Discovery of potential therapeutic targets and biomarkers for ischemic stroke by quantitative proteomics

Arnab Datta

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**DISCOVERY OF POTENTIAL THERAPEUTIC
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**A thesis submitted to the Nanyang Technological University
in partial fulfillment of the requirement for the degree of
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2012

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*To my sister, wife and parents for their love,
unconditional support and inspiration*

“The goal of mankind is knowledge... Now this knowledge is inherent in man. No knowledge comes from outside: it is all inside. What we say a man ‘knows’, should, in strict psychological language, be what he ‘discovers’ or ‘unveils’; what man ‘learns’ is really what he discovers by taking the cover off his own soul, which is a mine of infinite knowledge.”

Swami Vivekananda

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ABBREVIATIONS

%CV	Percent co-efficient of variation
2D-GE	Two dimensional gel electrophoresis
ACN	Acetonitrile
AD	Alzheimer's disease
ASIC	Acid-sensing ion channel
BBB	Blood brain barrier
BD	Bipolar disorder
CADASIL	Cerebral autosomal dominant arteriopathy with subcortical infarcts and
CCA	Common carotid artery
CERAD	Consortium to Establish a Registry for Alzheimer's disease
CIND	Cognitive impairment no dementia
CNS	Central nervous system
CoCl ₂	Cobalt chloride
CSF	Cerebrospinal fluid
CT	Computed tomography
ECA	External carotid artery
EF	Error factor
ER	Endoplasmic reticulum
ERLIC	Electrostatic Repulsion and Hydrophilic Interaction Chromatography
ESPRIT	European Australasian Stroke Prevention in Reversible Ischemia Trial
FA	Formic acid
FBS	Fetal bovine serum
FDR	False discovery rate
GABA	Gamma-aminobutyric acid
GDH	Glutamate dehydrogenase
Gln	Glutamine
Glu	Glutamate
GM	Geometric mean
GO	Gene Ontology
GSH	Glutathione
HI/HA	Hyperinsulinism/hyperammonemia
HIA	Hemispheric infarction area
HRP	Horseradish peroxidase
HT	Hemorrhagic transformation
HYP	Hypoxia
I/R	Ischemia-reperfusion
ICA	Internal carotid artery
ID-No	Identification number
IDA	Information-dependent acquisition
IF	Intermediate filament
IMM	Inner mitochondrial membrane
IS	Ischemic stroke
iTRAQ	Isobaric Tag for Relative and Absolute quantification

KEGG	Kyoto Encyclopedia of Genes and Genomes
LACI	Lacunar infarction
LDH	Lactate dehydrogenase
LSC	Low serum control
LVD	Large vessel disease
MACO	Middle cerebral artery occlusion
MAS	Malate- Aspartate shuttle
MMP	Matrix metalloproteinase
MPT	Mitochondrial permeability transition
MRI	Magnetic resonance imaging
MTT	3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
MudPIT	Multidimensional protein identification technology
MW	Molecular weight
NCI	No cognitive impairment
NGM	Normal growth media
NMDA	N-methyl-D-aspartate
OMM	Outer mitochondrial membrane
PAGE	Polyacrylamide gel electrophoresis
PANTHER	Protein Analysis Through Evolutionary Relationships
PD	Parkinson's disease
PDC	Pyruvate dehydrogenase complex
PGE ₂	Prostaglandin E ₂
PGI ₂	Prostacyclin
pI	Isoelectric pH
PMI	Post-mortem interval
PMV	Plasma Membrane Derived Vesicles
PI	Propidium iodide
PTM	Post-translational modification
SCX	Strong Cation Exchange
SD	Standard deviation
SDS	Sodium dodecyl sulphate
STAIR	Stroke Therapy Academia Industry Roundtable
SVD	Small vessel disease
TEAB	Triethylammonium bicarbonate
TIA	Transient ischemic attack
TOAST	Trial of Org 10172 in Acute Stroke Treatment
tPA	Tissue plasminogen activator
TTC	2, 3, 5-triphenyl tetrazolium chloride
TXA ₂	Thromboxane A ₂
VEC	Vascular endothelial cell
WB	Western blots
WebGestalt	WEB-based GEne SeT AnaLysis Toolkit

Note: All gene symbols and protein names from various databases (NCBI, IPI or Uniprot) are excluded from the list of abbreviations

ABSTRACT

Ischemic stroke resulting from the occlusion of cerebral vasculature is the major cause (around 80%) of stroke. Stroke is the second most common cause of death and the predominant cause of adult morbidity worldwide with limited therapeutic option. Despite decades of intensive research, the translation of hypothesis into effective therapies remains an insurmountable challenge without any success in clinical trials. In addition, despite the advancement of sophisticated clinical imaging, there remains ample scope to improve the diagnosis or prognosis of ischemic stroke. Evaluating the failures of previous clinical trials has resulted in a paradigm shift from a reductionist to a system-wide approach by accommodating the emerging concepts of a neurovascular unit instead of neurons only or from a single target to a multi-target therapy to tackle this multifaceted disease.

In this study, I applied an isobaric tag for relative and absolute quantification (iTRAQ)-based neuroproteomic-bioinformatic approach as an unbiased systems biological tool to progressively study an *in vitro* neuronal cell line model of ischemic penumbra, an *in vivo* rodent middle cerebral artery occlusion model of cerebral ischemia-reperfusion injury and autopsied ischemic human brain samples for discovering potential therapeutic targets by individual and comparative analysis of the three profiling data sets. Further, to complement the imaging tools with an alternative diagnostic approach, plasma from patients of lacunar infarction which is a poorly defined subtype of acute ischemic stroke and demographically matched control were quantitatively profiled by iTRAQ platform to identify differentially expressed proteins as candidate biomarkers for large-scale validation.

The unbiased quantitative proteomic approach revealed: 1) the presence of a dynamic equilibrium between mutually competing protective and deleterious pathways under sub-lethal hypoxic or ischemic stress in the *in vitro* model of neuronal penumbra, 2) the presence of several systems level properties (e.g. blood brain barrier damage or neuron-astrocyte cross-talk or deregulation of mitochondrial metabolism) involving multiple proteins from more than one cell-type of the neurovascular unit in the ischemic animal or human brain. Comparative analysis identified mitochondrial

enzyme glutamate dehydrogenase as a consensus candidate apart from the family of annexin or intermediate filament proteins, regulated in a similar fashion in both *in vitro* and *in vivo* models of ischemic stroke. Further data mining indicated probable involvement of glutamate dehydrogenase in both the central (e.g. lactic acidosis) and peripheral (e.g. stroke induced acute hyperglycemia) events of ischemic injury making it a potential multi-modal therapeutic target of ischemic stroke.

In addition, detection of numerous perturbed proteins novel in the context of ischemic stroke in every data set demonstrated the utility of this discovery approach. This opens up opportunities for further proof-of-concept studies to authenticate the therapeutic targets at the preclinical level and for large-scale validation of candidate biomarkers in the clinic. In conclusion, my study established an effective quantitative neuroproteomic approach for translational research to elucidate the pathophysiology of ischemic stroke as well as for discovering potential therapeutic and diagnostic targets.

1. INTRODUCTION

1.1 What is Stroke?

Stroke as defined by the World Health Organization in the 1970s, is a neurological deficit of cerebrovascular cause that persists beyond 24 hours or is interrupted by death within 24 hours. When the stroke symptoms persist for 24 h or less, it is called as transient ischemic attack (TIA).¹ Hippocrates (460 to 370 BC), an ancient Greek physician was first to describe the phenomenon of sudden paralysis that is often associated with ischemia. The word 'stroke' was used as a synonym for apoplectic seizure as early as 1599, and is a fairly literal translation of the Greek term.

1.2 Epidemiology of Stroke

Stroke causes 9% of all deaths around the world and is the second most common cause of death after ischemic heart disease.² Stroke together with ischemic heart disease is responsible for more than a fifth of all deaths worldwide.³ Stroke however remains the predominant cause of adult morbidity. In the developed countries, stroke mortality is on a decline during the past 50 years due to improved control of stroke risk factors (especially high blood pressure and smoking) along with a parallel improvement in the quality of life. However, a large proportion of this fall is attributable to a reduction in case-fatality rather than in incidence. There is insufficient data about trends in the developing countries like India. As late as 2000, India was ranked among the countries lacking sufficient research data on stroke.⁴ However, there is sufficient data to state that stroke is very much on the rise and poised to become a major threat.⁵ In the United Kingdom, mortality from stroke is higher among South Asians (people originating from Indian subcontinent) compared to European whites with a high frequency of lacunar stroke.⁶ Stroke is the leading cause of death in China presenting the largest number of stroke patients in the world.⁷ In Singapore, Stroke is the fourth leading cause of death.⁸ Overall, vascular disorder is the major cause of mortality and morbidity and accounts for more than one-quarter (29%) of the total years of life lost among Singaporeans.⁹ One in five Singaporeans would be ≥ 65 years of age by 2030, up from the recent proportion of 8% in 2004.⁹ This longer life expectancy in Singapore and other parts of the world along with the increased burden of diabetes and hypertension (major risk factors for stroke) is predicted to worsen the situation. Moreover, stroke is a risk factor for vascular dementia and several other chronic

neurodegenerative and neuropsychiatric disorders.^{10, 11} The prevalence of dementia is predicted to rise dramatically in the coming decades making stroke a direct as well as an indirect socioeconomic problem especially for the geriatric population.

Worldwide, stroke deaths are predicted to reach 10 million by 2020 which is double the estimate of 1990 with a dominant contribution from the less-developed world.^{12, 13} Worse, mortality data underestimate the burden of stroke as more often than death stroke causes disability in the surviving population. Despite worldwide consumption of 2-4% of total health-care costs by stroke, the allotment of research fund remains disappointingly and disproportionately low when compared with similar clinical and financial burdens of cancer and congestive heart disease.^{2, 13}

1.3 Ischemic stroke (IS) – A Subtype of Stroke

Strokes are either ischemic or hemorrhagic. About 80% of all strokes are IS. The rest are hemorrhagic in nature. IS is characterized by the occlusion of blood vessel(s) that results in the reduction or lack of blood flow in the affected area. Hemorrhagic stroke occurs when blood vessel(s) rupture inside the brain. The pathophysiology and clinical management of these subtypes are entirely different necessitating objective basic or translational studies on individual subtypes. The differential diagnosis of these subtypes has been revolutionized by the introduction of computed tomography (CT) and magnetic resonance imaging (MRI) techniques.

My current study is concerned about IS, also called cerebral ischemia or cerebral infarction. Acute IS can be classified following different classification systems such as Trial of Org 10172 in Acute Stroke Treatment [TOAST] criteria for clinical trials and The Oxfordshire Community Stroke Project classification for epidemiological studies. According to the TOAST criteria, there are five subtypes of IS: 1) large artery atherosclerosis, 2) Cardioembolism, 3) Small-vessel occlusion or lacunar stroke, 4) Stroke of other determined cause.²

1.4 Pathophysiology of IS

The area of the brain perfused predominantly by the blocked vessel is affected most severely following IS as rapid death of cells is observed. This is called as the core of

injury. The peripheral area surrounding the core has a milder deficit of blood flow due to the presence of collateral circulation. The neurons are electrically silent but metabolically viable in this hypo-perfused tissue present in between normal and critically compromised brain region. This area is named as penumbra by following the astronomical term indicating areas of half-light and half-shadow. The discovery of ischemic penumbra in a nonhuman primate brain in 1977 initiated the quest for an effective stroke therapy.¹⁴ It was hypothesized that ischemic penumbra is theoretically salvageable when treated at the right time with a suitable neuroprotective therapy. Subsequently, successive decades saw extensive physiologic, mechanistic and finally diagnostic characterization of the penumbra by evolving molecular biological, biotechnological and imaging tools. Today, it is understood that diminished oxygen and glucose following ischemia cause energy depletion, failure of neurogenic pump ($\text{Na}^+\text{K}^+\text{ATPase}$) and ionic homeostasis. Waves of depolarization (peri-infarct depolarizations) results in excess release of neurotransmitter glutamate (Glu), which coupled with impaired glial reuptake, leads to overactivation of Glu receptors (Glu excitotoxicity). The intracellular pH decreases as a result of anaerobic glycolysis with increased accumulation of lactic acid (lactic acidosis). Both Glu excitotoxicity and lactic acidosis are responsible for massive influx of calcium ion (Ca^{2+}) mediated through N-methyl-D-aspartate (NMDA) receptor and acid-sensing ion channel (ASIC) respectively, which instigates mitochondrial dysfunction, activates different kinases (e.g. PKC), proteases (e.g. calpains), phospholipases and endonucleases to damage essential proteins and lipids (lipid peroxidation). Parallel damage of the blood brain barrier (BBB) triggers inflammatory signaling as several cytotoxic mediators are released locally and by the accumulating leukocytes. Eventually, oxidative stress in concert with all the above mentioned deleterious events contributes to irreversible metabolic shutdown and death by apoptotic and necrotic pathways.^{15, 16}

1.5 Experimental models of IS

The basic and pre-clinical research is shaped by numerous models. None of them are ideal, but each of them represents certain aspects of the stroke pathology.

1.5.1 *In Vitro* Models of IS

The *in vitro* models generally use 1) primary neuronal or glial cultures from cortex, hippocampus and cerebellum of embryonic or perinatal rats and mice; 2) primary cultures of endothelial cells isolated from mammals including rat, mouse, pig, bovine or human; 3) organotypic slice cultures (mainly hippocampal) and 4) commercially available cell lines of various origins.^{17, 18} They have the advantage of being extremely flexible yet precisely controlled to incorporate specific aspects of stroke pathology in different cell types of brain. There are models to target Glu excitotoxicity, lactic acidosis, free radical stress or apoptosis individually to understand each of these deleterious mechanisms without systemic interference.^{19, 22} Other models tried to reproduce certain anatomical attributes of the brain, such as blood brain barrier¹⁸ or to understand hypoxic or ischemic preconditioning^{23, 24} or post-conditioning.^{25, 26} However, the most popular and widely used *in vitro* model combined oxygen and glucose deprivation (OGD) for specific duration to simulate the depletion of the two major nutrients in the brain following ischemia. Subsequently, to answer diverse pathological questions, the basic models were modified. These include 1) alteration of the media composition; 2) change of composition of the hypoxia-gas or experimental condition to differentiate hypoxia with anoxia;²⁷ 3) co-culture of different cell-types;²⁸ 4) application of custom-made set-up like transwell or semipermeable support;²⁹ 5) use of conditioned media of a specific cell line or cell type during, before or after the actual injury on another cell-type;³⁰ 6) incorporation of various chemicals (e.g. sodium azide or cyanide, cobalt chloride (CoCl₂) and 2-deoxy glucose) with or without specific agonists or antagonists;³¹ 7) genetic modification of one or more proteins as a proof-of-concept study among others. These models traditionally used a single cell type. However, use of conditioned media or secretome and multiple cell types in a single experimental design has been increased in the recent past to mimic cell-cell interaction in the ischemic brain.^{29, 30}

Commercially available cell lines are by far the most amenable starting material. It is robust and homogeneous in compared to the primary cultures which is difficult to prepare in a relatively pure form and cannot be subcultured. Absence or loss of synaptic connectivity is the major and common disadvantage for both primary and commercial cell lines, which can be supplemented by three dimensional organotypic

slice cultures. It maintains the neuronal morphology, intrinsic cellular and anatomical relations and network connections. It is heterogeneous and has the potential to partially replace animal experiments. In fact, the recent Stroke Therapy Academia Industry Roundtable (STAIR) VI recommendations suggested an expanding use of slice culture models.³² The *in vitro* models are applied to address several basic pathophysiological questions at the cellular or sub-cellular level and to study the preliminary efficacy of pharmacological or non-pharmacological approaches. The objectives are complementary across the studies and added to the growing knowledge about IS.

In the current work, I have selected a rat brain neuroblastoma cell line (B104)³³ and used OGD to recapitulate IS. B104 is long, thin and usually bipolar cells, cloned from tumors of rat central nervous system (CNS) (Figure 2.1C). It displays numerous neuronal characteristics including membrane excitability, expression of neurotransmitters and 14-3-2 neuron-specific protein.³³

1.5.2 *In Vivo* Models of IS

Most of the basic understanding about the pathology of IS is obtained using predominantly animal models. Although primates (e.g. rhesus monkeys, marmoset or baboon) are closer to human in terms of cerebrovascular system, gray to white matter ratio or behavior, the routine use of primates for basic or translational research is limited by the increased cost, lack of infrastructure and inadequate expertise. Most models use rodents (e.g. rat and mice), but cat, dog, pig and rabbits are also used.³⁴

Animal models have been developed by grossly following the etiological classification of IS, i.e. large vessel disease (LVD), embolic and small vessel disease (SVD). These subtypes of IS vary in terms of lesion size, location, pattern of infarct progression, severity and pathophysiologic mechanisms. Large vessel or cardioembolic stroke (non-lacunar stroke) may produce lesion with volume ranging from 28-80 mm³ and occupying 4.5-14% of the ipsilateral hemisphere.³⁵ Conversely, SVD such as lacunar stroke affects large (0.2- 0.5 mm) or small (<0.2 mm) arterioles with the infarct diameter generally less than 15 mm.³⁶ The location of the infarct is predominantly in the deep white or grey matter or pons while large vessel or cardioembolic strokes are mainly cortical in nature.¹⁰ Lacunar stroke is rarely fatal but causes different extent of disabilities, recurrent stroke or dementia in surviving patients.¹⁰ Non-lacunar strokes

are acute and often fatal with distinct pattern of recovery in the surviving patients. Spontaneous early lyses of the clot occur in 15–18.8% of all cases as assessed by serial monitoring. Thus, reperfusion is a common attribute for all non-lacunar subtypes that is induced either by clinical thrombolysis, intravascular clot retrieval device or by spontaneous reperfusion and collateralization.³⁵ Reperfusion is essential for recovery but paradoxically promotes injury through various mechanisms including inflammation. Animal models of stroke tried to address these differences when targeting specific IS subtypes with mixed success.

1.5.2.1 Animal Models of LVD or Cardioembolic Stroke

The models of non-lacunar strokes are much better developed than models of SVD. They are analogous to human stroke with respect to size, option to include or exclude reperfusion injury, pattern of recovery and various aspects of cellular and subcellular pathology. The models for non-lacunar stroke can be classified as embolic model, intraluminal suture middle cerebral artery occlusion (MCAO) model, photothrombosis model and endothelin-1 induced stroke model - all of which does not require craniectomy thus less invasive and easy to perform with minimal brain injury due to surgery.^{37, 38} The models using craniectomy include direct surgical occlusion of MCA alone or in combination with other cerebral vessels. Tamura's model or three vessel occlusion model are common examples of this class.³⁹ In addition, there are models where the basilar artery is targeted to produce hindbrain ischemia, the most severe form of human IS.⁴⁰ Of note, arterial occlusion is responsible for cerebral infarction in all the above mentioned models. Animal models of cerebral venous thrombosis have also been described by interfering with the circulation in jugular or cortical veins.^{38, 41} However, none of these models are ideal or truly representative of clinical IS, simply because IS is itself heterogeneous. Thus, each model has its own disadvantages that may be related to surgery, reproducibility or inherent nature of the model. For example, reperfusion injury cannot be produced in the microsphere-induced embolic model. Photothrombotic ischemic lesion lacks penumbra and cytotoxic edema because vasogenic edema and BBB breakdown occurs immediately after surgery.

Infarct volume is an objective indicator of brain tissue damage making it the most popular outcome measure necessary to validate these models. This is also used to determine the efficacy of the tested compounds in a terminal fashion. Hematoxylin-

eosin and 2, 3, 5-triphenyl tetrazolium chloride (TTC) staining methods are commonly used to calculate the infarct and edema area or volume. Diffusion weighted and perfusion weighted MRI are the most sensitive methods to evaluate the location or volume of infarct or penumbra in a non-invasive manner. The functional impairment of IS affected rodents is better assessed by motor deficits as neurological deficits are subjective and spontaneous recovery may happen rapidly. There are several tests to qualitatively examine the behavioral outcome including sensorimotor function (e.g. beam walking, rotarod) and cognition (e.g. Morris water maze). However, lack of correlation between different outcome measures is a confounding factor during the efficacy studies to unequivocally measure the therapeutic utility of the potential drug in a particular model.³⁸

1.5.2.2 Animal models of SVD

The animal models of SVD can be subdivided into four categories; 1) embolic, 2) hypoperfusion/ischemic injury, 3) hypertension-based injuries and 4) blood vessel damage.⁴² Although many of these models use similar methodology compared to that of non-lacunar stroke, they were modified to replicate certain key aspects of SVD not seen in non-lacunar strokes. The most-used model is the stroke-prone spontaneously hypertensive rat that is suggested to better resemble human lacunar stroke than other existing models. This is found to mimic both microvessel and parenchymal changes as endothelial dysfunction and BBB damage precedes arteriolar wall thickening, degeneration and perivascular tissue changes.^{43,44} However, this area is still immature and evolving gradually like the basic or translational research on SVD or lacunar stroke.

I have selected a rat intraluminal suture MCAO model of LVD for the present work. This is most widely used in the stroke fraternity because of its ease, flexibility, reproducibility and better analogy with clinical IS in the MCA territory. Focal infarct with large ischemic penumbra and evolution of both ischemic and reperfusion injury can be studied using transient MCAO model that is compatible with the quantitative proteomics guided discovery approach. Compared to an *in vitro* model, the *in vivo* model allows the capturing of multi-cellular response within the ischemic brain and offers better understanding of the systemic influences, none of which is amenable to single cell or slice culture models.

1.6 Therapy

The therapeutic approaches of acute IS can be broadly classified into four categories: 1) thrombolytics that dissolves the clot to restore blood flow in the ischemic area; 2) neuroprotectants that target various biochemical pathways by trying to inhibit cell death mechanisms and preserve or restore brain function in order to promote neuronal repair and recovery; 3) surgical removal of the occluding thrombus or emboli with the help of interventional device; and 4) hemicraniectomy.^{45, 46}

Of these interventions, Intravenous tissue plasminogen activator (tPA), a thrombolytic agent introduced in 1995 still remains the first line of treatment for acute IS and almost exclusively used worldwide at a dosage of 0.9 mg/kg. Initially the therapeutic window was 3 h from the onset of IS. This was extended to 4.5 h from 2008 onwards based on the evidence found in European Cooperative Acute Stroke Study III trial.⁴⁷ Aspirin is the major supportive therapy when used within first 48 h of IS. Warfarin for patients with atrial fibrillation, endarterectomy for symptomatic carotid stenosis, antiplatelet agents, and cholesterol reduction are proven secondary prevention strategies.² In the neuroprotectant category, edaravone, a multi-target free radical scavenger and citicholine, a phospholipid supplement are approved in some countries (e.g. Europe, Japan, and India) and used sparingly by clinicians.⁴⁸

The therapeutic uptake of tPA remained low as only 2-5% of all eligible patients on an average receive treatment with this thrombolytic. This is mainly attributable to the narrow window of intervention which is complicated by the low levels of knowledge among clinicians, poor recognition of symptoms due to diagnostic inefficiency, delays in emergency transport and lack of public awareness. In this context, management of IS patients in a specialized stroke unit has been found to improve the outcome by reducing mortality and morbidity.^{48, 49} In addition, because of the uncertainties about the time of onset, patients waking with stroke, who may constitute up to 25% of the total stroke population, remain ineligible for thrombolysis. To make the matter worse, symptomatic intracerebral hemorrhage is consistently associated with tPA treatment.⁴⁶

Owing to almost complete absence of effective therapies and the lure of substantial commercial success, the pharmaceutical industry invested heavily for the discovery of viable treatment options for IS in the last two decades. Despite intense efforts, 129

phase II and 60 phase III stroke trials ended without a single clinical success.⁵⁰ At present, clinical trials of varying scale are ongoing for several chemical (e.g. minocycline) or biological entities (e.g. erythropoietin or granulocyte colony stimulating factor) and other approaches (e.g. hypothermia or remote post-conditioning) apart from thrombolysis related trials. However, a single neuroprotective agent or better thrombolytics are yet to become available for worldwide clinical use.¹⁵

In light of the complete lack of neuroprotective therapies, the successive clinical failures along with a bleak epidemiological landscape and a relative neglect by the funding agencies, stagnancy has been descended in the area of basic, preclinical or clinical stroke research.

