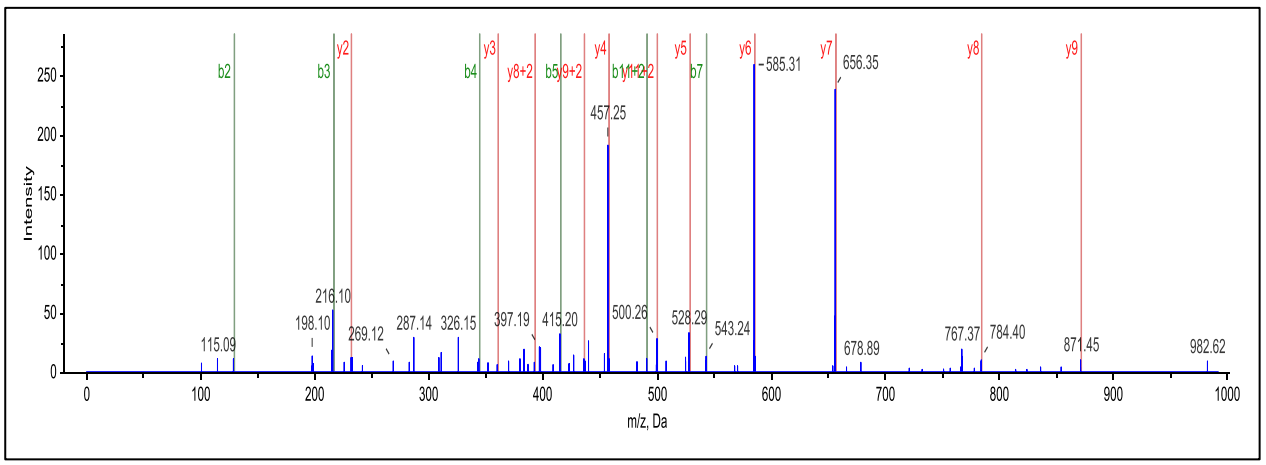

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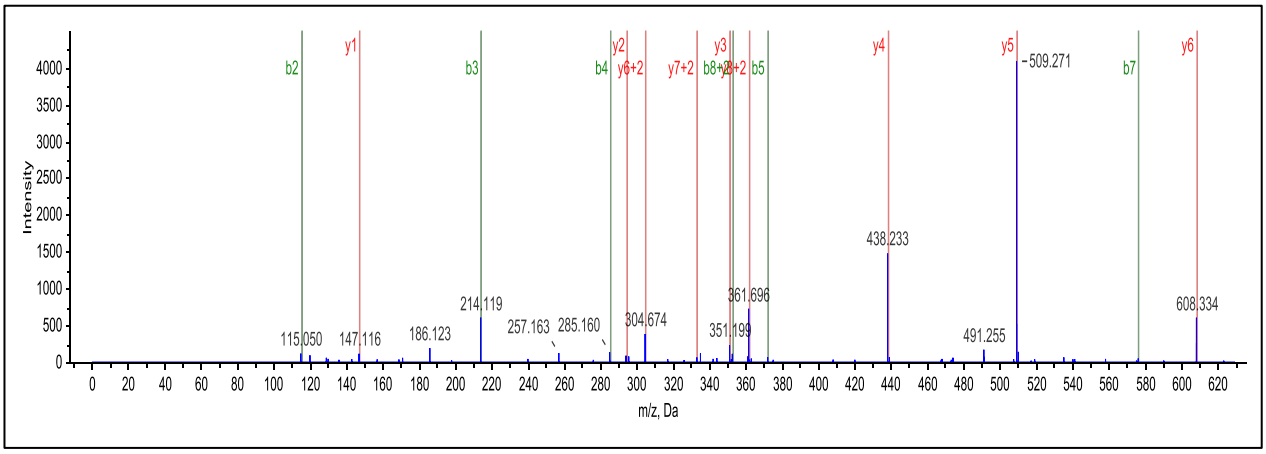