1.7 Emerging Concepts

Today the scientific community is standing at the crossroads and reevaluating and overhauling the existing strategies at every level in an effort to overcome this clinical menace. The introduction of STAIR guidelines during the late 90s with periodic reevaluation and upgrading have contributed significantly to shape the ideas by learning from the past failures while improving the quality of the IS research as a whole.^{32, 51} Several independent but useful concepts have emerged in the recent past to summarize the progress in this rapidly developing field. Neurons to a neurovascular unit or a combination therapy in favor of a monotherapy are among the novel concepts. Although each of these separately addresses crucial issues, they collectively underpin a paradigm shift from a reductionist to a systems approach in the area of IS.

1.7.1 Neurovascular Unit

Traditionally the neuron remains the center of focus for stroke research. Most of the therapeutic strategies tried to prevent injury to the healthy neuron or salvage the dying neuron and restore neuronal function following IS. A simple online search in the Scopus database by combining the keywords 'stroke' and the individual components of brain like 'astrocyte', 'microglia', 'oligodendroglia' and 'vascular endothelial cell (VEC)' shows that more than a half of the reports (around 56%) are related to neuron in stroke. Contrary to popular beliefs, the number of non-neuronal cells is

approximately the same as the neuronal cells in the human brain.⁵² Oligodendroglia, the brain cells supporting the neuron with myelin sheath is the most infrequently studied (only 2.5%) cell type followed by microglia (<9%) and astrocyte (around 13%) in the context of stroke. A stereological study based on post-mortem neocortices of human brains reported that among all non-neuronal cells, only oligodendroglial cells showed a significant and correlative decrease with neurons during aging.⁵³ Loss or damage of oligodendrocyte will have most profound consequences in the function of myelinated CNS tracts, the white matter.⁵⁴ Some forms of SVD specifically affect cerebral white matter, whereas in LVD white matter is equally affected like gray matter. Oligodendroglia like neuron is also susceptible to oxidative stress and excitotoxicity and may die through necrosis or apoptosis. However, they do not express functional NMDA receptors like neurons – one of the reasons that probably contributed to the failure of NMDA receptor antagonists in the clinical trials of IS.⁵⁵ VEC are the major component of the BBB and due to its direct involvement in the events that predisposes to IS or in post-stroke inflammation or hemorrhagic transformation (HT), is relatively better studied (>19%).

Hence, to address the differential or cell type specific response of the heterogeneous brain following IS, the concept of neurovascular unit is envisaged. It emphasizes that neurons participate as part of an integrative brain response in which signaling between various cell types and between cells and matrix are important determinants of tissue outcome.⁵⁴ Accordingly, a steady rise in interest has been observed to understand the role of non-neuronal cells, cell-cell or cell matrix interaction in various IS models.⁵⁶ Search of therapeutic targets or strategies have been initiated that will protect multiple cell types or white and gray matter simultaneously. For example, the stress-activated protein kinase p38 not only mediates neuronal death but also may augment caspase-3-mediated cell death in cerebral VEC after hypoxic injury.⁵⁶ Similarly, 12/15-lipoxygenase, an enzyme of arachidonic acid cascade has been shown to contribute to Glu-induced oxidative cell death in both neurons⁵⁷ and oligodendrocytes.⁵⁸

1.7.2 Temporal Consideration for Target Evaluation

Reperfusion is essential either spontaneously or through intervention to salvage the ischemic tissue. However, despite restoration of circulation, areas of the affected brain

do not return to normal perfusion and increasingly more area becomes infarcted. This is called ‘non reflow phenomenon’ and later contributes to swelling or vasogenic edema. Not all types of cells or all cells of a particular cell type die immediately in the penumbra following IS. There is an ongoing process of degeneration continues through days, weeks and months leading to IS induced chronic complications such as vascular dementia. The recent advent of non-invasive imaging techniques shows the presence of viable neurons as late as 52 h after stroke. Randomized thrombolysis trials indicate potential benefits to some patients well beyond the 4.5 h of treatment window.⁵⁹

Figure 1.1 Schematic representation of the emerging concepts of stroke research. Each of them points towards the involvement of multiple dimensions either in the form of more than one component of brain or multiple organs or therapeutic targets. This necessitates the inclusion of a profiling strategy to obtain an overview of this multi-factorial disease.

Despite over activation of NMDA receptor in the hyperacute or acute phase contributes to Glu excitotoxicity and cell death, NMDA signals may mediate neuroplasticity and protection against apoptosis in the late phase of ischemic injury. A similar biphasic role of matrix metalloproteinase (MMP) family of proteins have been observed where a transitioning from injurious to protective role takes place with

time.⁶⁰ The dual role of astroglia by promoting regeneration or by amplifying injury may also follow defined temporal pattern in the post-ischemic brain.⁶¹

Considering ample evidence about the presence of several chronic processes and the phasic regulation of potential targets, understanding the injury-to-repair transition in a spatiotemporal fashion for all known molecular events in the ischemic brain is essential. Rather than unilateral and sustained modulation, the dosing regimen of future therapeutic strategies should be planned to inhibit the deleterious processes without adversely affecting the endogenous recovery processes.⁶⁰

1.7.3 IS – A Coordinated Multi-organ Response

IS specifically affects the CNS. However, elevated risk of IS with metabolic disorders like diabetes or obesity and circulatory aberrations like hypertension or hyperlipidemia clearly indicate that deregulation of non-CNS organs such as pancreas or heart can predispose to this brain-specific disease. Under normal physiological condition, the brain controls the activity of all extracranial organs through the peripheral nervous system while endocrine mediators communicate through systemic circulation between brain and distant organs. Thus, it is expected that peripheral organs will sense any perturbations in the brain and may respond accordingly.

Focal IS modulates neuroendocrine and immune system in rat as reflected by alteration of the proteome of pituitary, adrenal gland and splenic lymphocytes.⁶² Many of the pathophysiological processes in stroke are a direct result of peripheral immune infiltration into the brain. The spleen responds to ischemic injury by releasing stored immune cells in the bloodstream, which then extravasate into the brain to promote a secondary inflammatory response. Accordingly, splenectomized rats have significantly reduced neurodegeneration following ischemic insult by MCAO. It is suggested that the spleen could be targeted to manage stroke induced neuroinflammation.⁶³ However, post-stroke immunosuppression is associated with potentially fatal systemic infections. Increased muscular activity during exercise has found to enhance the resistance to ischemic injury and neurodegeneration in animal models. This is mediated by upregulating the number of endothelial progenitor cells and by releasing vascular endothelial growth factor or endothelial nitric oxide synthase in the circulation while enhancing the post-ischemic cerebral perfusion and protection of the BBB.¹⁵ The

animal studies show that IS can cause the mobilization of bone marrow derived stem cells (e.g. endothelial progenitor cells, hematopoietic and mesenchymal stem cells) in the peripheral circulation that eventually reaches the site of injury through leaky BBB and provides protective or restorative action.⁶⁴ These encouraging preclinical results form the basis of stem cell or regeneration therapy for the treatment of IS. The sex hormone estrogen has also shown neuroprotective efficacy. The dramatic rise in IS incidence in elderly and post-menopausal women is a testimony to this endocrine connection of IS.⁶⁵ Taken together, in spite of clear emphasis on the central events, understanding the systemic or neurohumoral influences through bidirectional communication of brain and peripheral organs is equally important and may provide novel avenues to counteract IS.

1.7.4 Combination Therapy instead of Single Target

The presence of redundancy, cross-talk and compensation confers an enormous flexibility to the biological system of the CNS. There are always multiple ways to produce or destroy the same protein due to several checkpoints. The whole network is stochastic and non-linear yet dynamically controlled to precision. IS originates in the brain vasculature and affects multiple components of the neurovascular unit causing deregulation of this flexible molecular network. The parallel activation of cell type specific protective and deleterious pathways having transcellular cross-talk with participation of immune system and extracranial organs is an expected mode of compensatory mechanism constituting a coordinated endogenous response. This picture combined with the failure of all single target-specific therapeutic candidates argues strongly against the 'one disease- one target- one drug' concept.

Recent developments indicate an increased impetus from a single target to a combination therapy during preclinical or clinical trials. It has been suggested based on the preclinical evidence that combination of a neuroprotective agent (e.g. NMDA receptor antagonist or oxygen radical scavengers) with thrombolytics might offer better outcome when compared with the neuroprotectants alone. Intravenous tPA has been safely combined with two neuroprotectants, clomethiazole or lubeluzole in separate clinical studies.⁵⁴ An alternative approach is to combine glycoprotein IIb/IIIa receptor agonist that inhibits clot formation by promoting fibrinolysis with a lower

than usual dose of tPA.⁶⁶ Hypothermia has been combined with drugs and ultrasound has been used to aid tPA mediated thrombolysis.⁶⁶ Several advantages of combination therapies are apparent. For example, it might decrease doses of individual agents thus reducing adverse events. The therapeutic window of either of the individual candidates may also be expanded. If a combination therapy involving tPA becomes successful, it might enhance the therapeutic uptake of tPA by reducing the hemorrhagic complication and by expanding the current therapeutic window of 4.5 h.

1.7.5 Multi-target Drugs

The molecular structure with which the drug undergoes major and predominant biochemical interaction is generally considered as the 'target' for the specific drug. It is known that most drugs appear to interact with multiple targets over a range of affinities having either beneficial or deleterious effect. These interactions are usually far out of equilibrium and not energetically optimized. Generation of resistance or side-effects (off-target effects) or idiosyncratic variation in drug effect clearly indicate that systemic responses range beyond single target mechanisms and affect multiple biochemical pathways in different organs. Several examples of clinically used multi-target drugs can be found in various therapeutic areas.⁶⁷ Again a highly selective drug can also lead to unfavorable outcome as exemplified by the failure of cyclooxygenase-2 inhibitor Vioxx that adversely affects the balance between pro- and antithrombotic mediators.⁶⁸ Thus hypothetically, if a compound can favorably modify more than one deleterious event, e.g., apoptosis and lipid peroxidation in the context of IS then it will be an added advantage to counter this polycetiological disease. Of note, a multi-target free radical scavenger approved for clinical use in Japan for more than 10 years, is the only therapy in the 'neruoprotective agent' category to obtain clinical approval anywhere in the world. The pre-clinical and clinical data with edaravone provides an important proof-of-concept for the efficacy of an antioxidant strategy to treat IS as long as the drugs have multiple activities.⁶⁹ The example of the potential application of a synthetically modified curcuminoid, CNB-001 presents an ideal case study where the concept of a multi-target drug and a combination therapy has been envisioned. CNB-001 is a novel multi-target curcuminoid that has superior safety and pharmacokinetic profile compared to curcumin. It is proposed as a potential acute monotherapy that can be used in conjunction with thrombolytics for acute IS.⁷⁰

The drugs that were once called as 'dirty drugs', are now being considered as multi-functional or multi-target drugs. The promising new field of polypharmacology is emerging to integrate the concepts of systems biology with classical pharmacology, multi-target drugs, drug repurposing or combination therapy.⁷¹

In summary, the indications are obvious. All the emerging concepts described above emphasizes inclusion of multiple dimensions, that may be in the form of many components of the neurovascular unit or a combination of more than one therapy and so on to tackle this multifactorial disorder. An overview of the altered system as comprehensive as possible is the prerequisite to initiate such an approach. This formulates a conceptual framework for the introduction of proteomics as a systems biological tool for therapeutic target or biomarker discovery in the current project by delineating the underlying pathological processes of IS in suitable pre-clinical models or clinical samples.

1.8 Evolution of Proteomics and Quantitative Neuroproteomics - Isobaric Tag for Relative and Absolute quantification (iTRAQ)

The term 'proteome' was coined in 1995 by Marc Wilkins from the University of Sydney as an analogy of 'genome'.⁷² Proteomics is defined as the analysis of the whole complement of protein that is expressed by the genome of a cell, tissue or organism. Due to the inadequacies of genomic technologies, proteomics remains one of the fastest growing and most promising avenues of biomedical science in the post-genomic era.

The protein identification techniques in proteomics can be divided in two categories.

1.8.1 Top Down Approach – This approach is suitable for the analysis of Post-translational modification (PTM), mutation or for determination of alternative splice variants and protein isoforms. The intact protein is separated by either two dimensional gel electrophoresis (2D-GF), liquid chromatography or other biochemical methods and subsequently analysed by mass spectrometry (MS).⁷³ 2D-GF is the most commonly used technique, where isoelectric focusing in the first dimension separates proteins on the basis of their net

charge and irrespective of their masses. Mass following denaturation by sodium dodecyl sulphate (SDS) is the basis of separation for the second dimension. However, gel based approaches cannot detect highly acidic or basic proteins or proteins with very high or low molecular weight (MW) and proteins with low abundance. They cannot be automated and suffers from poor reproducibility and low throughput. Following separation, the accurate mass of the protein is determined by high resolution MS instrument during the first MS, when one charge state is selected for fragmentation during MS/MS to determine the sequence and to confidently identify the protein. Despite having various advantages, it remains a challenging task to characterize intact proteins having MW more than 20 KDa.⁷⁴

1.8.2 Bottom Up Approach – In this approach, the purified protein or mixture of proteins is reduced and alkylated before being digested with specific proteases (e.g. trypsin, glu-C). The peptides are analysed by MS to measure the masses which are searched in the protein database containing theoretical digest of all proteins specific to different proteases. This is called ‘peptide mass fingerprinting’ where each experimentally detected peptide and subsequently the parent protein gets a score based on the similarity between experimental and theoretical masses. The accuracy of the protein identification can be increased by sequencing of the selected peptides with MS/MS (i.e. tandem MS), where a peptide is fragmented in the MS machine into smaller ions by various fragmentation techniques such as collision induced dissociation (CID) or electron capture dissociation.

Bottom up approach can be classified as **gel based** and **gel free** methods. In gel based methods, the mixture of proteins is separated by one dimensional gel electrophoresis depending on the sample complexity. Each band or a part of the gel is digested *in situ* by in-gel digestion and the peptides are extracted for downstream analysis. Conversely, the sample containing multiple proteins can be digested directly by in-solution digestion in the gel-free methods. The resulting peptides’ mixture is fractionated by combination of various chromatographic methods (e.g. reverse phase, ion exchange, size exclusion,

affinity) before tandem MS analysis. An unbiased method for rapid and large-scale proteome analysis by multidimensional liquid chromatography, tandem mass spectrometry, and database searching, is named as multidimensional protein identification technology (MudPIT) or shotgun proteomics.⁷⁵ MudPIT is amenable to automation having a wider coverage of the proteome and compatible with various peptides' labelling approaches for quantization.

In the current study, a bottom up approach is adopted with a mixture of gel based and gel free methods. MudPIT or shotgun proteomics is used for the downstream fractionation of peptides for analysis with tandem MS.

Protein quantification is central to most of the biological applications, where relative or absolute expression level of protein is prerequisite in health or disease at different time or space to delineate their role. The quantification methods in proteomics are classified under three main categories.

1. **Gel and Staining Based** – This is more compatible with gel based bottom up approaches. Different fluorophores (e.g. propyl-Cy3 and methyl-Cy5) that emit light at different wavelengths and can form covalent bonds with the lysine or cysteine side chain of proteins are used for parallel labelling of two samples independently (2-plex). The mixture is later separated by 2D-GE and examined by CCD camera or fluorescence reader to perform comparative analysis of spots while the identification is done by downstream MS/MS analysis. This duplex method is called as difference gel electrophoresis.
2. **Label-free** – The relative abundance of the peptides between different samples can be compared either by counting the spectrum or using extracted ion current for each peptide in different samples processed identically and data acquired sequentially in the same MS instrument.
3. **Stable Isotope Based** – There are several approaches. The labelling can be performed on intact proteins *in vivo* (e.g. metabolic labelling or stable isotope labelling by amino acid in cell culture) or at the level of peptides (e.g. isotope coded affinity tag,⁷⁶ iTRAQ,⁷⁷ post-digest ¹⁸O labelling).

Here, I have combined the quantitative ability of iTRAQ with the unbiased identifying capability of MudPIT. This multiplexed iTRAQ can simultaneously quantify proteins from up to eight samples in a single experiment. These isobaric labels consist of amine

specific peptide reactive group and an isobaric tag with a mass of 145. The reactive group helps to form stable covalent links with N termini and lysine side chains of the peptides. Each isobaric tag contains a reporter (mass varies from 114-117 for 4-plex iTRAQ kit) and a balance group (mass varies from 28-31 for 4-plex iTRAQ kit). However, the mass of them is chosen in such a way that the total mass of all isobaric tags (i.e. $114 + 31 = 145$, $115 + 30 = 145$ and so on) remain same despite having unique reporters. Thus, the same peptide from two different samples when labeled with sample-specific isobaric tags, their total mass remains identical (i.e. $\text{peptide}_1 + 145[114 + 31]$ or $\text{peptide}_2 + 145[115 + 30]$, where $\text{peptide}_1 = \text{peptide}_2$). Thus, the peptides are isobaric and remain chromatographically indistinguishable and selected simultaneously as precursor ion after first MS. It is in the collision cell during MS/MS, the fragmentation by CID leads to neutral loss of the balance group as the signature reporter ions (e.g. 114 and 115) are detected in the low mass region of the individual spectra. The higher mass region provides the MS/MS spectral data for confident identification of the peptide whereas the ratio of the peak area of the reporter ions (e.g. 114/115 or 115/114) is used to generate quantitative information for a particular peptide (i.e. peptide_1 or peptide_2). The spectral information of sample-specific and individual peptides is collated to generate quantitative estimate of the proteome in different samples.

Neuroproteomics, the study of proteomes of nervous system is a late entrant in the field of proteomics.⁷⁸ It can be broadly classified among four categories in the line of proteomics.⁷⁹

1. Expression neuroproteomics – Deals with the qualitative and quantitative cataloguing or profiling of the neuroproteome of preclinical or clinical samples. When this aims to discover biomarkers or understand disease mechanisms using clinical samples, it is also called clinical neuroproteomics.
2. Structural neuroproteomics – Determination of the three-dimensional structure of a protein to understand mechanisms and properties of its action in the cell.
3. Functional neuroproteomics – Investigations of protein–protein(s) and protein–other molecule(s) interactions to understand complex physiological processes.
4. Neuroproteomic informatics – Related to the computational tools and databases necessary for handling and analysing proteomic data sets.

My current study belongs to the first and final groups, as iTRAQ based quantitative shotgun proteomic approach have been adopted for profiling of clinical or preclinical samples and the final conclusion was obtained through extensive use bioinformatic analysis.

1.9 Proteomics as an Effective Tool for Target Discovery and Translational Research – Rationale for Project Design

Translation research or the process of target and drug discovery has seen rapid evolution as it passed through successive technological/philosophical phases since its emergence in the 19th century.^{80, 81}

Historically, serendipitous biological or clinical observations lead to certain phenotypic drugs that were used as lead compounds and sequentially expanded by chemical diversification to provide effective compounds in different therapeutic areas. This can be ascribed as the ‘empirical or physiological phase’. With the beginning of ‘biochemical’ phase in early 50s, understanding the mechanisms of drug action at the level of tissue or intact animals became gradually important. Novel chemical entities used to be evaluated iteratively and optimized for potency, efficacy and target selectivity. Structure activity relationships were determined to streamline the synthesis of improved compounds. The advent of molecular biology and biotechnology during 90s with the launch of recombinant DNA technology led to the cloning of drug targets and subsequent mutation studies that provided better understanding of the individual targets at the molecular level. The target or drug discovery experienced a paradigm shift to embrace the ‘molecular’ approach instead of the traditional ‘systems’ approach where a combination of medicinal chemistry, microbiology, biochemistry and pharmacology were used to measure the therapeutic actions at the systems level such as organs or tissues.⁸² The launch of human genome project at the dawn of 20th century and subsequent mapping of complete genome on 2003 initiated the much-hyped era of genomics. The influx of novel targets coupled with high-throughput screening of combinatorial chemical libraries in genetically engineered cell lines was predicted to revolutionize target and drug discovery along with the help of bioinformatic tools. However, despite the sequencing of thousands of novel genes, the function of most

remained unknown as they were not assigned to any classical gene family. Traditionally gene function was studied by forward genetics approach in the pregenomic era by identifying the causative gene for a given phenotype using cell line or animal models. Considering the large number of uncharacterized genes, the reverse genetics approach became prevalent with the utilization of genetically modified cell line or animals to assign function to orphan genes. The trend was soon replicated in the area of therapeutic target discovery for identification and validation of targets. The number of druggable targets increased from ~500 in 1996 to 1446 as obtained recently from the therapeutic target database.⁸³ These include successful targets of already marketed drugs apart from the research targets for which novel chemical entities are tested by various pharmaceutical companies. However, despite the increase in the number of initial hits, rate of approvals of drugs against novel targets remained only 2-3 per year.⁸⁴ Clearly, drug discovery has experienced higher attrition rate in the post genomic era (2003-2010) with success rate in the clinical trial remained a paltry 10% in spite of increasing expenditure.⁸⁵ Worse, the field of neuroscience consistently lagged behind other important therapeutic segments like cancer in basic and translation research.⁸⁶

The hunt for targets based on genetic information has, until now, not delivered its early promise. In retrospect, putative drug targets have seldom been found to be nucleic acids. Only 3% of 1267 research targets and 2% of 268 successful therapeutic targets were classified as nucleic acids based on a report on 2006.⁸⁷ This is expected since protein, being the principle functional unit of the cells, continues to be the target for most clinically successful drugs. The poor correlation between expression of transcripts and proteins is well known.⁸⁸ The PTM, localization or degradation of proteins along with their functional implications cannot be predicted at the DNA or transcript level. The structural variations and protein-protein interaction cannot be accurately mapped based on the sequence information. Further, genomic especially microarray based techniques are not applicable to most of the body fluids (e.g. urine, saliva, tears, cerebrospinal fluid (CSF)) and even certain enucleated cell types (e.g. erythrocytes). All these contributed significantly to the ineffectiveness of genomics technologies as the driving tool for therapeutic target discovery. This implies that the emphasis should be given on the dynamic proteome rather than the static genome for target discovery.

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Identification of individual target genes and their extensive validation in genetically modified cell line or mouse models one by one is costly and time consuming and considered to be the bottleneck for the current ‘molecular’ or reductionist approach. In any case, animals are different from human and more importantly target biology may not reliably predict the drug biology.⁸⁴ For similar reasons, engineered cell lines or *in vitro* system alone are also going to of limited value for identification of viable targets. All these are especially true for an exceedingly complex disease like IS, where multiple subcellular mechanisms and systems level perturbations are involved. Thus, revisiting the traditional ‘systems’ approach is also important where observation is made from animal models as well as in related clinical samples.⁸² The emerging concepts discussed in the previous section also argues against a purely reductionist approach where a preconceived hypothesis is validated. This effectively downplays the chances of identifying unexpected or novel candidates, which is the key advantage of a hypothesis generating proteomic approach.^{86, 89} Accordingly, rather than individual targets, capturing of ‘target space’ should be tried that include certain pathways or protein networks for target prioritization.⁸⁴ Owing to the debatable superiority of either ‘molecular’ or ‘systems’ approach for the identification of targets, an integrative strategy should be the realistic choice. Hence, my dissertation adopts an integrative strategy with a ‘forward proteomics’ mode to quantitatively profile the proteome of preclinical models and clinical samples of IS to correlate phenotype and molecular mechanism with proteomic signature. Considering the limitation of preclinical models to precisely mimic human stroke, parallel proteomic exploration on relevant clinical tissue samples provides an opportunity for direct validation of the target space. The role of biomarkers is complementary as these are candidates accessible easily from body fluids unlike the tissue samples and can quickly provide a rough estimate about the ongoing pathological process happening in known or undisclosed location of the body. This is an added advantage of proteomics as genomic techniques cannot be applied for body fluids. Keeping this in mind autopsied human brain and plasma samples of stroke patients are also included in the experimental design. A detailed literature review indicates at the complete lack of studies using quantitative proteomics where a similar design has been implemented.

1.10 Specific Research Gaps and Objectives

The central hypothesis of my dissertation postulates that iTRAQ based shotgun quantitative proteomics can be used as an efficient tool for the identification of potential therapeutic targets and biomarkers in the area of IS.

The theoretical target for the IS therapy (i.e. penumbra) is known to have competing protective and deleterious pathways based on individual studies done on single targets. But, neither single target can control the future consequences nor will it decide the fate of multiple cell types. Similarly, each of the therapeutically targeted proteins evolves in temporally distinct ways in the ischemic brain, having a synergistic or an antagonistic consequence with respect to certain phenotype at the molecular or systems level. There is no single study to delineate them using a suitable animal model of IS. Taken together, although traditional reductionist approach has generated isolated hypotheses with target-specific strategy using both *in vitro* and *in vivo* models, these may fall short to explain the manifestation of a multifaceted disease like IS. The general predictive value of the *in vitro* and *in vivo* models has also been questioned following successive clinical failures as the targets tend to get lost during translation. Although the reasons are numerous and related to extreme heterogeneity of human stroke, absence of long-term environmental influence or co-morbidity or risk factors in the animals, flaws in clinical trial design or lack of quality of specific animal studies,⁹⁰ the need for direct analysis of clinical samples is increasingly being felt.⁸² Clearly, the majority of the animal studies used rodent that are evolutionally separated from human millions of years ago.⁹¹ African ape, which is the closest model organism to human albeit never used in stroke research, is separated from human by about 5 million years.⁹² Hence, even if pre-clinical studies involving various rodent species or models are highly correlative among themselves, it may not ensure success in the clinical trials. Better co-operation of basic or pre-clinical research with clinical research is warranted. A wealth of bedside-back-to-bench data is needed to exclude model specific artifacts and to identify IS specific 'target space' encompassing different pathological events and cell types with adequate attention to their temporal regulation.

In the clinical front, to offer optimum diagnosis and treatment, the importance of time is being understood increasingly.³² Considering that reasonably effective therapies will

become available in the near future, a delay in diagnosis may render them completely ineffective and sometime deleterious as seen for thrombolytic therapy with tPA.⁴⁶ Despite the recent advent of imaging tools (CT and MRI) to successfully diagnose and differentiate different forms of stroke, ample scope remains for the optimization and improvement of diagnostic modalities. The diagnosis of SVD or lacunar stroke represents an area where diagnostic precision or specificity is compromised in comparison with the large vessel and cardioembolic stroke. Even if IS is diagnosed in a timely manner, tools to predict prognosis and outcome of a specific therapy remain necessary. This is important because a significant proportion of patients do have a recurrence of vascular event or a decline in the cognitive function that contributes to the morbidity in the surviving population. Blood based protein biomarkers for diagnosis, prognosis and even for predicting the risk of IS have recently been proposed as a complementary tool.⁹³⁻⁹⁴ However, the discovery of novel candidates are limited and the research efforts are mainly concentrated on validation of already known candidates which have failed as biomarker for other vascular disorders or neuropathological conditions. Like therapeutic targets, the need for panel of biomarkers instead of a single candidate has recently been addressed to differentiate IS from symptomatically similar clinical conditions with high accuracy.⁹⁵

To summarize, we need to look at the overview from different levels and in parallel for drawing better analogy with clinical stroke and to propose realistic hypothesis having better translation potential or to discover biomarkers with sufficient predictive value. More than 10 years have been passed, since the suggestion of a multi-target therapy instead of a single-target therapy has surfaced.⁹⁶ Even the recent recommendations of the STAIR committee tried to highlight the utility of an integrative systems biology approach.³²⁻⁵⁶ However, lack of suitable technique having the desired depth and breadth has hampered the initiation of such study. This unmet pre-clinical need laid the foundation of the first phase (PHASE I) of the current project, where iTRAQ-based MudPIT was used on cell line, animal and autopsized human brain samples. Given most of the deleterious events take place in the brain parenchyma, capturing the perturbation of tissue proteome should precede the profiling of plasma proteome for the discovery of biomarkers.⁹⁵ This may be more realistic for a disorder like IS, where an alteration of the BBB permeability or vascular dysfunction occurs before or during the evolution of the disease. Thus, the first part

focusing on therapeutic target discovery acts as the prelude for the second phase (PHASE II) of the project that addresses the clinical need for biomarkers in lacunar infarction (LACI), the most poorly understood and diagnosed subtype of IS.

Figure 1.2 Schematic overview of the dissertation. The dissertation is divided in two phases, where iTRAQ-based shotgun proteomics approach was used on various pre-clinical and clinical samples for the discovery of therapeutic targets and biomarkers.

Accordingly, the workable hypotheses and corresponding objectives of each chapter are as follows:

PHASE I

- I. Competing pro- and anti-survival pathways are expected to be present in the ischemic penumbra in a cell-type specific manner. Identification of these in a neuronal model mimicking ischemic penumbra could reveal potential therapeutic targets for neuroprotective intervention.

Accordingly, I propose to apply the iTRAQ-2D-LC-MS/MS approach to identify the competing protective and deleterious pathways in the ischemic penumbra using a validated *in vitro* model of simulated neuronal penumbra using B104 neuroblastoma cell line.

- II. The dynamic response of the brain to ischemia and reperfusion changes temporally and spatially and involves a combined response of the neurovascular unit comprising of neurons, astrocytes, and vasculature

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among others. An animal model instead of a single-cell model is needed to understand the temporal evolution of cerebral ischemia-reperfusion (I/R) injury and to delineate the perturbation of systems level properties.

Accordingly, I propose to temporally profile the affected hemispheric proteome of the ischemic brain in a validated *in vivo* rat MCAO model of transient focal cerebral I/R injury to understand the regulation of potential therapeutic targets during acute and sub-acute phase of I/R injury using quantitative shotgun proteomics.

- III. Rodent models will always offer only an approximation of the human IS. Additionally, the *in vivo* experimental study proposed above would not encompass the spatial pattern of proteomic heterogeneity due to the use of the whole ischemic hemisphere. Quantitative comparison of the ethnicity-age-sex-location matched ischemic and control proteome may reveal better pathophysiological insight about IS and help to validate the preclinical data.

To justify this hypothesis, I propose to use iTRAQ based quantitative proteomics to compare the proteomic landscape of autopsied chronic ischemic human brain with ethnicity-age-sex and location matched control brain tissue from a group of seven individuals.

PHASE II

- IV. LACI being chronic in nature than non-lacunar subtypes of IS offers a longer and more realistic window of opportunity for diagnosis. Being quantitative, plasma based biomarkers may be complementary to the existing imaging tools which are qualitative in nature.

To establish quantitative proteomics as a useful tool for biomarker discovery, the proteome of pooled plasma from a group of LACI patients and age-matched control is compared to propose and prioritize a list of candidate biomarkers for large scale validation study.

This project is expected to identify potential therapeutic targets for basic or translational stroke research and candidate biomarkers for large-scale validation. I

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believe that expanding neuroproteomics to include multiple models or clinical specimens with the integration of other -omics technology-derived (e.g. genomics, lipidomics, metabolomics) information will generate hypotheses free of model or sample specific inconsistencies to greatly facilitate the daunting challenge of druggable target and biomarker discovery for IS.

2. Phenotyping of an *In Vitro* Model of Ischemic Penumbra by iTRAQ-Based Shotgun Quantitative Proteomics

2.1 INTRODUCTION

Extensive physiologic, mechanistic and diagnostic characterization of the ischemic penumbra has taken place since its conception in the late 70s¹⁴ following a reductionist approach of focusing on one or few proteins at a time. The basic deleterious events such as lactic acidosis, Glutamate excitotoxicity, calcium overload or free radical stress have been identified and studied in detail. However, owing to the failure of a plethora of therapeutic targets focusing on individual pathways or proteins, it became essential to map not only the wide array of injurious pathways but also to identify the endogenous protective events that activate in the ischemic brain simultaneously. Capturing the global protein landscape in a suitable model of ischemic penumbra would be an appropriate approach to unravel the cause-effect relationship between multiple mutually competing mediators. This exposes the drawback of the traditional molecular biological approaches which lack the power of comprehensiveness that is an inherent strength of the upcoming proteomic approach.

The recent advances in the field of proteomics and bioinformatics have made it possible to profile the comprehensive protein expression levels of the whole tissue, cells and even sub-cellular organelle in health and disease. More importantly, the proteome from different conditions or cell lines can now be quantified in a single experiment with the multiplexed iTRAQ technology.⁷⁷ It has a diverse range of application in mammalian and other systems to effectively capture the dynamic proteome in a quantitative manner. Despite its popularity, only a few applications of this high-throughput technique have been documented in the area of neuroproteomics.⁹⁷ Neuroproteomics of tissue suffers from the inherent heterogeneity of the brain thus making it difficult to distinguish the origin of a protein coming from the various cell types.⁹⁸ For the same reason, using primary cultured cells or organotypic cultures may not be the best option for mechanistic studies by a quantitative proteomic approach. This results in a dearth of reliable quantitative proteomic data related to cerebral ischemia.

To address this research gap, I applied iTRAQ-based shotgun proteomics (iTRAQ-2DLC-MS/MS) on an *in vitro* penumbral model of neuronal hypoxia and ischemia. As

neurons are the pivotal but the most vulnerable component of the neurovascular unit in the CNS during IS, the B104 neuroblastoma cell line was chosen for the generation of an appropriate *in vitro* model.⁹⁹ To mimic the salvageable part of the ischemic brain, various functional and cytometric assays, including 3-(4,5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) cell viability assay, lactate dehydrogenase (LDH) efflux assay, flow cytometric assays for mitochondrial permeability transition (MPT) pore opening and apoptosis were performed on glucose-deprived hypoxic cells to establish the correct time-point and conditions to mimic the pathology of ischemic penumbra. Afterwards, 4-plex iTRAQ was applied to obtain a quantitative fingerprint of the pathologically relevant proteome to understand the neuroprotective mechanisms.

2.2 MATERIALS AND METHODS

2.2.1 Reagents

Unless indicated, all reagents used for the biochemical methods were purchased from Sigma-Aldrich (Sigma-Aldrich, WI).

2.2.2 Cell Culture

The B104 rat neuroblastoma cell line (kindly provided by Dr. Kazuhiro Ikenaka; Okazaki National Research Center, Aichi prefecture, Japan) was cultured in Dulbecco's Modified Eagle Medium with high glucose (4500 mg/lit) and L-alanyl-L-glutamine (862 mg/l), supplemented with 1% PenStrep (Streptomycin (10000 µg/ml) and Penicillin (10000 units/ml)), and 10% fetal bovine serum (FBS). Cells were grown as a monolayer at 37 °C in a humidified incubator containing 95% air/5% CO₂. All experiments were performed within 8-16 passages of the cells. All media and supplements were bought from Gibco, Invitrogen (Invitrogen, Carlsbad, CA).

2.2.3 *In Vitro* Hypoxia- Ischemia Model

Cells were cultured for at least 24 h to achieve a confluency of 50-70% before subjecting to four different conditions (Figure 2.1C), such as:

- i. Normal growth media (NGM): Normal high glucose media, 10% FBS and 1% penstrep
- ii. Low serum control (LSC): Normal high glucose media, 1% FBS and 1% penstrep
- iii. Hypoxia (HYP): Same as LSC, but with oxygen deprivation
- iv. OGD: Glucose-pyruvate free media, 1% FBS and 1% penstrep with oxygen deprivation

To induce HYP and OGD, cells were washed with deoxygenated normal and glucose-free media respectively for HYP and OGD condition. They were transferred into a humidified airtight chamber (Modular Incubator Chamber (MIC-101), Billups-Rothenberg, Inc., Del Mar, CA) and flushed with hypoxia gas (95% N₂/5% CO₂). Subsequently, the chamber was kept at 37 °C in the humidified incubator for different durations (2, 4, 6 and 8 h). The control (LSC and NGM) plates were directly put inside the humidified incubator without being exposed to hypoxia (Figure 2.1A, B and D).

Figure 2.1 Schematic representation of the in vitro model of hypoxia- ischemia. (A) Dual controls having NGM and low serum (LSC) media. (B) Hypoxia (HYP) and ischemia (OGD) under low serum condition. (C) Bright field microscopic view (magnification: 20X) of B104 cell line cultured in NGM. (D) Photograph of MIC-101. B104 cells under HYP and OGD were placed inside this chamber and flushed with hypoxia gas. All conditions were kept at 37 °C in the humidified incubator.

2.2.4 Preproteomic Model Validation

The cytometric and plate-based assays were repeated with three independent biological samples; three experimental replicates were performed for each sample.

2.2.4.1 MTT Assay

The metabolic activity of the cells (cell viability) was assessed using MTT/formazan-assay.¹⁰⁰ Cells in serum-free media were incubated for 2 h with MTT (0.5 mg/ml) at 37 °C, 5% CO₂, to allow the yellow MTT to be metabolized to purple formazan. It was then dissolved in DMSO and the optical density was measured at 570 nm using a monochromator microplate reader (Safire², Tecan Group Ltd, Männedorf, Switzerland). The optical density was normalized with the number of cells (determined by trypan blue staining in a hemocytometer) for each sample after subtracting the background obtained using a cell-free control. Viability of the cells in NGM was set as 100% to calculate the relative metabolic activity as a percentage of control.

2.2.4.2 LDH Efflux Assay

LDH activity of the culture medium was assessed¹⁰¹ colorimetrically using the Cytotoxicity Detection Kit^{PLUS} (Roche, Mannheim, Germany) following the manufacturer's method. Cell death was measured with respect to the LSC sample which was set at 100% after subtracting the background that consisted of normal or glucose-free media.

2.2.4.3 Flow Cytometric Assessment of MPT Pore Opening

This experiment was performed with the MitoProbe Transition Pore Assay kit (Invitrogen, Eugene, Oregon) following the manufacturer's protocol.¹⁰² In brief, pre-warmed PBS-washed cells were resuspended in Hanks' Balance Salt Solution with calcium (10⁶ cells/ml). Three aliquots (10⁶ cells/sample) were incubated with calcein AM (2 µM), calcein AM+CoCl₂ (80 mM) and calcein AM+CoCl₂+ionomycin (0.1 mM) respectively at 37 °C for 15 min in dark. The first and third one acted as control whereas the change in the fluorescence intensity for the second tube (calcein AM+CoCl₂) between different conditions indicated the continuous activation of MPT

pores. Data acquisition (10^4 events/sample) was performed using the CellQuestPro software in BD FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA).

2.2.4.4 Apoptosis Measurement by Flow Cytometry

This experiment was performed using the commercial BD Pharmingen FITC Annexin V Apoptosis Detection kit I (BD Biosciences, San Diego, CA). Briefly, the trypsin-detached and washed cells from each condition were resuspended in binding buffer (10 mM HEPES/NaOH (pH 7.4) 140 mM NaCl, 2.5 mM CaCl_2) (10^6 cells/ml).¹⁰³ Aliquots of 10^5 cells were stained simultaneously with 5 μl of Annexin V-FITC and propidium iodide (PI) (50 $\mu\text{g/ml}$) for 15 min at room temperature in dark. The percentage of intermediate or late stage of apoptosis was determined from the number of Annexin V (+)/PI (+) cells using the CellQuest Pro software.

2.2.5 Proteomics

2.2.5.1 Experimental Design

The hypoxia-ischemia experiment was performed three times using three different passages of B104 cell line ranging between passage-9 to 12. Each passage was considered as one biological replicate, whereas different dishes of cells in the same passage used for the experiment were considered as experimental replicates. There was at least three experimental replicates for each condition (namely NGM, LSC, HYP, and OGD). My objective was to measure the community effect and to get rid of the erratic individual variations.¹⁰⁴ Thus, cells for the same condition obtained from separate experiments were pooled in order to collate sufficient quantity of cells and at the same time to normalize biological and experimental variations. Two iTRAQ experiments were conducted from the same pooled cell lysate using an anti-parallel labeling strategy (Figure 2.2). This was adopted purposefully to minimize variations due to the potential non-uniform labeling efficiency for the same target peptide among different labels. Technical replicate with respect to mass spectrometry was set at three (Experiment 2, see Figure 2.2) as multiple injections give better coverage of the target proteome with superior statistical consistency. This is especially true for single peptide proteins as more MS/MS spectral evidence was obtained from multiple injections

leading to a higher confidence of peptide identification and quantification¹⁰⁵. The design is depicted in the Figure 2.2.

Figure 2.2 Schematic representation of the experimental design showing biological and technical replicates.

2.2.5.2 Sample Preparation

The harvested cells were lysed at 4 °C with an ice-cold lyses buffer (0.5% SDS, 0.5 M triethylammonium bicarbonate (TEAB), Complete Protease Inhibitor Cocktail(Roche) and phosphatase inhibitor cocktail (PhosSTOP, Roche)) with intermittent vortexing and sonication using a Vibra Cell ultrasonic processor (Jencon, Leighton Buzzard, Bedfordshire, UK). The lysate was centrifuged at 20000 g for 45 min at 4°C and supernatant was stored at -20 °C. Protein quantification was performed using 2-D Quant kit (Amersham Biosciences, Piscataway, NJ). Accordingly, 200 µg of protein was taken from each condition for the subsequent iTRAQ experiments.

2.2.5.3 iTRAQ Labeling

iTRAQ labeling was carried out using commercial reagents (iTRAQ Reagent Multi-Plex kit, Applied Biosystem, Foster City, CA) based on the manufacturer's protocol

with minor modifications. Briefly, the cell lysates were reduced, alkylated and diluted 10 times before digesting overnight with sequencing grade modified trypsin (Promega, Madison, WI) with a mass ratio of 1:50 (trypsin : protein) at 37 °C. The dried peptides were then labeled (Figure 2.2) with respective isobaric tags, incubated at room temperature for 2 h before being combined and vacuum centrifuged to dryness.

2.2.5.4 Strong Cation Exchange (SCX) Chromatography

800 µg of iTRAQ-labeled peptide was reconstituted into 200 µl of Buffer A (10 mM KH_2PO_4 , 25% acetonitrile (ACN), pH 2.85) and fractionated using a 200 X 4.6 mm, 5 µm particle size, 200 Å pore size PolySULFOETHYL A SCX column (PolyLC, Columbia, MD) on a Shimadzu HPLC system (Kyoto, Japan) at a flow rate of 1 ml/min. Buffer B consisted of 10 mM KH_2PO_4 , 25% ACN, 500 mM KCl at pH 3.0. The 50 min HPLC gradient was composed of 100% buffer A for 5 min; 0-20% buffer B for 15 min; then 20-40% buffer B for 10 min; followed by 40-100% buffer B for 5 min and finally 100% buffer A for 10 min. The chromatogram was recorded at 218 nm. A total of 21 fractions were collected after pooling, dried in a vacuum centrifuge and desalted with Sep-Pak Vac C18 cartridges (Waters, Milford, Massachusetts). The desalted and dried sample was reconstituted with 0.1% formic acid (FA) for LC-MS/MS analysis.

2.2.5.5 LC-MS/MS Analysis Using Q-STAR

Each sample was analyzed three times using a Q-Star Elite mass spectrometer (Applied Biosystems/MDS SCIEX, Foster city, CA), coupled to an online Shimadzu micro-flow HPLC system. For each analysis, 30 µl of peptide mixture was injected and separated on a home-packed nanobored C18 column with a picofrit nanospray tip (75 µm i.d. x 15 cm, 5 µm particles) (New Objectives, Wubrun, MA). The separation was performed at a constant flow rate of 30 µl/min with a splitter to get an effective flow rate of 0.2 µl/min. The mass spectrometer was set to perform data acquisition in the positive ion mode, with a selected mass range of 300-2000 m/z . Peptides with +2 to +4 charge states were selected for MS/MS. The three most abundant peptides above 5 count thresholds were selected for MS/MS and each selected target ion was dynamically excluded for 30 s with ± 30 mDa mass tolerances. Smart information-dependent acquisition (IDA) was activated with automatic collision energy and

automatic MS/MS accumulation. The fragment intensity multiplier was set to 20 and maximum accumulation time was 2 s. The peak areas of the iTRAQ reporter ions reflect the relative abundances of the proteins in the samples.

2.2.5.6 Mass spectrometric Raw Data Analysis

The data acquisition was performed using the Analyst QS 2.0 software (Applied Biosystems/MDS SCIEX). Protein identification and quantification were performed using ProteinPilot software 2.0.1, Revision Number: 67476 (Applied Biosystems). The Paragon algorithm in the ProteinPilot software was used for the peptide identification which was further processed by Pro Group algorithm where isoform-specific quantification was adopted to trace the differences between expressions of various isoforms. User defined parameters were as follows: (i) Sample Type: iTRAQ 4-plex (Peptide Labeled); (ii) Cysteine alkylation: MMTS; (iii) Digestion: Trypsin; (iv) Instrument: QSTAR Elite ESI; (v) Special factors: None; (vi) Species: None; (vii) Specify Processing: Quantitate; (viii) ID Focus: biological modifications, amino acid substitutions; (ix) Database: concatenated 'target' (IPI rat; version 3.40; 40389 sequences and 20549266 residues) and 'decoy' (the corresponding reverse sequences); (x) Search effort: thorough. For iTRAQ quantitation, the peptide for quantification was automatically selected by Pro Group algorithm to calculate the reporter peak area, error factor (EF) and *p*-value. The resulting data set was auto bias-corrected to get rid of any variations imparted due to the unequal mixing during combining different labeled samples.