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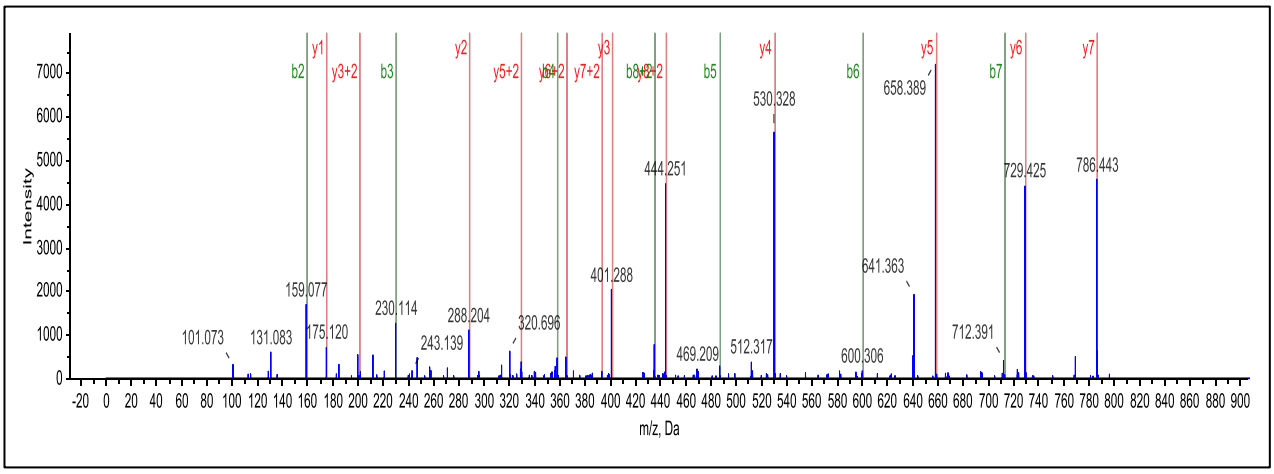
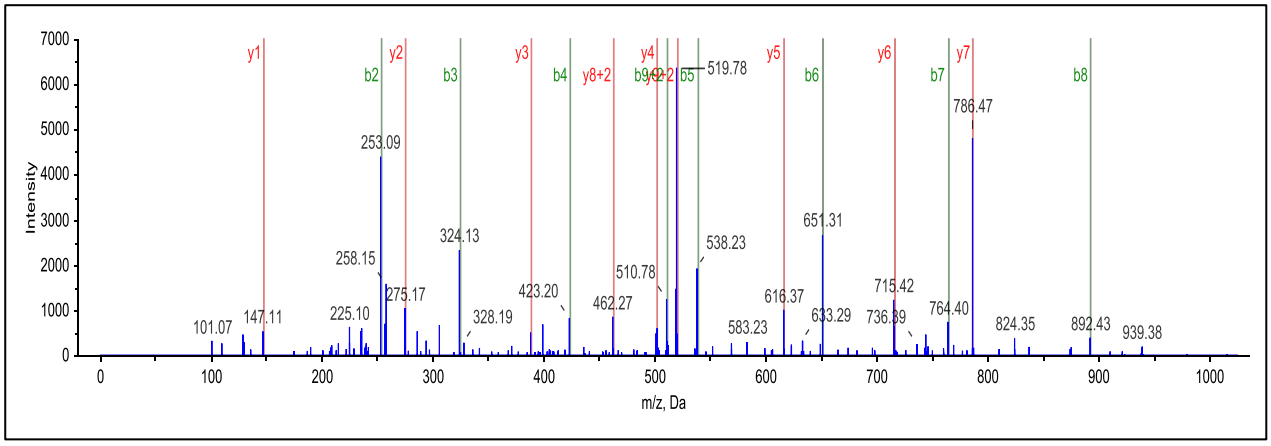
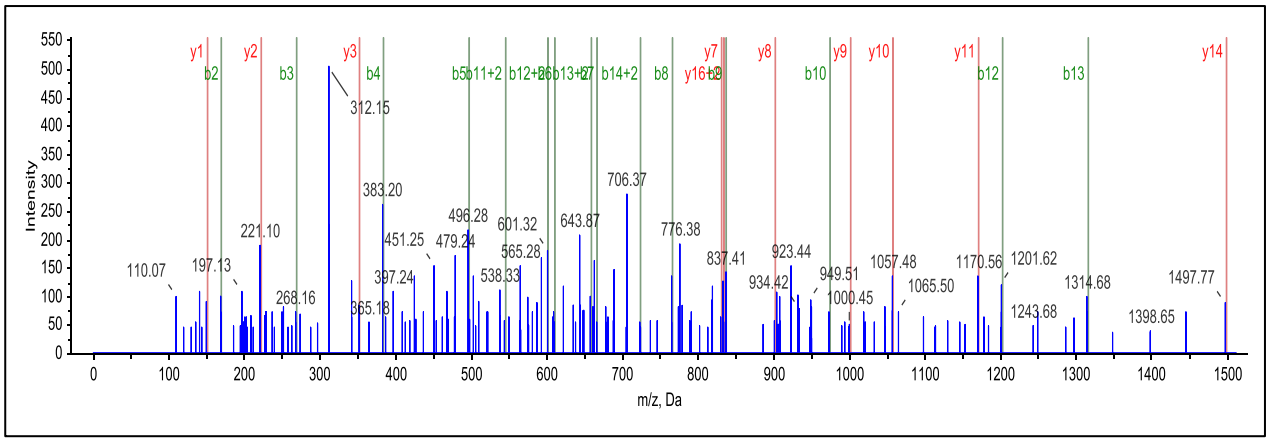