2.2.6 Postproteomic Data Verification with RT-PCR

Total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's protocol and quantified with NanoDropND-1000 (NanoDrop Technologies, Wilmington, DE) to measure the concentration in µg/µl. Specific primers were designed by using open-source primer 3.0 software for Elongation factor 2 (EF-2), Anxa1, glutamate dehydrogenase or GDH (Glud1) (Table 2.1). α -Actinin was used as internal control to check the efficiency of cDNA synthesis and PCR amplification. Two µg of RNA was used for cDNA synthesis and amplified using primers for EF-2, annexin 1, GDH and α -Actinin. PCR products were separated by 1.5% agarose gel electrophoresis.

Table 2.1 Primer sequences for RT-PCR

Protein name	Gene symbol	NCBI number	PCR product (bp)	T _m (°C)	Primer sequence
Annexin A1	Anxa1	NM_012904	175	60.4	5'-aaaggggacagacgtgaatg-3' 5'-ggcacacttcacaatggttg-3'
GDH, mitochondrial precursor	Glud1	NM_012570	243	62.4	5'-ggcgteccataatgacctaca-3' 5'-cctatgggtgctggcataggt-3'
Eukaryotic translation elongation factor 2	Eef2	BC066661	236	62.4	5'-cgcttetatgccttcggtag-3' 5'-gtagtgatgggtgccggtctt-3'
Alpha Actinin	Actn1	BC074001	212	60.4 62.4	5'-cgagtgcacaagatctccaa-3' 5'-ctctgacaccacaggagcaa-3'

2.2.7 Statistical Analysis

Experimental data were presented as the mean of each condition $\pm$ SEM. The n value indicates the number of replicate readings from same or different experiments. One-way ANOVA followed by post hoc Dunnett's multiple comparison test was performed for comparing HYP or OGD condition with NGM or LSC depending on the experiment. Statistical significance was accepted at * $p < 0.05$, ** $p < 0.01$.

2.3 RESULTS AND DISCUSSION

The affected brain tissue in cerebral ischemia suffers from partial deprivation of oxygen, glucose and serum. There exist a gradient of injury radiating out from the epicenter of the affected area (called core of injury) to the adjacent tissue in decreasing insult severity. The penumbra represents the intermediate electrically silent but metabolically viable tissue and is in a transitional phase where competing protective and deleterious pathways remain in a dynamic equilibrium until the latter predominates to seal the fate of the affected cell. Therefore, the penumbra is theoretically salvageable, if the equilibrium could be shifted by stimulating the protective pathways. Here I aim to elucidate the proteomic changes in stroke pathophysiology as a mean to discover therapeutic targets for clinical neuroprotective or neurorestorative therapy.

The *in vitro* OGD or HYP were induced in partially serum-deprived media containing 1% serum (10% normal supply) to mimic the partial depletion of blood supply. NGM (high glucose with 10% serum) that simulates healthy brain tissue with normal supply of serum and nutrients served as the central control. LSC (high glucose with 1% serum) was included to detect protein expression changes due to serum deprivation only. As most of the reported *in vitro* ischemia experiments were performed in a time scale between 0.5-10 h,¹⁰⁶⁻¹⁰⁸ I employed mutually complementary functional and cytometric assays to determine the optimal time-point for unraveling the endogenous protective mechanisms. This not only served to validate the model by avoiding batch variations between replicate biological experiments (due to difference in cell age, culture or experimental conditions) but also helped in selecting the most relevant time-point to recapitulate the penumbral condition for the proteomic experiment.

Table 2.2 Summary of the Preproteomic Model Validation Using Functional and Cytometric assays

Time-point	% LDH cytotoxicity Mean $\pm$ SEM		% MTT viability Mean $\pm$ SEM		Intermediate/end stage of apoptosis or necrosis (Annexin V ⁺ /PI ⁺)		MP1 pore status	
	HYP	OGD	HYP	OGD	HYP	OGD	HYP	OGD
4 h	111.4 $\pm$ 10.5	114.5 $\pm$ 2.1	83.2 $\pm$ 1.9	82.0 $\pm$ 3.5	5.0 $\pm$ 0.4	3.3 $\pm$ 0.1	closed	closed
6 h	105.1 $\pm$ 3.7	139.9 $\pm$ 8.7	66.2 $\pm$ 2.1	66.5 $\pm$ 2.2	NA	NA	closed	closed
8 h	97.8 $\pm$ 1.6	147 $\pm$ 6.7	77.7 $\pm$ 3.3	72.0 $\pm$ 1.2	NA	NA	closed	p/o

p/o : Partially open

2.3.1 MTT and LDH Assay

Time dependent MTT assay (Table 2.2 and Figure 2.3) was performed to measure the metabolic activity (mitochondrial dehydrogenase activity)¹⁰⁹ of the cells following *in vitro* hypoxic or ischemic stress. MTT assay indicated significant decrease in metabolic activity after 4, 6 and 8 h of hypoxic and OGD stress. I then assessed the LDH activity of the culture supernatant after 4, 6 and 8 h of induced stress as an indicator of the overall cytotoxicity and relative membrane integrity of the neuronal cells. As shown in Table 2.2 and Figure 2.3, LDH release progressively increased in OGD to reach ~147% post 8 h of stress, while LSC and HYP did not show significant increase in membrane permeability for LDH up to 8 h.

Figure 2.3 Temporal characterizations (2 – 8 h) of *in vitro* HYP and OGD (ischemia) using functional assays. (A) MTT Assay: Time-dependent changes (2, 4, 6, 8 h) in the mitochondrial redox potential, indicating a significant decrease in metabolic activity at time points post 4 h. (B) LDH Assay: cell death as measured by LDH efflux into the surrounding medium. Note the gradual increase in cell death following 4, 6 and 8 h of OGD. Data represented as mean $\pm$ SEM (n = 8), calculated from at least 3 experimental runs where $^*p < 0.05$, $^{**}p < 0.01$ vs. NGM or LSC using one-way ANOVA test.

2.3.2 Flow Cytometric Detection of MPT Pore Activation and Apoptosis

To further confirm the presence of significant cellular damage after 6 h of OGD stress as indicated above, cytometric assays to assess the mitochondrial function and cellular apoptosis were performed.

Opening of MPT pore indicates a transition from reversible to irreversible cellular damage that induces cell death by apoptosis and necrosis after I/R injury.¹¹⁰

¹¹¹Mitochondrial function was assessed with the MitoProbe Transition Pore Assay Kit to characterize the temporal profile of MPTpore opening induced by the dissipation of the electrochemical proton gradient after HYP and OGD. As seen in the Figure 2.4A-C, OGD caused a gradual increase in MPT pore opening starting at 6 h and continued till 8 h. In contrast, HYP did not elicit a MPT response as indicated by the lack of changes in the calcein fluorescence (Figure 2.4D). These observations which were consistent with the results of the LDH assay suggest that OGD become more cytotoxic than HYP with increasing duration of stress.

Figure 2.4 Temporal analysis of MPT pore opening with the MitoProbe Transition Pore Assay Kit using flow cytometry. CoCl₂ specifically quenches the fluorescence of cytosolic calcein. The decrease in calcein fluorescence (x-axis) indicates opening of the MPT pore. The ionophore ionomycin was included as a positive control to trigger MPT pore opening and subsequent loss of mitochondrial calcein fluorescence in the presence of CoCl₂. (A - C) Gradual opening of the MPT pore following OGD with increasing duration of induced stress. (D, E) Overlays of HYP and OGD at different time-points.

As irreversible mitochondrial damage measured by MPT assay began at 6 h of OGD stress, 4 h of OGD should induce a stressed but salvageable cellular state that might be equivalent to the penumbral condition in cerebral ischemia. To evaluate this hypothesis, the extent of apoptosis at 4 h post OGD was assayed using Annexin V-FITC Apoptosis Detection Kit. Consistent with the observations of LDH and MPT pore assay, neither HYP nor OGD induced significant apoptosis (<5.0%) after 4 h of exposure to stress (Table 2.2, Figure 2.4). Based on the above results (Table 2.2), 4 h was selected as the most suitable time-point for hypoxia-ischemia experiment to mimic the penumbra for the proteomic experiment as the ischemic cells did not show significant membrane damage (<15% increase in LDH release), MPT pore formation or apoptosis.

Figure 2.5 Detection of the apoptotic B104 cell population after 4 h of HYP and OGD. (A) Representative scatter plot showing the distribution of cell populations into four quadrants. (B) Bar diagram of Annexin V-FITC/PI staining after 4 h. Note that there is no significant difference in percentage of intermediate or late apoptosis (annexin+/PI+) for HYP and OGD condition at 4 h. All conditions were compared to NGM ($p < 0.05$) using one-way ANOVA.

2.3.3 iTRAQ Results

To understand the global proteomic changes that occur in the penumbral state, protein extracts from the control cells (NGM and LSC) and cells exposed to 4 h of OGD or HYP were processed as described in the Materials and Methods. Total four MS runs were compared from two replicate iTRAQ experiments (iTRAQ-1: 1 MS run and iTRAQ-2: 3 MS runs, See Figure 2.2). The three technical replicates from iTRAQ-2 were combined during database searching to obtain the iTRAQ-2 data set. This was formally analyzed, whereas the iTRAQ-1 data set was kept as a reference to measure the experimental variation.

The quality of the sample preparation was determined by plotting the distribution of the proteins according to their theoretical MW and theoretical isoelectric pH (pI) values as depicted in Figure 2.6. The presence of proteins covering a wide MW spectrum from high (>200 kDa) to low (<15 kDa) and also a wide pI range from acidic (pI<4) to basic (pI>11) character indicated an extensive representation of the whole proteome in mydata set.

Figure 2.6 Distribution of identified proteins from iTRAQ-2 data set (unused prot score ≥ 3) according to their MW (MW, kDa) and theoretical pI (pI).

To minimize the false positive identification of proteins, a strict cut-off of unused ProtScore ≥ 2 , was used as the qualification criteria, which corresponds to a peptide confidence level of 99%. With this filter, the corresponding false discovery rate (FDR) was calculated from the decoy hits. iTRAQ-1 and iTRAQ-2 were able to identify 1566 and 1796 proteins respectively with a FDR of $\leq 1.0\%$. 40% of the proteins have more than 5 peptides in iTRAQ-2 data set whereas only around 6% comprised of a single peptide having confidence level of 95%.

Figure 2.7 Correlation between two iTRAQ experiments (iTRAQ-1 and iTRAQ-2) and determination of experimental variation using 210 and 139 common proteins (p -value ≤ 0.05) found in both data sets for HYP and OGD respectively. (A) Plotting of the ratios for OGD and HYP from iTRAQ-1 and iTRAQ-2 data set. The correlation coefficient, R^2 was 0.88 and 0.84 and the slope was 0.90 for both HYP and OGD respectively. (B) Percent variation in ratios between the same proteins found in both different % variation that was plotted in the horizontal axis. The secondary vertical axis represents the cumulative % of the counted proteins (lines) where 100% equals to 210 and 139 proteins for HYP and

OGD respectively. More than 90% of the proteins had less than 20% of variation justifying the regulation cut-off of 1.2 fold.

A total of 1081 proteins were found to be common in both iTRAQ experiments. Among them, 210 and 139 proteins for HYP and OGD condition respectively had significant p -values (<0.05).¹¹² The p -value assigned by the Protein Pilot software measures the confidence of the real change in protein expression level. The corresponding ratios of these high confidence proteins were plotted to calculate the experimental variation (R^2), which was found to be 0.88 and 0.84 respectively for HYP and OGD (Figure 2.7A). It should be mentioned here that OGD corresponds to 117/114 and 114/117 whereas HYP corresponds to 116/114 and 115/117 in iTRAQ-1 and iTRAQ-2 experiment respectively (Figure 2.2) thus involving all iTRAQ labels into consideration.

Subsequently, the meaningful cut-off for the up- or down-regulation was finalized by using the experimental replicate method.^{101, 112, 113} The average variation between two iTRAQ experiments was $\pm 8\%$. The experimental variation was less than $\pm 20\%$ for 92% and 97% of the commonly identified proteins with significant ratios ($p < 0.05$) for HYP and OGD stress. In fact, 68% and 79% of the commonly identified proteins had less than $\pm 10\%$ of experimental variation indicating the reliability of the proteomic data (Figure 2.7B). Based on this, the regulation cut-off was set at 1.2 fold; ratio >1.20 or <0.83 was considered as up- or down-regulated.

2.3.4 Verification by RT-PCR

Figure 2.8 RT-PCR results of selected proteins from the iTRAQ data set. EF-2 was down-regulated and annexin I, GDH were up-regulated that were consistent with the iTRAQ results. Alpha actinin that did not show any change in the iTRAQ data set was used as a control. mRNA was collected from the same batch of cells that were used for the iTRAQ experiments.

Some of the differentially expressed proteins were verified by RT-PCR. Three proteins representing distinct functional groups in the data set were chosen. The EF-2 had shown down-regulation, whereas Anxa1 and GDH were up-regulated at the mRNA level in HYP and OGD (Figure 2.8) which were in conformity with the results obtained from the iTRAQ experiment.

2.3.5 Unraveling the Ischemic Pathophysiology by analysis of iTRAQ Data Set

The significantly regulated proteins from iTRAQ-2 experiment were functionally analyzed and classified (Table 2.3) to interpret the molecular events relevant to the pathophysiology of cerebral ischemia.

2.3.5.1 Proteins Related to Chaperonic Response and Protein Metabolism

Chaperonic proteins showed a mixed response depending on their sub-cellular locations (Table 2.3, Figure 2.9A). There were a general decline in the expression of translation initiation (eIF-5A-1, eIF-4E) and chain elongation (EF-2) proteins during HYP and OGD stress (Table 2.3, Figure 2.9B).

Multiparametric imaging in animal models has revealed that protein synthesis in the penumbra was inhibited to conserve ATP supply for the basal metabolic activities.¹¹⁴ The results were consistent with the metabolic penumbral model as indicated by the down-regulation of the initiation and elongation factors (eIF-4E, eIF-5A-1 and EF-2) (Figure 2.9B) without a critical decrease in metabolic activity (as seen in MTT assay in Figure 2.3A) after 4 h of HYP and OGD.

On the other hand, parallel down-regulation of the energy-intensive translation with the co-activation of stress protein expression is indicative of a global stress response. Increasing the chaperonic activity makes the cells better equipped to maintain the critical proteins in optimally folded state under stress while the supply of the newly synthesized proteins is restricted to reduce the workload for the chaperones.¹¹⁵ This protective mechanism was reflected in the result, as down regulation of many cytosolic HSPs (CRHSP24, HSP90 alpha, HSP90 beta) along with initiation and elongation factors were accompanied with an upward trend for mitochondria and endoplasmic

reticulum (ER) -based chaperones (mtHSP70, HSP75, GRP78) (Figure 2.9A,B). Mortalin (mtHSP70/Grp75), the heat un-inducible mitochondrial chaperone has been reported to be up-regulated following focal brain ischemia in response to oxidative injury and glucose deprivation.¹¹⁶ GRP78 acts as a quality control chaperone for ER and is central to the unfolded protein response in ER.¹¹⁷

Figure 2.9 Box whisker plots showing the fingerprint of the functionally important proteins after 4 h of OGD stress. Both data sets (iTRAQ-1 and iTRAQ-2) having a total of 4 separate MS runs were combined to get mean, SEM and SD for each ratio. The gene symbols were plotted in the horizontal axis. The vertical axis represents the corresponding iTRAQ ratios. (A) The chaperonic cluster showed an upward trend for mitochondrial and ER resident chaperones (Trap1, Hspa9a and Hspa5) unlike cytosolic chaperones which were unaffected or down-regulated. (B) Proteins related to the protein metabolism were down-regulated. (C) Most of the anti-oxidative enzymes were down-regulated. (D) Anti-inflammatory (Anxa1, Anxa2) and anti-apoptotic (prohibitin, Bcl2l2) proteins were moderately upregulated. (E) An overview of mitochondrial proteins, where chaperonic response, oxidative phosphorylation (Atp5f1, Atp5c1, Ndufs1, and Ndufs3) showed an upward trend unlike proteins involved in the MPT pore (Vdac1, Ppid, Bcl2l2) which showed a mixed trend. The marker for mitochondrial acidosis, GDH (Glud1), was significantly upregulated. (F) Regulated proteins implicated in other neurological disorders.

Table 2.3 Functional Classification of Regulated Proteins^a

Unused prot score	% Cov (95)	Gene symbol	Protein name	LSC: NGM	HYP: NGM	OGD:N GM	Subcellular location [‡]
Chaperonic response							
41.4	33	Hspa4	Heat shock 70 kDa protein 4	0.92	0.79	0.86	Cytosol
102.8	56.9	Hsp90ca	Heat shock protein HSP 90-alpha	1.00	0.74	0.83	Cytosol
102.8	56.9	Hspcb	Heat shock protein HSP 90-beta	1.04	0.77	0.87	Cytosol
6.1	34.7	Carhsp1	Calcium-regulated heat stable protein 1(CRHSP24)	0.81	0.60	0.78	Cytosol
9.0	15.7	Hspbp1	Hsp70-binding protein 1(HspBP1)	0.89	0.70	0.77	Ubiquitous
26.8	30	Trap1	Heat shock protein 75 kDa, mitochondrial precursor(HSP75)	1.14	1.29	1.20	Mitochondria
47.6	39.3	Hspa9a	Hspa9a, predicted Stress-70 protein, mitochondrial precursor (GRP75/mitHSP70)	1.05	1.22	1.13	Mitochondria
74.5	48.5	Hspa5	78 kDa glucose-regulated protein precursor(GRP78)	1.07	1.24	1.14	ER lumen
Protein Metabolism							
86.1	39	Eef2	Elongation factor 2(EF-2)	0.98	0.71	0.81	Cytosol
16.0	47.4	Eif5a	Eukaryotic translation initiation factor 5A-1(eIF-5A-1)	0.91	0.68	0.73	Nucleus
7.5	27.7	Eif4e	Eukaryotic translation initiation factor 4E(eIF-4E)	0.94	0.67	0.80	Cytosol
14.2	30.9	Eif3j	LOC691947; Eif3s1_predicted Eukaryotic translation initiation factor 3 subunit J	0.84	0.69	0.72	Cytosol
Oxidative defense							
8.1	42.9	Txn1	Thioredoxin (TRX)	0.78	0.49	0.64	Cytosol
10.0	35.3	Ptdx6	Peroxisredoxin-6(PRDX6)	0.91	0.63	0.75	Cytosol, lysosome
13.3	24.9	Txn12	Isoform 1 of Glutaredoxin-3	0.88	0.71	0.81	Cytosol
5.8	15.4	Gsto1	Glutathione transferase omega-1(GSTO1)	0.98	0.67	0.78	Cytosol
6.1	21	Park7	Park7 protein	1.08	0.72	0.82	Cytosol, nucleus, mitochondria
8.8	20.3	Tmx2	Thioredoxin domain-containing protein 14 precursor	1.10	1.28	1.18	ER membrane (probable)
12.7	30.06	Akr1b8	Aldose reductase-like protein	0.97	0.77	0.83	Cytosol

Anti-inflammatory protein							
35.2	33	Anxa1	Anxa1 44 kDa protein, Annexin I	1.06	1.15	1.25	Ubiquitous
12.3	33.4	Anxa2	Isoform Short of Annexin A2, Annexin II	1.13	1.24	1.22	Secreted
25.3	49.2	Anxa5	Annexin A5, Annexin V	0.92	0.84	0.95	NA
Anti-apoptotic protein							
24.6	42.1	Phb2	Prohibitin-2	1.19	1.51	1.25	Mitochondria
19.9	51.1	Phb	Prohibitin	1.09	1.33	1.18	Mitochondria
27.0	33	Pabpn1	Bel2l2:Pabpn1 similar to Polyadenylate-binding protein 2	1.06	1.15	1.25	Mitochondrion membrane
Pro-apoptotic protein							
6.0	24.1	Clc4 [†]	Chloride intracellular channel protein 4 (CLIC4)	0.88	0.71	0.77	ER
Pro-inflammatory protein							
13.5	15.6	Ptgs1	Cyclooxygenase-1 (COX-1)	1.13	1.46	1.31	Microsome, Peripheral membrane or ER membrane
Energy homeostasis							
28.0	55.4	Ckb	Creatine kinase B-type	0.91	0.62	0.70	Cytosol
Cytoskeletal protein							
26.6	26.2	Dpysl3	Isoform 2 of Dihydropyrimidinase-related protein 3 (DPYSL3)	0.97	0.67	0.75	Cytosol

[†]The list contains quantitative information of the proteins from the iTRAQ-2 data set. These proteins have met the criteria (i.e. unused prot score >2.0, change in expression levels of at least 1.2 fold for HYP or OGD relative to NGM, *p*-value <0.05 and EF <1.4 for all ratios) as defined in the "Experimental Procedures".

‡ Source: <http://www.uniprot.org/uniprot>

^{††} <http://www.ncbi.nlm.nih.gov/sites/entrez>

^{†††} *p*-value >0.05

2.3.5.2 Proteins Related to Oxidative Defense

Free radical stress (oxidative and nitrosative) was one of the major factors causing cell death after ischemia of brain and other organs like heart or kidney.^{54, 56} Down-regulation of most of the anti-oxidative proteins (TRX, PRDX6, GSTO1, aldolase reductase-like protein) (Table 2.3, Figure 2.9C) positively correlates with the severity of the induced stress in this acute model. TRX is a small (13 kDa), multifunctional protein with a redox-active site having antioxidant, anti-apoptotic and neurotrophic activity.¹¹⁸ The immunoreactivity of this isozyme had been diminished in the injured region of the cortex and striatum in a neonatal rat model of hypoxia-ischemia after 4-16 h of injury¹¹⁹ and in the rat MCAO model.¹¹⁸ Similarly, PRDX6 has protective effect on the retinal ganglion cells from Glu and TNF- α induced cytotoxicity by reducing ROS level and NF-kappa- β activation and subsequent Ca²⁺ overload.¹²⁰

This down-regulation of the antioxidant enzymes renders the cells susceptible to the massive burst of free radicals generated during reperfusion thus making I/R a deadly combination with poor outcome in clinical situation. Hence, this result also supports the concept of neuro-protection by ischemic post-conditioning either through controlled reperfusion or non-injurious cycles of ischemia-reperfusion.¹²¹ This will allow the cells with sufficient time to revive their antioxidant machinery.

2.3.5.3 Inflammation and Apoptosis Related Proteins

Annexins belong to the superfamily of calcium and phospholipid-binding proteins having diverse biological functions that include signal transduction to inflammation. Three members of this family, Anxa1, Anxa2 and Anxa5 were localized in neurons and glial cells and have potent anti-inflammatory effects through inhibition of phospholipase A₂ (PLA₂) activity.¹²² Several reports have indicated the increased expression of annexins in various pathological conditions like hypoxic-ischemic injury¹²³ and spinal cord injury.¹²⁴ Anxa1, 2 and 5 were quantified in the current experiment, where the first two (Anxa1 and 2) showed the expected upward trend, consistent with the previous studies (Table 2.3, Figure 2.9D).

Prohibitin is present in the inner mitochondrial membrane (IMM) and plays a chaperonic role in the stabilization of newly synthesized subunits of mitochondrial respiratory enzymes.¹²⁵ Over-expression of this protein in cardiomyocytes suppressed H₂O₂-induced mitochondria-mediated apoptotic pathways by inhibiting the release of cytochrome c into the cytosol and the opening of the MPT pore.¹²⁶ This *in vitro* penumbral model showed an up-regulation of prohibitin-2 (51% in HYP, 25% in OGD; $p = 0.00$) and prohibitin (Table 2.3, Figure 2.9D), which may be indicative of an endogenous adaptive response. This finding provides the first illustration of the involvement of this class of chaperone in the pathology of cerebral ischemia to the best of my knowledge.

CLIC4 is a histamine H3 receptor interacting single pass membrane protein which was down-regulated by 29% and 23% (although $p > 0.05$) (Table 2.3, Figure 2.9D) in HYP and OGD respectively. Over-expression of this protein induces apoptosis in several human and mouse cell lines that may be mediated through TNF- α or p53.¹²⁷ I attribute this yet another survival strategy for the cells, where the simultaneous up-regulation of

the anti-inflammatory (Anxa1, Anxa2) and anti-apoptotic response (prohibitin, prohibitin2, Bcl-2 like 2) was complemented by the down-regulation of a pro-apoptotic protein (i.e. CLIC4).

2.3.5.4 Degradation of Key Proteins

The creatine kinase (CK) catalyses the reversible interconversion of creatine into phosphocreatine and is a central controller of the cellular energy homeostasis. Particularly, it plays an important role in tissues with large and fluctuating energy demands like the brain and the muscle. All CK isoenzymes are extremely susceptible to enzymatic inactivation by reactive oxygen and nitrogen species generated under ischemic stress.¹²⁸ Therefore, a significant down-regulation of the cytosolic isozyme creatine kinase type B (38% in HYP, 30% in OGD; $p = 0.00$)(Table 2.3) indicated a compromised oxidative state of the cells.

On the other hand, Glutamate excitotoxicity and oxidative stress can act as the up-stream contributing factor that leads to Ca^{2+} ion overload during ischemia. The deleterious consequences of the Ca^{2+} overload is well-known with respect to the stroke pathology, where it causes a wide-spread activation of proteases (including calpain and caspases), phosphatases, endonucleases and PLA_2 leading to uncontrolled degradation of key cytoskeleton proteins like actin, spectrin, myosin as well as DPYSL3 (Figure 2.10).¹²⁹

Figure 2.10 Flowchart of pathways involved in the degradation of DPYSL3. The encircled proteins were significantly regulated in the iTRAQ data set.

Simultaneous down-regulation of the calpain substrate, DPYSL3 (33% in HYP, 25% in OGD; p -value = 0.00), and up-regulation of COX-1 probably indicates an early sign of Ca^{2+} overload causing the activation of the calpain and PLA_2 -COX1 signaling pathways (Figure 2.10). It has been shown recently that DPYSL3 degradation in rat

cortical neuron can be directly mediated by oxidative stress or Glutamate excitotoxicity or through the downstream metabolic product of PLA₂-COX1 signaling, prostaglandin E₂ (PGE₂) which augments the toxic effects of Glu.¹²⁹ Thus, up-regulation of Anxa1, Anxa2 in this model may be compensatory in nature by interfering with the PGE₂ generation through the inhibition of PLA₂ and can constitute a novel way of protecting the essential cytoskeletal proteins (Figure 2.10).

2.3.5.5 Role of Mitochondria in Ischemic Stress Response

Mitochondria are central to the survival of the neuronal cells following ischemia by controlling the aerobic metabolism and calcium homeostasis. This was revealed by its active participation in the ischemic stress response with many mitochondrial proteins showed significant up-regulation (Table 2.4, Figure 2.9E).

The result indicated the presence of metabolic acidosis as seen by the compensatory increase in the expression of GDH at the level of protein (48% in HYP, 34% in OGD; *p*-value = 0.00) and transcription (Figure 2.8) that may enhance subsequent catabolism of glutamine (Gln).¹³⁰ Activation of proteins involved in the respiratory chain and chaperonic functions (Table 2.4) suggested an early stage of hypoxic-ischemic injury as oxidative phosphorylation will be inhibited if mitochondria are depolarized due to the influx of Ca²⁺ ions through an electrophoretic uniporter. This may reflect cell's need for maintaining the ATP supply to sustain the protein synthesis and cytosolic chaperonic activity, both of which were quite depressed as seen in the previous discussion.

Table 2.4 Functional Classification of Mitochondrial Proteins^a

Unused prot score	% Cov (95)	Gene symbol	Protein name	LSC: NGM	HYP :NG M	OGD :NG M
Metabolic acidosis						
36.7	32.1	Glud1	Glutamate dehydrogenase 1, mitochondrial precursor (GDH)	1.20	1.48	1.34
Chaperonic						
26.8	30.0	Trap1	Heat shock protein 75 kDa, mitochondrial precursor (HSP75)	1.14	1.29	1.20
47.6	39.3	Hspa9a	Hspa9a_predicted Stress-70 protein, mitochondrial precursor (GRP75/ mtHSP70)	1.05	1.22	1.13
24.6	42.1	Phb2	Prohibitin-2	1.19	1.51	1.25

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19.9	51.1	Phb	Prohibitin	1.09	1.33	1.18
Respiratory chain						
9.4	16.8	Atp5f1	ATP synthase subunit b, mitochondrial precursor	1.13	1.38	1.27
5.7	3.6	Atp5c1	ATP synthase gamma chain	1.12	1.26	1.25
31.3	29.7	Ndufs1	NADH-ubiquinone oxidoreductase 75 kDa subunit, mitochondrial precursor	1.01	1.26	1.21
14.7	34.5	Ndufs3	predicted NADH dehydrogenase (ubiquinone) Fe-S protein 3	1.06	1.20	1.17
MPT pore						
22.0	34.3	Vdac1	Voltage-dependent anion-selective channel protein 1 (VDAC1)	1.19	1.45	1.25
22.8	36.8	Ppid	40 kDa peptidyl-prolyl cis-trans isomerase, CypD	0.86	0.63	0.72
Apoptosis related						
27.0	33.0	Pabpn1/Bcl2l2	Bcl2l2(Bcl2-like-2), Pabpn1 similar to Polyadenylate-binding protein 2	1.06	1.15	1.25
6.0	24.1	Clcc4	Chloride intracellular channel protein 4 (CLIC4)	0.88	0.71	0.77

The list contains quantitative information of the significantly regulated mitochondrial proteins from the iTRAQ-2 data set. These proteins have met the criteria (i.e. unused prot score >2.0, change in expression level of at least 1.2 fold for HYP or OGD relative to NGM, p -value <0.05 and EF <1.3 for all ratios) as defined in the "Experimental Procedures" (p -value >0.05).

The role of mitochondria has been implicated in the intrinsic pathway of the apoptotic cascade where different apoptogenic molecules (e.g. cytochrome c, AIF, Smac/Diablo, DNaseG) are released into the cytosol following a sudden increase in outer mitochondrial membrane (OMM) permeability following the MPT.^{131, 132} Formation of the MPT pore takes place in response to the Ca^{2+} overload, oxidative stress and ATP depletion. The MPT pore complex composes of VDAC at OMM, ANT (Adenine nucleotide translocase) in the IMM and CypD located in the mitochondrial matrix. It has been speculated that over-expression of VDAC under conditions of acute hypoxia-ischemia will encourage its dynamic oligomerization at the OMM thus permitting the release of pro-apoptotic proteins from the inter-mitochondrial space.¹³³ Anti-apoptotic members of the Bcl-2 family of proteins (e.g. Bcl-2, Bcl-w) tend to prevent this MPT pore formation probably by blocking the VDAC homodimerization.¹³⁴ The increase in the expression of VDAC1 (45% in HYP, 25% in OGD; $p = 0.00$) in this model thus indicates an ominous signal for the cells that have been duly complemented by the increase in protective Bcl2l2 and prohibitin-2.

Brain mitochondria exert a higher calcium threshold to MPT induction compared to other types (heart or liver) of mitochondria.^{135, 136} This relative resistance might be a protective mechanism as neurons cannot be renewed in the passage of life. Intriguingly, CypD expression decreases progressively during the brain development indicating an inverse relationship with the MPT threshold for Ca^{2+} ion.¹³⁶ In addition, recent reports suggest involvement of CypD in necrotic cell death that is independent of the Bcl-2 family of proteins. CypD along with other components of MPT are also in contention as a promising therapeutic target for I/R injury of the brain.^{111, 137} Thus, the down-regulation (37% in HYP, 28% in OGD; $p = 0.00$) of this essential component of MPT following acute hypoxia-ischemia may constitute a unique survival strategy for the mitochondria in context of the current penumbral model.

Figure 2.11 Complex interplay of pro- and anti-survival mechanisms after 4 h of *in vitro* hypoxia and ischemia.

The quantitative proteomic result indicates the delicate balance between mutually competing numerous pro- and anti-survival mechanisms in the ischemic penumbra after 4 h of acute hypoxia and ischemia. The cells are actively trying to salvage themselves by up-regulating the anti-apoptotic and anti-inflammatory proteins including mitochondrial chaperons and enzymes for ammoniogenesis with a concomitant suppression of the protein synthesis. On the other hand, the collapse of the cellular antioxidant machinery and subsequent inactivation of creatine kinase type B, activation of COX1 and calpain mediated breakdown of the cytoskeleton protein, DPYSL3 indicates the accumulating stress thus exposing cell's vulnerability towards prolonged hypoxic-ischemic or I/R injury (Figure 2.11). This initial phase is crucial for the cells as the pro-death signals eventually outweighs the survival machinery in

the later timepoints when opening of MPT pore causes cell death via apoptosis or necrosis. Hence, it will be interesting to see how the novel or existing pharmacological approaches behave in this model when used alone or in compatible combinations at different time-points (4 h or 8 h).

2.3.6 Proteins Related to Chronic Neurological Disorder

I also discovered a set of novel proteins regulated in this acute penumbral model (Table 2.5, Figure 2.9F) that have an important implication in different chronic neurological disorders.

Table 2.5 Proteins Related to Chronic Neurological Disorders^a

Unused prot score	%Cov (95)	Gene symbol	Protein name	ISC: NGM	HYP: NGM	OGD: NGM
Alzheimer's disease						
26.0	65.7	Ywhaz	14-3-3 protein zeta/delta	0.76	0.63	0.82
5.8	15.4	Gstol	Glutathione transferase omega-1 (GSTO1)	0.98	0.67	0.78
9.5	17.0	Sugt1	Suppressor of G2 allele of SKP1 homolog	0.92	0.68	0.79
6.4	15.4	Pak1	Serine/threonine-protein kinase PAK 1	0.97	0.76	0.79
10.6	15.3	Pak2	Serine/threonine-protein kinase PAK 2	0.95	0.77	0.85
4.3	41.1	Hmgal	Isoform HMG-I of High mobility group protein HMG-I/HMG-Y	1.18	1.34	1.23
Parkinson's disease						
6.1	21.0	Park7	Park7	1.08	0.72	0.82
Bipolar disorder						
3.7	13.3	Vapa	Vesicle-associated membrane protein-associated protein A (VAP-A)	1.17	1.36	1.28

^aThe list contains quantitative information of the significantly regulated proteins related to chronic neurological disorders from the iTRAQ-2 data set. These proteins have met the criteria (i.e. unused prot score >2.0, change in expression level of at least 1.2 fold for HYP or OGD relative to NGM, *p*-value <0.05 and EF <1.3 for all ratios) as defined in the "Experimental Procedures".

Park-7 (also known as DJ-1), which was moderately down-regulated (28% in HYP, 18% in OGD; *p*-value= 0.00) in this penumbral model, was initially discovered as a protein involved in the pathology of Parkinson's disease (PD). Recent reports have indicated a significant protective role of DJ-1 in both *in vitro* and *in vivo* models of stroke, where a loss of DJ-1 increases the vulnerability to excitotoxicity and ischemia.^{138, 139} Similarly, the omega class of glutathione S-transferase (glutathione-dependent thiol transferase and dehydroascorbate reductase) was another down-regulated multifunctional enzyme in the data set having a protective role against

oxidative stress. It has been implicated in a variety of neurological disorders like early onset Alzheimer's disease (AD), PD, vascular dementia, cerebral atherosclerosis and amyotrophic lateral sclerosis.¹⁴⁰⁻¹⁴² Thus, my results corroborate previous studies which showed a general decline of the antioxidant machinery in the current model.

The down-regulated 14-3-3 protein zeta/delta is a highly abundant adaptor protein and reported as a hub in the genetic network of AD and aging.¹⁴³ Another down-regulated candidate, suppressor of G2 allele of SKPI homolog, has been suggested as a possible marker of degenerating neurons in AD based on its decreased immunostaining in the AD-affected cortex.¹⁴⁴ Similarly, a recent report relates PAKs (serine/threonine class of protein kinases), that was moderately down-regulated at the level of total protein, to synaptic loss and cognitive deficits in the AD patients.¹⁴⁵

The moderately up-regulated vesicle-associated membrane protein-associated protein A (VAP-A) (36% in HYP, $p = 0.002$; 28% in OGD, $p = 0.017$) is a single-pass type IV membrane protein that interacts with presynaptic proteins and is necessary for vesicular neurotransmission. Recently, polymorphism of VAP-A has been linked to bipolar disorder (BD).¹⁴⁶ The other member of this 'up-regulation' group, HMG-I/Y is a nucleus-resident non-histone protein, over-expression of which has been observed in the brain tissue of sporadic AD patients.¹⁴⁷

Although the down-regulation of the above proteins can be explained by the correlative decrease in the overall rate of protein synthesis or increased turnover, the significant up-regulation of some of them (HMG-I/Y, VAP-A) (Figure 2.9F) is a novel finding in context of this acute model, which necessitates further study to adequately understand their roles in the pathology of cerebral ischemia. Nevertheless, the result clearly indicates the complex interaction and presence of common signaling pathways between radically differing neuropathologies.

2.3.7 Possible Presence of Glucose Paradox

This result provides evidence to support the existence of a relative "glucose paradox", which is defined by the aggravation of post-ischemic outcome due to pre-ischemic hyperglycemia. This phenomenon has only been observed in numerous *in vivo* studies but a convincing explanation is not available yet. Tentatively, it has been attributed to

either lactic acidosis or to the involvement of stress hormones and glucocorticoids.¹⁴⁸ Here, I demonstrate a similar enigma as my result showed that glucose deprivation did not produce a synergistic damaging effect when coupled with oxygen deprivation (i.e. OGD) in the initial phase (4 h) of *in vitro* ischemia. This is evident in the trend of the absolute protein expression in the iTRAQ data set (Tables 2.3-2.5) as well as in the functional and cytometric assays. The expected trend was observed in the functional assays when the duration of stress was increased to 6 h or 8 h (Figures 2.3B and 2.5) as OGD becomes more lethal than HYP. This crucial finding, novel with respect to an *in vitro* system, may be related to the type of cell line, culture conditions or the experimental design. This further indicates the participation of cell-specific mechanisms as systems level properties, like hormonal interference, can be ruled out in an isolated cell system. Nevertheless, this *in vitro* penumbral model with the neuronal B104 cell line can be used for studying the mechanisms involved in the glucose deprivation-induced transient protection against ischemic insult.

2.4 CONCLUDING REMARKS

By applying a discovery-driven iTRAQ-based quantitative proteomic approach to a validated *in vitro* neuronal hypoxia-ischemia model I was able to capture the early stages of neuronal response to cerebral hypoxic and ischemic stress. This was achieved even when most of the functional assays fail to differentiate hypoxic and ischemic cells from the normal cells in absence of significant morphological or functional abnormalities. The preproteomic assays suggested the presence of a therapeutic window for the ischemic penumbra having a set of mutually competing protective and deleterious pathways. Therefore promoting the protective and suppressing the deleterious pathways in a compatible combination could provide a novel strategy for neuroprotective intervention (Figure 2.11). The sheer number of detected proteins (634 and 739 proteins for OGD and HYP respectively) with quantitative information having acceptable levels of confidence (i.e. p -value < 0.05) also makes this iTRAQ-based 2D-LC-MS/MS approach as the preferred technique to investigate the underlying molecular mechanisms of cerebral ischemia at the systems level. Moreover, these short-listed proteins can act a starting point for the follow-up experiments to characterize individual proteins by RNA interference (RNAi) to identify potential neuroprotective targets for interventional studies. My findings also indicate the

probable presence of a relative glucose paradox under *in vitro* condition. Furthermore, many proteins involved in chronic neurological disorders (AD, PD and BD) were changed in this acute neuronal injury model, suggesting the involvement of some common pathways between deleterious neuronal diseases. Concluding, these results show that the iTRAQ-based shotgun proteomic approach not only constitutes a novel way of investigating the pathophysiology of complex diseases in validated models but can also act as a screening tool in discovery-driven translational research.

3. Quantitative Neuroproteomics of an *In Vivo* Rodent Model of Focal Cerebral Ischemia Reperfusion Injury Reveals a Temporal Regulation of Novel Pathophysiological Molecular Marker

3.1 INTRODUCTION

Therapeutic or spontaneous reperfusion following focal IS is essential for better recovery of the affected tissue.^{152–155} However, it can also lead to free radical injury apart from hemorrhagic complications.^{149–150} Recently, post-conditioning through controlled therapeutic reperfusion has gained importance in the clinical setting with the finding that uncontrolled thrombolysis can lead to reperfusion injury.¹⁵¹ The instigation and progression of several subacute and chronic events along with the presence of viable neurons way beyond the narrow therapeutic window (i.e. 4.5 h) of the lone drug, viz tPA indicates that the damage is not all done in the first few hours. Conversely, this establishes time as an importance determinant in the evolution of cerebral I/R injury. Failure of Glu receptor antagonists in clinical trials was a testimony of ignoring the biphasic role of Glu in the ischemic brain as well as the hyper-acute nature of Glu excitotoxicity in the pathological cascade of stroke.^{60, 152} In addition, biphasic response of many other proteins (e.g. vascular endothelial growth factor, Src kinase, MMP family of proteins), with a transition from a pro- to an anti-survival role, or vice-versa, during the progression of the secondary injury following I/R indicates the presence of a target-specific time-window of opportunity.^{60, 152, 153} Hence, the pressing need in IS research is not only the finding of realistic target space instead of a single target but also to determine their regulation during the course of temporal evolution of the disease. However, the traditional reductionist approach is not exhaustive enough to generate a comprehensive temporal signature of the stroke-affected brain in order to characterize multiple prospective targets in a suitable *in vivo* model of cerebral ischemia.

Despite recent advances in proteomic technologies and its extensive application in other therapeutic areas, neuroproteomics of cerebral ischemia still remains in its infancy.¹⁵⁴ Some of the earlier neuroproteomic studies on experimental ischemic brain samples would fall in the category of efficacy studies,^{155–157} whereas the others, that were mechanistic in nature, were either related to ischemic preconditioning^{158–159} or used a longer duration of ischemia without reperfusion.^{156, 160} Moreover, irrespective of their specific objectives, they were technically dependent on traditional 2D-GE-MS/MS.^{157, 158, 160, 161} Common subset of proteins (e.g. spectrin α II chain, heat shock proteins, and dihydropyrimidinase-related protein 2) identified by 2D-GE-MS/MS as

regulated proteins was proposed as potential pathological markers for specific events of the ischemic pathophysiology. In comparison with the gel-based methodologies, the LC-MS/MS-based MudPIT⁷⁵ combined with iTRAQ⁷⁶ provides an alternative approach for quantitative proteomic profiling. In the previous chapter, I have successfully applied the same strategy for the first time in the area of IS to study a validated *in vitro* model of ischemic penumbra.¹⁶²

To extend its application into a clinically relevant *in vivo* model, here I used the iTRAQ-MudPIT approach in an extra-cranial transient MCAO model of focal cerebral I/R injury for quantitative temporal profiling of the ischemic hemisphere. First, focal ischemia for 2 h alone or in combination with an acute (i.e. 4 h) and subacute duration (i.e. 24 h) of reperfusion were included in the experimental design to make it analogous to the clinical cerebral I/R injury. Neurological scoring of the affected animals, TTC staining of the brain and western blots (WB) of representative hypoxia markers were performed to confirm the success of this MCAO model. Subsequently 4-plex iTRAQ experiment was used to generate a list of the pathologically important and regulated proteome from the ischemic hemisphere. Finally, RT-PCR and WB were used to measure the mRNA and proteins levels of some the perturbed candidates to better understand the source of regulation. In addition, a few of the selected proteins were spatiotemporally mapped by WB analyses to scrutinize deep into their functions during the evolution of cerebral I/R injury. The result shows for the first time a phasic regulation of several novel proteins during the course of I/R injury thus indicating the importance of a temporally optimized therapeutic strategy to extract maximum clinical benefit.

3.2 MATERIALS AND METHODS

3.2.1 Reagents

Unless indicated, all reagents were purchased from Sigma-Aldrich (St. Louis, MO).

3.2.2 Animals

6 – 8 weeks old, male Sprague-Dawley rats weighing 225 – 275 g were cage acclimated for 3 – 6 days prior to surgery in a temperature-controlled environment (24

$\pm 3^{\circ}\text{C}$) on a 12 h light: 12 h dark cycle, with food and water *ad libitum*. Animal protocols were approved by the Nanyang Technological University Institutional Animal Care and Use Committee. Before undergoing the experimental procedures, all animals were clinically normal, free of any infection or inflammation and did not show any neurological deficits.

3.2.3 Induction of Cerebral I/R Injury

Focal cerebral ischemia was induced by extracranial intraluminal MCAO following the previously described method of Zea Longa³⁷ with minor modifications.¹⁶³ Anesthesia was induced with 4% Isoflurane (Nicholas Piramal, Mumbai, India) in oxygen and was maintained at 2% during the procedure. Body temperature was monitored with a rectal probe and was maintained within normal limits with a feedback-controlled homeothermic blanket (Harvard Apparatus, Massachusetts, USA). Briefly, the left common carotid artery (CCA), external carotid artery (ECA) and internal carotid artery (ICA) were sequentially exposed (Figure 3.1A, D). The pterygopalatine artery was not occluded. A 30 mm length of 3-0 polyamide monofilament non-absorbable surgical suture (Ethicon, Johnson and Johnson, USA), coated with poly-L-lysine solution (0.1% (w/v), in deionised water), was inserted via ECA into the ICA (Figure 3.2B, D). When the suture was advanced 19–22 mm from the bifurcation of ECA-ICA till the origin of the left MCA and anterior cerebral artery, resistance could be felt, indicating the blockade of circulation at the left MCA territory (Figure 3.1C, D). The incision was closed and the animal was allowed to recover from anesthesia on the blanket. Once the animal regained consciousness, it was caged with free access to food and water. After 2 h of MCAO, blood flow was restored (reperfusion) by withdrawal of the inserted suture. Sham operation was performed identically as described above, including reanesthetizing the sham animals after 2 h of surgery, except for the brief introduction of the filament into the ECA. The animals were monitored at regular intervals as a part of post-operative care during the course of the experiments and normal saline (2 ml) was administered when necessary. Special attention was paid to ensure the accessibility of food and water.