APPENDIX FIGURE 8: COMPLEMENT C4-A PEPTIDE SEQUENCE SPECTRA

AEMADQAAAWLTR



ANSFLGEK



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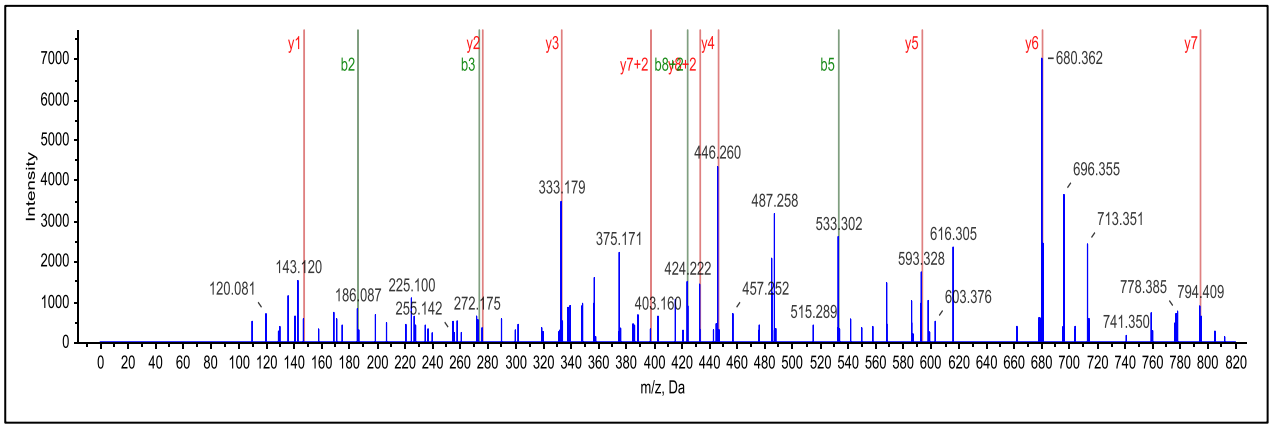