Figure 3.1 Establishment of MCAO model of focal IS. Representative photographs showing (A) isolation of CCA, (B) insertion of poly-L-lysine coated 30 mm 3-0 polyamide monofilament suture through ECA (arrow), (C) the end of the inserted suture after MCA occlusion, (D) Schematic diagram of the vascular anatomy showing different branches of ICA and the position of the inserted suture after MCA occlusion. PPA, pterygopalatine artery; HTA, hypothalamic artery; AchA is anterior choroidal artery; PCOM is posterior communicating artery; ACA is anterior cerebral artery.

3.2.4 Preproteomic Validation

3.2.4.1 Neurological Evaluation

Postischemic motor and behavioral deficits were evaluated on a scale of 0–4 adopted from a previously reported neurological score with slight modifications.¹⁶⁴ Tests were performed on all animals before and after occlusion, 1 h post occlusion, before and after reperfusion to ensure the success of the MCAO model.

- i. Grade 0: No detectable deficits.
- ii. Grade 1: Forelimb flexion. Held gently by the tail and suspended 1–2 m above the ground, animal showed consistent flexion of the forelimb contralateral to the ischemic hemisphere (in this case, the lesion was at the left hemisphere), varying from wrist flexion and shoulder abduction to severe thorax twisting (Figure 3.4A).

- iii. Grade 2: Stimulated circling towards the paretic side. Animal was lifted by the tail to align it at about 45° with the floor plane and was observed for circling behavior (Figure 3.4B).
- iv. Grade 3: Spontaneous circling towards the paretic side. Animal was allowed to move about freely and was observed for circling behavior (Figure 3.4C).
- v. Grade 4: No spontaneous motion; absence of exploratory behaviors; falling on the paretic side following lateral push or without lateral push; unconsciousness or death.

Cumulative scoring (on a 10(=0+1+2+3+4) point scale) was performed to determine the success of surgery as mentioned in the selection criteria. In case of partial or incomplete manifestation of any of the grades, the animals were scored 0.5 less than the respective grade.

3.2.4.2 TTC Staining

TTC staining was performed as described to confirm the presence of infarct in the MCA territory.¹⁶⁵ Rats were euthanized under deep anesthesia after 24 h of reperfusion. The brains were sectioned into coronal slices (2 mm thick), 3 mm from the frontal lobe using a rodent brain matrix (ASI Instruments, Inc., city, MI, USA). Slices were immersed in 1% TTC solution at 37 °C for 15 min and then fixed in buffered 4% formaldehyde solution overnight. The normal tissue stains red whereas the infarct tissue appears white due to the loss of mitochondrial enzymatic activity. They were transferred to a transparent film and scanned at a resolution of 600 dpi using an Epson scanner. The infarct area of each section was traced and measured using open source image analysis software (Scion Image, Alpha 4.0.3.2, Scion Corporation, Frederick, MD, USA). The following parameters were calculated: (1) % area of infarction at each coronal level (% hemispheric infarction area, %HIA), (2) total area of infarction, expressed as the % of the left hemisphere (3) % edema at each coronal level of the infarcted hemisphere (%HE), (4) % edema of the left hemisphere. The %HIA was corrected for the effect of edema by applying the following formula: %HIA = [(right hemispheric area – left hemispheric area + measured infarct area)/left hemispheric area] × 100 as described previously.¹⁶⁶ Edema of the left hemisphere, %HE, was calculated as follows: %HE = [(left hemispheric area – right hemispheric area)/right hemispheric area] × 100.

3.2.5 Proteomics

3.2.5.1 Experimental Design

The experimental design is described in Figure 3.2 and Table 3.1. All animals were randomly selected for age and body weight. The duration of MCA occlusion was fixed at 2 h to obtain reproducible infarction with lesser intergroup variation.^{163, 167} This 2 h MCAO model is characterized by maximum infarction and vasogenic edema during 24 h - 48 h post-reperfusion. Thus, to evaluate the proteome regulation due to ischemia, acute and subacute phase of reperfusion injury, three groups of rats ($n = 3$ per group i.e. biological replicate = 3) with only 2 h of ischemia (i.e. 2 + 0 h) followed by two different lengths of reperfusion (i.e. 2 + 4 h and 2 + 24 h) were included in this experimental design. Sham surgery models of 2 h, 6 (= 2 + 4) h and 26 (= 2 + 24) h were designed to mimic the surgical trauma of 2 + 0, 2 + 4 and 2 + 24 groups respectively. The final ischemic area as a result of occlusion of the MCA is rather consistent with this model. But the salvageable brain tissue decreases continuously due to the expansion of injury from the core to the penumbral area during reperfusion thus making it difficult to select specific area of affected hemisphere for temporal profiling. My aim was to elucidate the evolution of I/R injury as a function of time by using the quantitative proteomic strategy. Thus, the whole left or ischemic hemisphere excluding the olfactory lobe and cerebellum (hereafter called as ipsilateral hemisphere) was selected in this temporal proteomic experiment and comparison was made between groups of sham and I/R-affected animals. Moreover, pooling the ipsilateral hemisphere from three different animals minimized the surgical and biological variations between individual subjects. Injection for LC-MS/MS was performed thrice (technical replicate = 3) as multiple injections give better coverage of the target proteome with superior statistical consistency. This is especially true for single peptide proteins as more MS/MS spectral evidence was obtained from multiple injections leading to higher confidence of peptide identification and quantification.¹⁶⁸ The same pooled extracts were used for postproteomic data validation using WB analysis. RT-PCR was also performed in duplicate using a separate set of animals without pooling ($n = 2$, 4 groups). Finally, the whole hemispheric target identification strategy guided the selection of perturbed candidates for postproteomic spatiotemporal profiling by WB analysis with three biological replicates for each group (except sham, where $n = 1$).

Figure 3.2 Schematic illustration of the experimental design. It is divided into iTRAQ based quantitative proteomics, data verification by complementary techniques and subsequent spatiotemporal profiling of selected candidates.

Table 3.1 Summary of the Group Allocation for Different Experiments

Experiments	Purpose	Group of Animals			
		Sham	2 + 0 h	2 + 4 h	2 + 24 h
iTRAQ-2D-LC-MS/MS	Temporal profiling	3	3	3	3
WB	iTRAQ data Validation	Same pooled protein samples from iTRAQ experiment			
RT-PCR	Estimation of mRNA level	2	2	2	2
WB	Spatiotemporal profiling	1	3	3	3

3.2.5.2 Selection Criteria

The animals showing a consistent neurological score of at least 1, 2 but not more than 3 (cumulative neurological score of 3 - 6) during the occlusion period were included in this study and randomly allocated between different groups. The animals with a score <3 were termed as 'surgical failure' and were excluded. In addition, surgery

duration was kept similar in all four groups (25 ± 5 min). Any animal with the surgery duration beyond the above-mentioned time span was excluded to avoid the differential interference of the inhalational anesthetic, isoflurane.¹⁶⁹ The suture was withdrawn seconds before the starting of trans-cardiac perfusion for animals of the '2+0' group to ensure complete perfusion and to avoid any reperfusion injury. The perfused brains with a visible hematoma or hemorrhagic spot near the MCA territory or those that were not perfused properly were excluded to avoid unnecessary interference of the circulatory proteins.

3.2.5.3 Sample Preparation

The perfused ipsilateral cerebral hemispheres were obtained immediately after termination, briefly washed with PBS solution, snap-frozen in liquid nitrogen and stored at -80°C until use. Frozen samples were homogenized with liquid nitrogen before lyses at 4°C with ice-cold lyses buffer [2% SDS; 0.5 M TEAB with Complete Protease Inhibitor Cocktail (COMPLETE, Roche, Mannheim, Germany) and phosphatase inhibitor cocktail (PhosSTOP, Roche)] by intermittent vortexing and sonication (amplitude, 23%; pulse: 5 s/ 5 s for 5 min) using a Vibra Cell high intensity ultrasonic processor (Jencon Scientific Ltd, Leighton Buzzard, Bedfordshire, UK). The lysates were centrifuged at $20\,000 \times g$ for 30 min at 4°C . The supernatant was collected and stored in aliquots at -80°C (longer term) or at -20°C (shorter duration). Protein quantification was done using Bicinchoninic Acid Protein Assay kit.

3.2.5.4 In-Gel Tryptic Digestion and Isobaric Labeling

The samples were subjected to a denaturing polyacrylamide gel electrophoresis (PAGE) for the purpose of removing the non-protein interfering substances. Briefly, 500 μg of protein from each condition were run on an 8% stacking – 25% separating gel. Proteins which migrated into the 8% layer were retarded by the 25% layer, thus concentrating them in a narrow strip at the end of the stacking gel. The diced gel bands were then reduced (5 mM tris-(2-carboxyethyl) phosphine, 60°C , 1 h) and alkylated (10 mM methyl methanethiosulfonate in isopropanol, room temperature, 15 min) before being digested with 10 ng/ μl of sequencing-grade modified trypsin (Promega, Madison, WI) for overnight at 37°C . The peptides were extracted with 50% ACN and vacuum centrifuged to dryness. The dried peptides were reconstituted into 0.5 M

TEAB and ethanol, and labeled with respective isobaric tags of 4-plex iTRAQ Reagent Multi-Plex kit (Applied Biosystems) as follows: sham, 114; 2 + 0, 115; 2 + 4, 116; 2 + 24, 117 (Figure 3.2). The labeled samples were combined after 2 h and dried in a vacuum centrifuge.

3.2.5.5 SCX Chromatography

The dried iTRAQ-labeled peptide was reconstituted in Buffer A (10 mM KH_2PO_4 ; 25% ACN; pH 2.85) and fractionated using a PolySULFOETHYL A SCX column (200 $\times$ 4.6 mm; 5 μm ; 200 Å) (PolyLC, Columbia, MD) as mentioned previously¹⁶² on a Prominence HPLC system (Shimadzu, Kyoto, Japan) in a 50 min gradient with Buffer B (10 mM KH_2PO_4 , 25% ACN, 500 mM KCl (pH 3.0)). Eluted fractions were collected in every 1 min, and then pooled into 25 fractions, depending on the peak intensities, before drying them in a vacuum centrifuge. The dried fractions were desalted through Sep-Pak C18 SPE cartridges (Waters, Milford, MA) and stored at -20 °C till MS analysis.

3.2.5.6 LC-MS/MS Analysis using QSTAR

The iTRAQ-labeled desalted peptides were reconstituted with 0.1% FA for MS analysis. Each sample was analyzed three times using a QSTAR Elite Hybrid MS (Applied Biosystems), coupled to an online HPLC system (Shimadzu). For each analysis, 30 μl of peptide solution was injected, concentrated in a C18 peptide trap, and was separated on a home-packed nanobored C18 column with a picofrit nanospray tip (75 μm i.d. $\times$ 15 cm, 5 μm particles) (New Objectives, Wubrun, MA). Mobile phase A (0.1% FA in 2% ACN) and B (0.1% FA in 100% ACN) were used to establish a 90 min HPLC gradient with an effective flow rate of 0.3 $\mu\text{l}/\text{min}$, obtained from a constant flow of 30 $\mu\text{l}/\text{min}$ using a splitter. The mass spectrometer was set to perform data acquisition in the positive ion mode. Precursors with a mass range of 300 – 1600 m/z and calculated charge of +2 to +4 were selected for fragmentation. The three most abundant peptide ions above a 5 count threshold were selected for each MS/MS spectrum. The selected precursor ion was dynamically excluded for 30 s with a 30 mDa mass tolerance. Smart HDA was activated with automatic collision energy and automatic MS/MS accumulation. The fragment intensity multiplier was set to 20 and

maximum accumulation time was 2 s. The peak areas of the iTRAQ reporter ions reflect the relative abundance of the proteins in the samples.

3.2.5.7 Mass Spectrometric Raw Data Analysis

The spectral data acquisition was performed using the Analyst QS 2.0 software (Applied Biosystems). ProteinPilot software 3.0, Revision Number: 114732 (Applied Biosystems) was used for peak list generation, protein identification and quantification against the International Protein Index rat database (version 3.40; 79354 sequences; 41861410 residues).¹⁷⁰ A concatenated target-decoy database search strategy was also employed to estimate the FDR. FDR was obtained by calculating the percentage of decoy matches among total matches. The user defined parameters of the software were configured as described previously with minor modifications (ID Focus: biological modification). The ProteinPilot software employed Paragon and Pro Group algorithm for the peptide identification and isoform-specific quantification respectively. Details of the quantification algorithm can be found in the supplier's manual. The resulting data set were auto bias-corrected to get rid of any variations imparted due to the unequal mixing during combining different labeled samples. Subsequently background correction was also performed to eliminate any background ion signal due to non-target peptides, co-eluting with the target peptide.

3.2.6 Bioinformatics Analysis

Batch search was adopted by uploading the gene IDs of the proteins of interest using PANTHER 7.0 (Protein Analysis Through Evolutionary Relationships) classification system¹⁷¹ against the NCBI (*Rattus norvegicus*) data set. The regulated genes were categorized according to the molecular functions or biological processes or protein classes or pathways.

3.2.7 WB Analyses

WB was performed after SDS-PAGE by probing with specific horseradish peroxidase (HRP)-conjugated primary antibodies directed against proteins at the indicated dilutions: hypoxia-inducible factor-1 (Hif1a) (clone H1alpha67, 1:500; Novus Biologicals, Littleton, CO), Tf (1:2000, Abcam Ltd., Cambridge, UK), heat shock

protein 70 (Hsp70, 1:5000; Abcam Ltd.), Slc1a3 (1:1000; Cell Signaling, Danvers, MA), Caskin1 (1:1200; Santa Cruz Biotech, Santa Cruz, CA), Vcl (1:4000; Millipore, Billerica, MA), Vim (1:2000; Millipore), Gfap (1:10000; Millipore), Actn1 (clone C4, 1:4000; Millipore), β -tubulin (clone B7, 1:2500; Santa Cruz). Immunoreactivity was detected by using an HRP chemiluminescent substrate reagent kit (Invitrogen, Carlsbad, CA). The membranes were stained with Ponceau Sand later stripped and reprobed against the Actn1 or β -tubulin antibody to confirm uniform transfer and equal loading.

3.2.8 RNA Isolation and RT-PCR

Table 3.2 Primer Sequences of the Genes Selected for RT-PCR from the Regulated List of Proteins following iTRAQ Experiment

Protein name	Gene symbol	NCBI number	PCR product (bp)	T _m (°C)	Primer sequence
Complement C3	C3	NM_016994	237	62.4	Sense 5'cagcagacctcagtgaccaa 3'
				62.4	Antisense 5'gecttctctctagccgaat 3'
Glial fibrillary acidic protein	GFAP	NM_017009	188	58.4	Sense 5'agaaaaacgcateacattc 3'
				62.4	Antisense 5'gaacacctcacatcacatcc 3'
CASK interacting protein 1 (caskin1)	Caskin1	NM_080690	242	62.4	Sense 5'ccccgcgatggtagacaact 3'
				60.4	Antisense 5'acttggtctggeggttaatg 3'
Transferrin	Tf	NM_001013110	163	62.4	Sense 5'gtgtggccagtttctctc 3'
				62.4	Antisense 5'ccacatctccacctccatct 3'
Neurofilament light polypeptide	Nefl	NM_031783	205	62.4	Sense 5'gccgaagagtggttcagag 3'
				60.4	Antisense 5'tgtctgcattctctgttctcc 3'
Tropomyosin 3, gamma	Tpm3	NM_057208	151	58.4	Sense 5'atccagctgaagggaagcaaa 3'
				64.5	Antisense 5'ctctctccagctcagaacac 3'
Alpha Actinin	Actn1	BC074001	212	60.4	Sense 5'cgagtgcacaagatctccaa 3'
				62.4	Antisense 5'ctctgacacacacaggagcaa 3'

Specific primers were designed by using open-source primer 3.0 software for C3, Gfap, Caskin1, Nefl, Tpm3, and Actn1 (Table 3.2). Total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's protocol and quantified in $\mu\text{g}/\mu\text{L}$. Two μg of RNA was used for the RT reaction with the RevertAid H Minus Moloney Murine Leukemia Virus Reverse Transcriptase kit (Fermentas Life Sciences, Hanover, MD) following the manufacturer's instructions. Actn1 was used as internal control to

check the efficiency of cDNA synthesis, PCR amplification and uniform loading. PCR products were electrophoresed through 1% agarose gels.

3.2.9 Spatiotemporal Profiling by WB Analyses

In the MCAO model, subcortical regions (preoptic area and striatum) are first and more severely affected as a result of microvascular injury with higher chances of BBB damage-induced edema and HT.¹⁷² Conversely, the cortex in the transient MCAO model is affected in the later stage thus making it suitable to study the spatial trend of the secondary injury.³⁵ Hence, 6 mm coronal section of the ipsilateral hemisphere starting 7 mm caudal to the frontal lobe was divided into two parts; the cortical (part I) containing somatosensory cortex and the subcortical (part II), containing caudoputamen or striatum, to understand the spatiotemporal pattern of the selected proteins from the temporal proteomic experiment. The dissection was guided by the knowledge of the vascular architecture of the rat brain, by the TTC staining done during the model development and was based on the reports on the evolution of the infarct in this MCAO model.^{35, 173}

3.2.10 Statistical Analysis

The physiologic variables (e.g. temperature, body weight) were presented as mean $\pm$ standard deviation (SD) to report the variability of the observations. The ordinal variable (i.e. cumulative neurological score) was presented as median $\pm$ inter quartile range. The n value indicated the number of replicate readings from same or different experiments. The possible correlation between non-parametric variables was determined using Spearman rank correlation coefficient (time vs. cumulative neurological score). One-way analysis of variance (ANOVA) followed by post hoc Dunnett's multiple comparison test or non-parametric Kruskal-Wallis test followed by chi-square test was performed for comparing scale (physiological variables) or ordinal variables (neurological score) respectively involving at least three groups. Being a non-parametric method, the Kruskal-Wallis test does not assume a normal distribution, unlike the analogous one-way ANOVA. Statistical significance was accepted at $*p < 0.05$ and $**p < 0.01$, respectively.

3.3 RESULTS

3.3.1 Preproteomic Validation of the *In Vivo* Model of Focal Cerebral Ischemia

3.3.1.1 Physiological Monitoring

Figure 3.3 Changes in physiological variables of Sprague Dawley rat in MCAO model of cerebral I/R injury and sham group. Data was presented as mean $\pm$ SD ($n = 6$, surgery; $n = 3$, sham). $^*p < 0.05$, calculated using one way ANOVA followed by Dunnett's Multiple Comparison Test, where body weight at 0 h postreperfusion and pre-operative rectal temperature were used as respective controls. (A) Significant increase in weight loss at 4 h and 24 h postreperfusion due to MCA ischemia whereas the weight loss of the sham group is attributed to the ECA territory ischemia. (B) Hyperthermia was observed due to the ischemia of the hypothalamus that peaked after 2 h of MCAO and persisted till 24 h of reperfusion.

Rectal temperature and body weight were monitored at periodic intervals till the sacrifice of the animals. The starting body weight of the animals varied between 225–275 g (250.9 ± 16.6 g). The baseline rectal temperature (37.1 ± 0.3 °C) was within normal limits. Additionally, pre-operative mean body weight or rectal temperature did not differ statistically ($p > 0.05$, one-way ANOVA) among the four participating groups (Table 3.3). Post-operative weight loss occurred for sham and all surgical groups partly because of the MCAO surgery (ECA territory ischemia) that resulted in impaired mastication and swallowing function. The brain edema or final lesion size due to MCAO was not affected by this extracerebral ischemia.¹⁷⁴ There was a statistically significant increase in weight loss at 24 h postreperfusion for the stroke affected rats ($11.79 \pm 2.5\%$) in comparison with the sham rats ($5.22 \pm 1.8\%$) (Table 3.4,

Figure 3.3A) that was attributed to the severity of the I/R injury. Hyperthermia was observed in all the groups due to the occlusion of the hypothalamic branch of the ICA that peaked (38.8 ± 0.5 °C) at the end of 2 h of occlusion.¹⁶⁷ Thereafter the rectal temperature showed a decreasing trend till 24 h of reperfusion, although some of the animals remain slightly hyperthermic (38.2 ± 0.6 °C) (Table 3.4, Figure 3.3B) .

3.3.1.2 Neurological Deficits

The neurological scoring of the animals was one of the easiest ways of deciding the success of the surgery in a non-terminal manner and monitoring the functional impairment, at least for the earlier part of the reperfusion episode. It is also more accurate as compared to the assessment based on the animals' spontaneous activity. Neurologic functions were evaluated mainly on two aspects, i.e. postural reflex and sensorimotor integration with respect to visual, tactile and proprioceptive stimuli.¹⁶³ Neurological deficits were observed to be maximal during 60-120 min (6.0 ± 0.9) postreperfusion, i.e. before reperfusion for rats of group 2+4 and 2+24, and before termination for rats of group 2+0. Spontaneous and partial recovery of neurological deficits was observed till 24 h postreperfusion (Figure 3.4D).¹⁶³ This may be partly because of the use of young adult animals, a situation also encountered for younger human population with IS.³² Spearman rank correlation test revealed marked negative correlation between cumulative neurological score and duration of I/R injury (Spearman's rho $r = -0.893$; $p < 0.05$). The groups considered for proteomics and subsequent validation experiments were not significantly different (Kruskal Wallis test, $p > 0.05$) with respect to the cumulative neurological score during the occlusion period (data not shown).

Table 3.3 Summary of the Physiological Parameters of the Four Participating Groups in the iTRAQ Experiment^a

Physiologic Parameter	Sham			2+0			2+4			2+24		
	Mean	SD	SEM	n	Mean	SD	SEM	n	Mean	SD	SEM	n
Body Weight (g)	259.8	19.1	7.8	6	243.6	13.6	5.1	7	256.0	15.1	5.3	8
Rectal Temperature (°C)	37.2	0.3	0.1	6	37.0	0.32	0.1	8	37.2	0.4	0.1	8

^aPre-operative mean body weight or mean rectal temperature did not differ statistically ($p > 0.05$, one-way ANOVA) among the four participating groups.

Table 3.4 Regulation of Physiological Variables during the Course of Cerebral I/R Injury in Sprague Dawley Rats^a

Physiologic parameter	Condition	Groups			
		2 + 4 h		2 + 24 h	
% Decrease in Weight (g)	Surgery	3.7 ± 1.6		11.8 ± 2.5	
	Sham	1.5 ± 0.4		5.2 ± 1.8	
Temp (°C)		0 h	2 + 0 h	2 + 4 h	2 + 24 h
	Surgery	37.1 ± 0.4	38.8 ± 0.5	38.6 ± 0.4	38.2 ± 0.6
	Sham	37.2 ± 0.3	37.2 ± 0.6	37.5 ± 0.3	37.4 ± 0.6

^aData was presented as mean ± SD ($n \geq 6$, surgery; $n \geq 3$, sham). Body weight at 0 h post-reperfusion and pre-operative rectal temperature (0 h) were used as respective controls for the statistical test (one way ANOVA followed by Dunnett's Multiple Comparison Test). Significant increase in % loss of body weight and rectal temperature was observed following the MCAO model of cerebral I/R injury in comparison to the sham group of animals.

Figure 3.4 (A-C) Photographs demonstrating postures and movements of a representative rat during neurological score evaluation. (A) score 1: forelimb flexion & thorax twisting. (B) score 2: stimulated contralateral circling. (C) score 3: spontaneous circling. The MCA territory of left hemisphere of the brain was affected. (D) Evolution of cumulative neurological score during the course of cerebral I/R injury. Data was presented as median $\pm$ interquartile range ($n = 7$). Partial functional recovery was observed at 24 h postreperfusion spontaneously.

3.3.1.3 Mortality

Subarachnoid hemorrhage (SAH) due to excessive insertion pressure of the intraluminal filament and inconsistency in infarct development are common drawbacks of this model necessitating the use of sufficient animals and stricter selection criteria to avoid inter and intragroup variations.¹⁷⁵ Post-operative mortality was around 3.6% due to SAH, whereas another 5.5% animals were excluded during autopsy due to the presence of hemorrhagic spot at the base of the brain. Another 12.7% of animals were termed as surgical failure due to an insufficient neurological score during the course of occlusion and early reperfusion probably due to premature or intermittent reperfusion and was excluded from the study.¹⁷⁵ Thus, around 78% of the total population was progressed into the next phase for the molecular validation.

3.3.1.4 TTC Staining

The terminal TTC staining was performed for all four groups as part of a preproteomic model validation that indicated a massive infarction in the striatum and the overlying cortex after 24 h of reperfusion (Figure 3.5). There was only insignificant amount of detectable necrotic tissue in the striatum at 4 h postreperfusion. However, the infarct spreads gradually thereafter into caudo-putamen and subsequently in the dorsolateral cortex in the next 20 h as seen in the 2+24 group. The overall corrected% HIA was found to be around 35% after 24 h of I/R injury which is similar to the previously published reports.¹⁶⁷ The ipsilateral hemisphere at the level of bregma was the most affected one, where around 74% of the area became necrotic by 24 h of reperfusion. Hypothalamic ischemia was also apparent in the 2 + 24 group as a result of 2 h of MCAO, as reported previously.¹⁶⁷

Figure 3.5 TTC staining of the representative coronal sections with a corresponding schematic diagram and respective antero-posterior position to bregma (0 mm). White indicates infarction; red indicates normal tissue. Infarct area was maximum at the level of bregma (-0.8 mm posterior to bregma) after 24 h of I/R injury. No ischemic lesion was observed in the sham group.

3.3.1.5 Molecular Validation by WB Analyses

Hif1 α and Hsp70 were selected as representative pathological markers for completing the validation process of this rat MCAO model at the molecular level.¹⁷⁶ The anti-apoptotic stress protein Hsp70 is generally regarded as an early response chaperone and a marker of metabolic deprivation in cerebral ischemia that increases the expression of many downstream proteins as a part of the survival response.¹⁷⁷ In contrast, the transcription factor, Hif1 α is induced following a reduction in oxygen supply but not due to the inhibition of mitochondrial respiration and it controls the glycolysis and angiogenesis in the ischemic brain.¹⁷⁸ During the entire 26 h (2 + 24 h) of I/R experiment the increased expression of Hsp70 and the transient up-regulation of Hif1 α (with a peak at 4 h of reperfusion) compared with the sham hemisphere (Figure 3.6) indicated the specific stress response by the viable cells confirming the success of the rat I/R model.

Figure 3.6 Molecular validation of the rat MCAO model of I/R injury by WB analysis. Pooled lysate from each group were probed with monoclonal antibodies against Hsp70 and Hif1 α along with actin as loading control. Hsp70 expression was high till 24 h of I/R injury, whereas expression of Hif1 α peaked at 4 h post-reperfusion.

3.3.2 Proteomics

Group allocation for the proteomic and spatiotemporal profiling experiments were finalized after carefully screening the animals to comply with the selection criteria as mentioned previously. The groups were similar ($p > 0.05$, one way ANOVA) with respect to the measureable parameters like pre-operative body weight or rectal temperature (Table 3.3) and pre- or postischemic cumulative neurological score (data not shown).

3.3.2.1 iTRAQ Results

To understand the global proteomic changes that occurred in the ipsilateral hemisphere at different time-points, pooled protein extracts from each group ($n = 3$, biological replicate) were labeled and analyzed thrice (technical replicate = 3) as described in the experimental procedure.

3.3.2.2 Quality Control of iTRAQ Data set

To minimize the false positive identification of proteins, a strict cutoff of unused ProtScore ≥ 2 was used as the qualification criteria, which corresponds to a peptide confidence level of 99%. With this criterion, 2242 proteins were identified with a FDR of 0.33%. The average number of unique peptides (having a confidence level of $>95\%$) detected per protein was 9.23 and 37% of the proteins had ≥ 5 unique peptides which was similar to the *in vitro* data set (section 2.3.3).¹⁶² The ratios for each condition were sorted using a p -value cut-off of 0.05 to obtain the list of proteins with significant ratios.

3.3.2.3 Estimation of Cutoff for Confidently Defining Perturbed Proteins

Next, the cut-off for up- or down-regulation was determined based on the analytical variation between three technical replicates (Figure 3.7).¹⁶² The cut-off for up- or down-regulation was determined based on the analytical variation between three technical replicates as previously reported with slight modifications. The bias and background corrected datasets from the individual replicates were taken after removing the low confident proteins (unused score < 2.0). A total of 1166 proteins were found to be common among three technical replicates having quantification information for all iTRAQ labels. Subsequently, Geometric mean (GM) and ratio-specific (2+0: sham, 115:114; 2+4: sham, 116:114; and 2+24: sham, 117:114) SD was calculated using log10 space for all the proteins using triplicate values from three MS runs. The average SD in log space was 0.086, 0.080 and 0.080 for three ratios. The frequency histogram and the corresponding cumulative (%) was plotted (Figure 3.7) for all three ratios (115:114, 116:114 and 117:114) to measure the distribution of variation. About 93% and 99.2% of the commonly quantified proteins had a SD lesser

than 0.2 and 0.4 respectively among all technical replicates. Based on this, the regulation cut-off was set at 1.4 fold: ratio >1.40 or <0.71 was considered as up- or down-regulated.

Subsequently, the final list of regulated candidates was obtained separately for each time-point by applying the p -value and regulation cut-off. Thus, 24, 28 and 37 proteins from 2 + 0, 2 + 4 and 2 + 24 group (having overlap between different time-points) respectively showed significant perturbation with a magnitude beyond the specified cut-off of 1.4 fold. Combining all time points, only 61 candidates (2.72% of total hits) remained having at least one significant ratio ($p < 0.05$) with the expression level >1.40 or <0.71 (Supplemental Table 3A). This data set was advanced to the next phase for rigorous validation by complementary techniques.

Figure 3.7 Determination of ratio-specific technical variation using 1167 commonly quantified proteins (unused prot score >2.0) from three technical replicates. The primary vertical axis represents the absolute number of proteins (bars) for different conditions ('Frequency') categorized into groups of 'SD' values plotted in the horizontal axis. The corresponding 'Cumulative (%)' was plotted in the secondary vertical axis where 100% equals to 1166 proteins. The relative expression value for '2+0', '2+4' and '2+24' group with respect to sham was represented by 115:114, 116:114, and 117:114 respectively. The SD was calculated using $\log_{10}$ of ratios. The magnitude of 'SD' was less than 0.2 for 93% of the proteins on an average, irrespective of conditions. More than 99% of the log ratios had a variation less than 0.4 between replicates justifying the regulation cutoff of 1.4-fold.

3.3.3 Postproteomic WB and RT-PCR of Selected Proteins

Six proteins (i.e. Tf, Slc1a3, Caskin1, Vim, Vcl and Gfap) out of 61 candidates representing distinct events in the pathology of IS were chosen from the data set. Tf is a circulatory protein whereas Slc1a3 (excitatory amino acid transporter) is an

astrocyte-specific membrane-bound high-affinity sodium-dependent aspartate/Glu transporter. Three intermediate filament-related structural proteins (Gfap, Vim and Vcl) were chosen as they may be representative of the neurorestorative response in the ischemic brain. As seen in Figure 3.8 and Table 3.6, WB showed similar trends with the corresponding iTRAQ ratios.

In addition, RT-PCR of five genes selected from the list of perturbed proteins (C3, Caskin1, Gfap, Tpm3, and Nefl) showed similar trends to the iTRAQ results (Figure 3.9).

Figure 3.8 Postproteomic validations of the selected proteins by WB analysis using the same pooled extract from the iTRAQ experiment. Cytoskeletal proteins (Gfap, Vim, and Vcl) and Tf peaked 24 h after I/R injury. Slc1a3 expression was highest at 4 h post-reperfusion while Caskin1 showed a gradual decline till 24 h post-reperfusion. Actin1 was used as loading control. All of them showed consistent trends with the iTRAQ results. Data was presented as mean $\pm$ standard deviation for densitometric measurements. Statistical significance was accepted at $^{*}p < 0.05$ and $^{***}p < 0.01$, respectively.

Figure 3.9 Representative RT-PCR images of the selected genes at identical time-points as the iTRAQ experiment. C3 showed an increasing trend from 4 h onwards whereas Gfap exhibited a late up-regulation after 24 h of reperfusion injury. In contrast, Caskin1 was down-regulated at 24 h post-reperfusion. Nefl showed a biphasic response with increase at 2+0 and 2+24 group. Tpm3 was up-regulated in all three groups except sham. A separate set of animals in duplicate were used without pooling ($n = 2$, 4 groups).

3.3.4 Temporal Regulation of Perturbed Proteins following Focal Cerebral I/R Injury

The gene IDs of the significantly regulated proteins from the filtered and validated data set were classified by PANTHER 7.0 using 'pathway' as ontology. Most of the genes were assigned to more than one category thus making the total number of hits greater than the number of genes uploaded. They were assigned to 18 different pathways (Table 3.5). Among total pathway hits, 14.2% and 14.3% of the gene hits were associated with glucose metabolism (7.1% each for ATP synthesis and glycolysis) and Glu neurotransmission (7.1% for the ionotropic Glu receptor pathway and 3.6% each for the metabotropic Glu receptor group III pathway and Gln/Glu conversion) respectively. Blood coagulation (14.3%), inflammation (3.6%) and angiogenesis (3.6%) were featured among the other perturbed pathways that were consistent with the vascular nature of this disorder. Strikingly, a few of the regulated genes (*Uchl1*, *Apba1*, *Dync1h1*) were also assigned to various chronic neurodegenerative disorders (e.g. PD, AD and Huntington's disease). Guided by the above trends, regulated proteins were manually classified (Table 3.6) with incorporation of additional candidates based on their identification information only considering their association with the regulated proteins (see discussion).

Sustained up-regulation of energy metabolism related proteins following I/R injury signified an adaptive response of the stressed brain that includes glycogen mobilization (i.e. *Pygb*) to glycolysis (e.g. *Pgk1*, *Pgam1*) and the oxidative phosphorylation (e.g. *Atp5b*, *Atp5a1*) (Figure 3.10A, B). Early up-regulation was seen for two glial Glu transporters (*Slc1a3* and *Slc1a2*) having a neuroprotective role in the Glu-mediated excitotoxicity.¹⁷⁸ Persistent increase in *Glul*, an enzyme with almost exclusive astrocytic localization, and significant up-regulation of *Glud1* or *GDH* were observed after 2 h of ischemia. Considering I/R injury as a combination of the hypoxic and metabolic stress, active involvement of sirtuins could be possible as an up-stream modulator of the deregulated *Hif1a* and *Glud1* in this current model.^{179, 180} Accordingly, two isoforms of sirtuins (*Sirt2* and *Sirt5*) were identified in the iTRAQdata set whose expression is reported to be higher in the adult brain relative to the fetal brain unlike other isoforms of sirtuins, thus supporting the above assumption.¹⁸¹

Figure 3.10 Histograms showing the relative temporal expression patterns of regulated proteins in the ischemic hemisphere during the course of cerebral I/R injury. Natural logarithm of iTRAQ ratios of the proteins with respect to sham group were plotted on the vertical axis. The gene symbols were plotted on the horizontal axis. (A) Proteins related to glycolysis and glycogen mobilization showed a persistent up-regulation. (B) Proteins taking part in the Glu excitotoxicity were mostly up-regulated especially after 4 h of reperfusion except the neuronal subtype of Glu transporter, Slc1a1 that showed a downward trend. (C) Increasing trend of all circulatory proteins (except Hbb) signified a gradual leakage of the BBB causing progressive vasogenic edema. (D) The synapse related proteins (Mtap6, Epb41l1, Caskin1, Shank3, Cnmd2, and Apha1) exhibited a generalized downward trend implying the compromised synaptic function. Notably all of them were novel in the pathology of cerebral ischemia.

Table 3.5 Pathway Analysis of the Regulated and Validated Proteins from the iTRAQ dataset by PANTHER 7.0

S/N	Name of Pathway	# genes	Percent of gene hit against total # genes	Percent of gene hit against total # Pathway hits
1	ATP synthesis	2	3.6	7.1
2	Metabotropic glutamate receptor group III pathway	1	1.8	3.6
3	Apoptosis signaling pathway	2	3.6	7.1
4	Ionotropic glutamate receptor pathway	2	3.6	7.1
5	Angiogenesis	1	1.8	3.6
6		1	1.8	3.6
7	Cell contraction mediated by chemo-kines and cytoskeleton signaling pathway	1	1.8	3.6
8		3	5.4	10.7
9	Cytoskeletal regulation by Rho GTPase	1	1.8	3.6
10	Nicotinic acetylcholine receptor signaling pathway	1	1.8	3.6
11	Cadherin signaling pathway	1	1.8	3.6
12	Muscarinic acetylcholine receptor 2 and 4 signaling pathway	1	1.8	3.6
13	Blood coagulation	4	7.1	14.3
14	Glutamine glutamate conversion	1	1.8	3.6
15		1	1.8	3.6
16	Heterotrimeric G-protein signaling pathway-Gq alpha and Go alpha mediated pathway	1	1.8	3.6
17	Heterotrimeric G-protein signaling pathway-Gi alpha and Gs alpha mediated pathway	2	3.6	7.1
18	Glycolysis	2	3.6	7.1

Table 3.6 Quantitative Information of the Significantly Regulated Proteins Obtained from the Bias and Background Corrected iTRAQ Data Set Showing a Temporal Pattern in the Transient MCAO model of Cerebral I/R Injury ^a

N	Unused	q Con (95)	Gene Symbol	Protein Name	Unique Peptides (95%)	Total Peptides (95%)	115:114 (2 + 0:sham)	116:114 (2 + 4:sham)	117:114 (2 + 24:sham)
Energy Metabolism									
29	65.7	41.6	Pygb	brain glycogen phosphorylase	47	154	3.08	1.60	2.73
38	60.5	53.5	Atp5b	ATP synthase subunit beta, mitochondrial precursor	83	414	3.63	3.37	3.53
48	58.5	31.4	Hk1	Hexokinase 1 ^d	50	220	1.36	0.85	1.43
56	54.5	44.7	Atp5a1	ATP synthase subunit alpha, mitochondrial precursor	67	463	2.42	1.84	1.84
65	49.4	43.4	LOC688509	similar to Alpha-enolase	50	159	1.39	1.47	1.72
91	42.5	36.0	Ndufs1	NADH-ubiquinone oxidoreductase 75 kDa subunit, mitochondrial precursor ^d	31	87	1.46	1.21	1.71
119	38.0	49.2	Pgk1	Phosphoglycerate kinase 1	34	176	2.56	2.36	2.56
179	29.0	26.2	Pygm	Glycogen phosphorylase, muscle form ^d	23	50	1.33	0.97	1.11
216	25.6	60.2	Tpi1	Triosephosphate isomerase	20	50	1.58	1.37	1.38
333	18.8	28.5	Ndufs2	NADH dehydrogenase (Ubiquinone) Fe-S protein 2 ^d	10	22	1.27	1.33	1.15
357	17.7	37.0	Pgam1	Phosphoglycerate mutase 1	11	38	2.56	1.53	1.84
Glutamate Excitotoxicity									
8	169.6	47.1	Cltc	Clathrin heavy chain	177	804	1.02	1.94	1.41
115	38.8	41.8	Glud1	Glutamate dehydrogenase 1, mitochondrial precursor	27	67	4.25	2.42	2.56
120	37.9	21.4	Slc1a2	Isoform Glt-1A of Excitatory amino acid transporter 2 ^d	39	289	1.10	1.82	1.28
152	31.8	36.5	Glu1	Glutamine synthetase ^e	30	87	1.80	1.84	1.85

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243	24.0	21.9	Slc1a3	Isoform GLAST-1 of Excitatory amino acid transporter 1 (EAAT1) ^d	31	168	1.36	1.64	1.18
1387	4.0	6.5	Slc16a1	Monocarboxylate transporter 1 (MCT1) ^d	3	7	1.39	1.58	1.37
2070	2.0	4.8	Slc1a1	Excitatory amino acid transporter 3 ^d	2	3	0.86	0.79	0.91
Neuro-inflammation and Iron Metabolism									
42	60.1	54.9	Alb	Serum albumin precursor	45	176	2.21	18.20	24.89
131	36.8	29.7	Tf	Isoform 1 of Serotransferrin precursor ⁷²	23	125	2.09	6.25	9.55
458	14.2	58.5	Hbb	Hemoglobin subunit beta-1	9	28	3.47	2.96	1.69
491	13.5	5.1	C3	Complement C3 precursor (Fragment)	6	16	1.08	7.59	7.18
619	10.5	6.5	Cp	121 kDa protein	5	11	0.85	2.54	4.13
734	8.7	4.3	Pzp	Pregnancy-zone protein, 167 kDa protein	4	7	0.73	7.31	6.49
978	6.1	6.7	Hpx	Hemopexin precursor	3	4	1.63	3.47	5.20
1217	4.5	5.3	Fetub	Fetub protein	2	2	3.60	7.59	14.59
1242	4.3	5.7	Mug1	Isoform 1 of Murinoglobulin-1 precursor	7	14	1.04	6.55	6.67
2239	2.0	16.3	S100b	Protein S100-B ^d	3	37	1.45	2.05	2.10
Cerebral Plasticity									
Synapse Related Proteins									
81	44.5	39.0	Mtap6	STOP protein	25	72	0.69	0.45	0.51
84	43.6	18.4	Epb4.111	Isoform 1 of Band 4.1-like protein 1	36	94	0.77	0.74	0.55
133	36.0	19.4	Caskin1	Caskin1 ⁷⁶	22	44	0.61	0.59	0.48
155	31.4	13.1	Shank3	Isoform 1 of SH3 and multiple ankyrin repeat domains protein 3	15	36	0.81	0.94	0.57
269	22.4	11.4	Ctnd2	similar to Catenin delta-2	11	31	0.82	0.69	0.66
974	6.1	5.7	Apha1	Amyloid beta A4 precursor protein-binding family A member 1	4	7	0.70	0.73	0.59
Structural Proteins									
1	396.7	63.2	Spna2	Spectrin alpha chain, brain	405	1957	0.83	1.15	1.43
2	287.7	36.7	Dync1h1	Dynein heavy chain, cytosolic	207	706	0.64	0.90	1.01
4	198.5	28.6	Plec1	Plectin 3	105	273	0.56	0.96	1.19
6	175.6	45.3	Spnb3	Spectrin beta	142	524	0.65	0.88	0.94

				chain, brain 2					
7	173.8	42.0	Mtap1a	Microtubule-associated protein 1A	122	396	0.94	0.63	0.94
11	153.1	42.6	Myh10	Myosin-10	105	321	0.57	0.77	1.39
111	39.3	46.1	Nefl	Neurofilament light polypeptide ^a	39	127	1.94	1.04	1.87
141	33.4	47.7	Gfap	Isoform 1 of Glial fibrillary acidic protein ^{b,c}	27	92	1.15	0.88	1.66
185	28.3	16.9	Vcl	vinculin ^b	15	43	0.84	1.25	1.56
186	27.8	37.5	Vim	Vimentin ^b	19	60	1.10	0.79	1.66
238	24.3	42.0	Gap43	Neuromodulin	22	48	5.97	3.77	5.01
403	15.8	25.4	Tpm3	Isoform 1 of Tropomyosin alpha-3 chain	8	18	2.99	2.99	3.22
Ubiquitin Proteasome System									
44	59.1	34.1	Ube1x	Ubiquitin-activating enzyme E1, Chr X	56	228	1.39	1.02	1.74
599	10.9	31.4	Uchl1	Ubiquitin carboxyl-terminal hydrolase isozyme L1	9	15	2.81	1.85	1.77
Nucleocytoplasmic Transport									
169	30.01	22.03	Kpnb1	Importin subunit beta-1	16	44	1.33	1.18	1.50
197	27.07	17.4	Ipo7	predicted importin 7 ^{a,d}	13	54	1.13	0.95	1.34
Housekeeping Gene (Control for WB, RT-PCR)									
40	60.09	39.9	Actn1	Brain-specific alpha actinin 1 isoform ^{b,c}	37	136	1.02	0.98	1.05

^aMost of these proteins have qualified through the pre-set selection criteria (i.e. unused prot score >2.0, *p*-value <0.05 for at least one ratio, magnitude of expression changes of at least 1.4 fold compared to the sham group), as mentioned in the "Results" section. They were classified according to their participation in the key molecular events of stroke pathophysiology. The ratios with significant *p*-value (<0.05) are shown in **bold**. ^bProteins validated by WB. ^cProteins whose of transcripts were measured by RT-PCR. ^dProteins incorporated in the list due to their close association with the regulated proteins although they did not meet the above mentioned pre-set selection criteria (i.e. unused prot score >2.0 but *p*-value >0.05). For example, Hk1, Ndufs1 and Ndufs2 (energy metabolism) are important enzymes of glucose catabolism. Pygm was the unperturbed muscle isoform of glycogen phosphorylase, whose brain isoform was significantly up-regulated after 2 h MCAO. Similarly, Slc1a2, Glul, Slc16a1, Slc1a1 are related to Glu neurotransmission and metabolism.