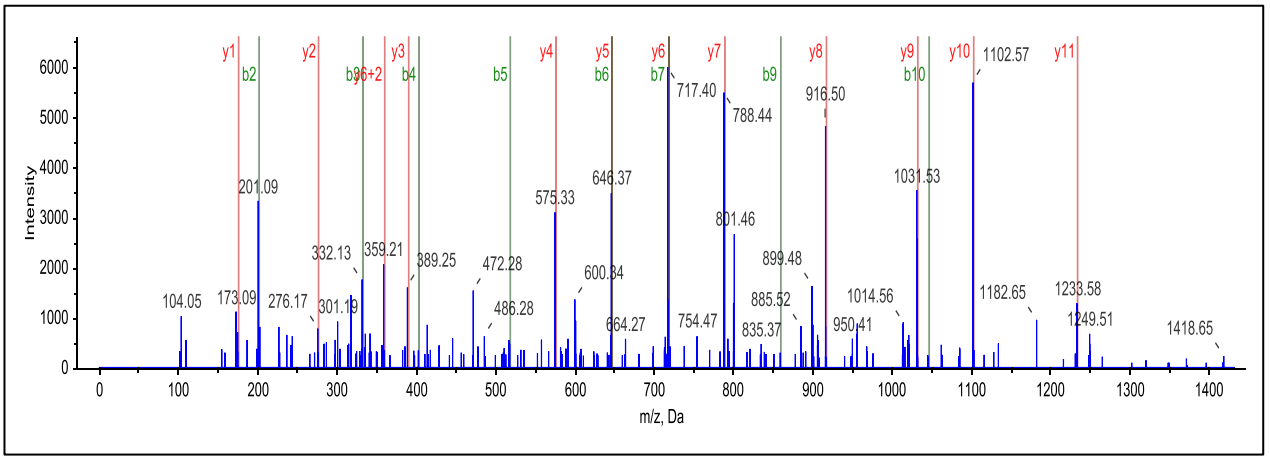
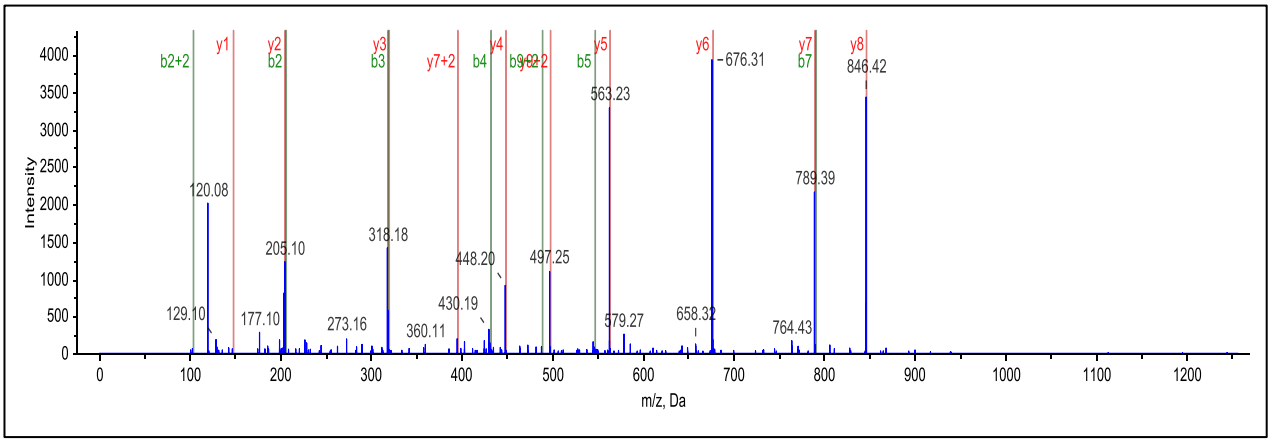


DHAVDLIQK



FGLLDEDGK



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