The significant presence of several acute phase proteins in the brain parenchyma after 2 h of ischemia (Figure 3.10C) indicated a hyper-acute opening of the BBB as reported recently.¹⁸² All candidates of this group except Hbb (beta-1 subunit of hemoglobin) showed an ascending trend of absolute expression signifying a progressive neuro-inflammation and vasogenic edema as a result of the I/R injury. The precipitous elevation in the overall expression of albumin can be explained by the complementary

de novo expression in the ischemic hemisphere when albumin mRNA and protein expression was elevated after 2 h of MCAO and 22 h post-reperfusion.¹⁸³ To evaluate the *de novo* component on the overall expression of the plasma-derived candidates, C3 was chosen as a representative and its expression level was checked at the transcript level. Gradual increase in the mRNA level of C3 after 24 h (Figure 3.9) indicated the participation of brain cells in complement generation that is most likely supplemented by the massive infiltration of circulatory leukocytes and direct leakage of C3 from the circulation.

A significant decrease in the levels of essential structural and cytoskeletal proteins (e.g. Myh10, Plec1, Dync1h1, and Spnb3) was observed 2 h after MCAO that was probably related to an irreversible activation of intracellular proteases (Table 3.6). All of them recovered while some others showed significantly increased levels (e.g. Gap43, Gfap, Vim, Vcl, Nefl, and Tpm3) at the end of 24 h of reperfusion indicating the presence of cerebral plasticity in the ischemic brain. This may be a part of the endogenous recovery response happening in parallel with the worsening of the secondary injury as exhibited by the generalized downward trend of the synapse related proteins (e.g. Mtap6, Caskin1, Ctnd2), the other subclass under the category of “cerebral plasticity” (Figure 3.10D). These contradictory trends of the growth-promoting (e.g. Gap43, Gfap or Vim) and synaptic proteins (e.g. Mtap6, Caskin1, Shank3) can be attributed to the heterogeneity of the brain substructures or to the differential cellular or sub-cellular origin of the candidate proteins. Detailed immunohistochemical studies are needed to elucidate the underlying molecular complexities.

The regulated list also consists of several proteins (e.g. Caskin1, Shank3, Apba1, Ctnd2, Ube1x, and Kpnb1) novel in context of the pathophysiology of cerebral ischemia highlighting the utility of this discovery approach. Hence, the individual proteins can further be explored through functional studies in similar animal or simpler cell line models.

3.3.5 Spatiotemporal Profiling of the Validated Candidates

Two of the validated candidates (Tf and Gfap) from the iTRAQ data set, representing two distinct events of stroke pathophysiology (Table 3.6) were chosen to define this model by spatiotemporal profiling. As seen from Figure 3.11, the presence of Tf

peaked at 4 h in the striatum (I) followed by a plateau or decrease at 24 h, whereas in the overlying cortex (II), a gradual but detectable increase with time was observed as inflammation and vasogenic edema spread into the ischemic area. The well studied astrocytic marker, Gfap showed a late up-regulation in part I and recovered from an early decrease in the part II at 24 h post I/R injury, indicating the presence of reactive gliosis in the MCA territory.

Figure 3.11 Spatiotemporal profiling of the validated candidates (Tf and Gfap) by WB analysis in the striatum (part II) and in the overlying cortex (part I) of the ipsilateral hemisphere. ActinI was used as a loading control. Each time-point has three biological replicates except sham. Sham was used to normalize intensities of the other time points during densitometric analysis of the respective blots. Data was presented as mean $\pm$ standard deviation. Statistical significance was accepted at $*p < 0.05$ and $**p < 0.01$, respectively. Early opening of the BBB was evident by significant leakage of the circulatory Tf at 4 h post-reperfusion in the striatum (Part II). Gfap showed a reverse trend in the striatum at the same time signifying the astrocytic damage. Elevation of the Gfap level in both parts after 24 h of I/R injury was indicative of the reactive gliosis, constituting a recovery response.

3.4 DISCUSSION

I report an overview of the perturbation of ischemic proteome in the first 24 h of cerebral I/R injury showing the deregulation of fundamental mechanisms (failure of energy metabolism, Glu excitotoxicity, and inflammation) that orchestrate the cell

death following IS. Most of the potential therapeutic interventions tried to target one or more of these deleterious events without a single clinical success. Recent evidence indicated that each of these mechanisms may have some neurorestorative role at some point of time during the evolution of cerebral I/R injury.^{60, 152} Thus temporal considerations are important as terminating the therapy in time may be equally important as its well-timed initiation to extract maximum clinical advantage. Further, understanding the underlying molecular mechanisms and evaluating the efficacy of the therapy will remain a serious challenge in absence of any standard diagnostic protocol to demarcate and measure the ischemic penumbra, core and the normal tissue as they usually remain scattered and highly heterogeneous.^{59, 184} Animal models of stroke are more uniform and can be controlled precisely to represent specific types of human stroke. MCA, being one of the most common sites of human large artery stroke, makes transient MCAO, a clinically relevant and the most widely accepted rodent model for understanding the temporal complexities of the cerebral I/R injury.¹⁵³

3.4.1 Unraveling the Temporal Evolution of Ischemic Pathophysiology in the transient MCAO model by Analysis of the iTRAQ Data set

The final list of regulated proteins (Figure 3.6) was functionally analyzed to interpret the molecular events relevant to the pathophysiology of cerebral ischemia in a chronological way.

3.4.1.1 Alteration of Energy Metabolism and Glu Excitotoxicity – Neuron-Astrocyte Cross-talk

Focal ischemia is associated with an acute release of a massive amount of Glu by the glutaminergic synapses that represent >85% of the cortical synapses.¹⁸⁵ In neurons, GDH plays an essential role in generating releasable Glu stored in the synaptic vesicles. Increased *in vivo* release of Glu has been observed after neuronal depolarization in transgenic mice over-expressing Glud1.¹⁸⁶ Thus, significant up-regulation of Glud1 following 2 h of MCAO indicated the excitotoxic release of Glu in the ischemic brain by the depolarized neurons. The excess Glu in the synaptic cleft is quickly taken up by the specific high-affinity excitatory amino acid transporters

(mainly Slc1a3 and Slc1a2) under physiological conditions to prevent overstimulation of the neuronal Glu receptors (Figure 3.12). Their predominant astrocytic localization justifies the significant up-regulation (Figure 3.6, Figure 3.10B) following 4 h of reperfusion as seen with cultured astrocytes upon Glu exposure.¹⁸⁷ Additionally, the up-regulation of ubiquitous Glud1 in astrocytes would complement this Glu uptake by using the neurotransmitter as an energy substrate to replenish α -keto glutarate along with NH_4^+ to sustain the TCA cycle. Immunoreactivity of Glud1 in the astrocytic processes surrounding the glutaminergic nerve endings had been observed providing indirect evidence to the above event.¹⁸⁸ Depletion of the negative regulator, GTP or increase in the positive regulator, ADP under ischemic conditions will promote the ammoniogenesis through the forward reaction by Glud1, having a pH-stabilizing effect on the lactic acidosis, the well known early event in the pathophysiological cascade of stroke. Increased catabolism of Glu by stabilization of Glud1 mRNA in the rat kidney following metabolic acidosis¹⁸⁹ or its up-regulation both at the mRNA and protein levels in the rat brain neuroblastoma cell line under conditions of simulated hypoxia or ischemia was suggestive of the above phenomenon.

It is conceivable that acute shortage of blood-borne glucose and oxygen following 2 h of ischemia will shift the brain energy metabolism from an aerobic to an anaerobic mode with active participation of astrocytes due to their ability to use stored glycogen. The brain-specific isoform of glycogen phosphorylase, Pygb with its predominant astrocytic localization will supplement glucose-6-phosphate from glycogen and can further save one mole of ATP thus making it an attractive substrate under ischemic conditions.¹⁸⁹ Predictably, a glycogenolysis-mediated rapid decrease in brain glycogen level has been observed in a model of *in vitro* ischemia using a guinea pig hippocampal slice culture.¹⁹⁰ Hence, a concomitant up-regulation of Pygb and several glycolytic enzymes (Tpi1, Pgam1, Pfkfb1) after 2 h of MCAO (Figure 3.10A) indicated for the first time an increased mobilization of astrocytic glycogen in an ischemic brain. The relative decrease of all regulated glycolytic enzymes along with Pygb after 4 h of reperfusion compared to 2 h of ischemia (Figure 3.6) was attributed to the blood supply induced restoration of glucose-driven aerobic metabolism in most parts of the ischemic territory causing a compensatory decrease on glycogen dependency. Therefore, detailed understanding of these astrocyte-mediated survival responses could

provide important insights about the evolution of cerebral I/R injury and metabolically targeted stroke therapeutics.

3.4.1.2 Neuro-inflammation and Failure of Iron Homeostasis

This study provided evidence that breakdown of the BBB starts in parallel with the early events like failure of the energy metabolism or Glu excitotoxicity and precedes serious tissue damage.¹⁸² This hyper-acute opening of the BBB may cause a leakage of the brain-specific proteins to the circulation that could be potential serum-specific prognostic biomarkers for predicting the therapeutic outcome or progression of the disease. Reportage of many brain specific proteins from this data set as potential serum biomarkers for stroke (Gfap, Uchl1, S100b)^{93, 191} is consistent with the above hypothesis. Conversely, concentration of the extravasated acute phase proteins in the circulation may be reduced as seen for Tf in stroke patients,¹⁵⁰ or synthesis by the target organ (e.g. liver) may be increased as a part of a compensatory response to provide surrogate markers for stroke. Nonetheless, some of the novel candidates (Pzp or Mug1) from this group (Figure 3.6) may be useful for prospective validation studies to improve the sensitivity or specificity of the commonly used predictors of long term mortality after acute IS. Thus, tissue-based proteomic profiling may be a complementary strategy for the discovery of stroke-biomarkers due to the presence of a permeable BBB.

Peaking of Hbb after 2 h of ischemic injury (Figure 3.6) was contributed by the serum-free hemoglobin originated from the intravascular hemolysis as erythrocytes (diameter: 5 μ m) cannot extravasate into brain parenchyma at an early stage.¹⁹² In turn, it reconfirms the leakage of BBB immediately after ischemic injury. Recently free hemoglobin in serum has been found to be associated with atherosclerosis and stroke and proposed as a potential biomarker for IS.¹⁹³ The reperfusion probably restored the normalcy partly by decreasing the intravascular hemolysis although the leakage of serum-free hemoglobin increased due to progressive opening of BBB. Hence, Hbb was continued to be detected till 24 h post-reperfusion with a decreasing magnitude.

Unilateral increase of multiple acute phase proteins (e.g. Tf, Cp, Hpx) (Figure 3.10C) in the affected hemisphere that are involved in the mobilization of iron to and from the brain cells (transportation and transit from the circulation as well as the intracellular

astrocyte, blue; and vasculature, red border) of the ischemic brain. The gene symbols with red color were identified in the current experiment, whereas the one with bold red had at least one of the quantification ratio with a significant *p*-value. Multiple cell-specific roles of Glu in the form of a neurotransmitter, metabolic intermediate and as a signaling molecule are shown that acted as a connecting link between the Glu excitotoxicity and the altered energy metabolism. Up-regulation of ubiquitous Glul would help in this neurometabolic coupling. Both Glul and Pygb have predominantly an astrocytic localization. Pygb would complement the anaerobic glycolysis under ischemic conditions by utilizing the stored glycogen in the astrocytes. Astrocytic Glu uptake through up-regulated transporters (e.g. Slc1a3, Slc1a2) is coupled with an increased Na⁺-driven glucose uptake stimulating the glycolysis in astrocytes during early reperfusion. In the vessel lumen, vascular dysfunction and reactive oxygen species (ROS) induced hemolysis would produce serum free hemoglobin (Hbb), heme and iron. Hemopexin (Hpx) and ceruloplasmin (Cp) - Tf system would act to keep free heme or iron within normal limits. Vascular dysfunction and free radical-mediated leakage of BBB would cause the accumulation of several circulatory proteins (e.g. Tf, Cp, Hpx, Hbb, and Alb) inside the brain parenchyma with an increasing presence till 24 h of I/R injury. ETC is electron transport chain; Hk1 is hexokinase-1; Glc-6-P is glucose-6-phosphate; α -KG is α -keto glutarate; TCA is tricarboxylic acid.

3.4.1.3 Presence of Stroke-Induced Synaptic Plasticity

Increased expression of cell-specific markers (e.g. neuronal Gap43, glial Gfap) indicated activation of specific components of the neurovascular unit (neuron and astrocyte respectively) that constitute the survival response of the I/R injured brain by remodeling and re-establishing new connections. Although Gap43 is predominantly a pre-synaptic neuron-specific phosphoprotein, a small population of Gfap-positive astrocytes in the cerebrum was found to express this protein following I/R injury in a similar transient MCAO model.¹⁹⁶ Thus, early and sustained up-regulation of Gap43 till 24 h signified a hyper-acute initiation of axonal sprouting and a persistent regeneration of nerves with specific involvement of astrocytes.¹⁹⁷ Up-regulation of Gfap and Vim at 24 h post-reperfusion (Figure 3.6) was hallmarks of reactive gliosis in the postischemic brain. These proteins participate in the formation of the intermediate filament network in astrocytes that is a part of the cytoskeleton. The stress induced activation of astrocytes may contribute in the repair or regeneration of the BBB, the neurovascular unit, and the neuron.¹⁹⁸ Thus my study corroborate with others who showed the presence of viable neurons following 24 h or at later time points (48 h or 52 h) using imaging techniques.⁵⁹ All these proteins may be accounted for the spontaneous recovery in the neurological impairment, observed at 24 h post-reperfusion.

3.4.1.4 Identification of Novel Candidates in Stroke Pathology

A host of proteins never reported in the context of stroke was also regulated during the course of I/R injury (Figure 3.10D). The down-regulated proteins like, Caskin1 or

Epb4.111 had direct interaction with a synaptic scaffolding protein, CASK of the membrane-associated guanylate kinase family. Similarly, both Caskin1 and Shank3 were located in the post synaptic density having multidomain scaffolds. Intriguingly, their domain composition was found to be similar amid differences in precise number, type, arrangement and sequence of certain domains. This probably reflects a similar scaffolding function and explains the concomitant down-regulation in the current model.¹⁹⁹ Thus, parallel down-regulation of all these proteins (e.g. Epb4.111, Caskin1 and Shank3) at 24 h post I/R injury is closely associated and may indicate a general decline in the normal synaptic functions. Recently, shank proteins, that organize the Glu receptors at excitatory synapses, were altered in AD-affected brains due to abnormal activity of the ubiquitin-proteasome system with Shank3 showing a significant down-regulation.²⁰⁰ Similar mechanisms can also be implicated here as two proteins related to ubiquitin-proteasome system were up-regulated (Uchl1 and Ube1x) indicating its abnormal regulation. Uchl1, a deubiquitinating enzyme present throughout the brain, has also been proposed as a potential biomarker for I/R injury using a 2 h MCAO model.¹⁹¹

It is fascinating that many of the regulated synaptic proteins (Mtap6, Apba1, Shank3, and Ctnd2) have been implicated in chronic neurodegenerative or psychiatric disorders.^{201, 200, 202, 203} Mtap6 is a class of microtubule associated calmodulin-binding and calmodulin-regulated protein. Recent studies with Mtap6^{-/-} null mice have indicated that suppression of Mtap6 can cause synaptic defects associated with severe behavioral disorders like schizophrenia.²⁰³ Thus, its significant down-regulation at 4 h and 24 h post-reperfusion could be related to the compromised state of synaptic plasticity. Hence, further studies are necessary to determine the participation of these proteins in the pathology of stroke-induced motor, cognitive or neuropsychiatric disorders like post-stroke or vascular dementia²⁰⁴ and stroke-induced depression, anxiety or psychosis, conditions where IS acts as a predisposing factor.²⁰⁵

3.4.2 Transferrin, a Marker of Neuro-inflammation

The involvement of Tf in the neuro-inflammation and deregulated iron homeostasis¹⁵⁰ had prompted me to check its spatiotemporal distribution in the affected area of the ipsilateral hemisphere to find the correlation between the Tf level and BBB failure.

Myresult displayed that the BBB opening is not an all-or-none phenomenon; rather it is a gradual and progressive event. The overlap in the pattern of infarct expansion and Tf presence can potentially make it a marker for BBB breakdown in similar experimental settings to understand the neuro-inflammatory processes.

The other selected candidate, Gfap has a brain-specific origin unlike Tf and has been suggested as a sensitive serum biomarker of brain damage in patients with smaller lacunar lesions or minor strokes²⁰⁶ or traumatic brain injury.²⁰⁷ This study provides a unique opportunity to compare the regional and temporal heterogeneity of two potential biomarkers in the ischemic hemisphere having an extra-cranial and brain-specific origin. The reverse expression trends of Tf and Gfap or a pathological increase in the ratio of Tf/Gfap in the Striatum during early reperfusion before the appearance of detectable tissue damage could act as a pathological marker for irreversible brain lesion in the post-stroke scenario (Figure 3.11). Hence, a similar approach can also be adopted for other regulated proteins to generate a pathological fingerprint of specific substructures of the brain to propose event-specific spatiotemporal markers.

3.5 CONCLUDING REMARKS

By applying iTRAQ coupled with 2D-LC-MS/MS, I capture for the first time the global-temporal protein expression profile of the affected hemisphere in a validated *in vivo* model of transient focal cerebral ischemia. This regulated list will offer a reference data set for the future studies with analogous rodent or primate models, especially in the absence of a comparable data set from the human ischemic brain samples. It revealed brain's response to ischemia and reperfusion injury simultaneously to better understand the temporal dynamics of the key facets of stroke pathophysiology unfolding concurrently in an interconnected manner. The detection of several perturbed proteins (Caskin1, Shank3, Kpnb1, Uchl1, Mtap6, Eph4.111, Apha1, Ube1x) novel in the context of stroke pathology, demonstrated the advantage of this discovery approach. The regulated proteins can be exploited for their validity as serum-specific prognostic biomarkers or pharmacodynamic markers for checking the consequence of drug-target interactions or for reporting the efficacy of the prospective therapeutic candidates on the progression of I/R injury.¹⁵ The detection of systems-

level emergent properties, like the identification of a metabolic cross-talk between neurons and astrocytes and the breakdown of the BBB in the ischemic brain, is only amenable by an *in vivo* profiling approach. The active involvement of astrocytes as a savior of neurons during the acute phase of I/R injury substantiates the changing priorities of stroke research from neuron-specific to cerebro-specific approaches that includes the whole neurovascular unit.³⁶ BBB damage, despite being reported as a hyper-acute event, evolved gradually along with the iron-mediated neurotoxicity, providing a wider therapeutic window. In addition, late recovery or up-regulation of expression of several structural (Myh10, Plec1) and regenerative proteins (Gfap, Vim, Gap43) indicates the presence of putative pleiotropic mechanisms with ample opportunities to promote brain repair by favorably modulating these subacute or chronic events. The phasic response of multiple players during the first 24 h of cerebral I/R injury argues against the conventional approach of sustained and unilateral modulation of certain targets. In turn, the phasic response supports a combination therapy involving multiple targets and addresses the need for careful designing of the therapeutic strategy during future clinical trials depending on the temporal profile of the respective targets. In conclusion, I provide a new strategy in the area of stroke-neuroproteomics for unraveling the hitherto novel molecular mechanisms of cerebral I/R injury.

4. A Quantitative Neuroproteomic Study of Autopsied Human Ischemic Brain Specimens

4.1 INTRODUCTION

I have successfully applied the iTRAQ-based quantitative neuroproteomics on an *in vitro* model of ischemic penumbra¹⁶² and an *in vivo* rodent model of transient focal stroke²⁰⁸ in the previous two chapters. The biological insight provided by these data indicated the diversified capabilities of this strategy that is beyond the routine use of proteomics as a mere tool for biomarker discovery.²⁰⁹ This unbiased discovery approach not only provided a better understanding of the complex pathophysiology of cerebral ischemia, but also detected several potential therapeutic targets for further exploitation.²⁰⁸ Noteworthy, due to the catastrophic effect of ischemia on brain cells in a short time, the therapeutic targets of IS emphasizes on restricting the deleterious secondary events, or promoting the endogenous protective pathways irrespective of cell types that will be beneficial to alleviate the morbidity or IS induced chronic complications (e.g. vascular dementia or AD).

In spite of encouraging results presented with the pre-clinical models of IS, they have limited clinical value due to the evolutionary divergence and the enormous genetic diversity found in human population.⁹¹ Rat has lissencephalic brain, whereas human brain is gyrencephalic having higher proportion of white matter relative to the rat brain. The behavioral and functional component of the human brain, most of which are high-end systems-level emergent properties and far advanced than rodents makes the comparison even more complicated. More importantly, human stroke is exceedingly multifaceted due to the existence of physiological (e.g. age) or habitual factors (e.g. smoking) apart from the presence of accompanying diseases like hypertension, atrial fibrillation, diabetes mellitus, and the use of concomitant medications.³² Additionally, due to the emphasis on the temporal evolution, the whole hemisphere was used in the *in vivo* study²⁰⁸ that resulted in the normalization of signals from individual brain regions and was unable to determine region-specific molecular signatures. In spite of that, spatiotemporal profiling of a couple of selected proteins (i.e. TF and GFAP) showed unique and dynamic pattern of expression during first 24 h of I/R injury.²⁰⁸ Demonstration of spatial pattern of differential vulnerability in IS patients using cerebral blood flow and infarction volume has made it imperative to study the spatial differences of molecular response at the proteome level.²¹⁰ Thus, to overcome the

translational challenges and to recapitulate a disease as complex as IS, a comprehensive profiling of region-specific human ischemic samples is necessary.

Use of autopsied human brain specimen is confounded by several pre-analytical or ex-vivo stresses that can potentially affect the result.²¹¹ This is expected due to the presence of various logistic constraints like finishing the formalities in time for a terminal collection, keeping the subject in proper place before post-mortem, shortening the duration of post-mortem and storing of the samples under proper condition. Additionally, for a pathological condition like IS, the samples are more precious as only specific locations have to be targeted on an individual basis that needs considerable neurosurgical and diagnostic expertise. Unsurprisingly, although several published methodological studies target different sub-structures of human brain (e.g. frontal cortex,²¹² thalamus,²¹³ dorsolateral prefrontal cortex,²¹⁴ substantia nigra²¹⁵ or neocortex²¹⁶) for profiling the whole proteome or to identify sub-proteome (e.g. phosphoproteome of cortex²¹⁷), very few tried to address pathological questions especially in the context of IS. One study analyzed the cerebral microdialysate from the contralateral, non-infarcted hemisphere of three stroke patients using 2D-GE-MALDI-MS/MS to identify ten exclusively expressed proteins in comparison to CSF. However, no physiological control was present due to obvious ethical reasons and no comparative or quantitative information was provided.²¹⁸ To my knowledge, only a couple of studies tried to follow a quantitative strategy using human ischemic brain tissue as a starting material. One of them focused on MMP family of proteins using a combination of Searchlight Protein Array and Laser Microdissection thus was not considered as an unbiased profiling.²¹⁹ The other one used off-line 2D-GE-MALDI-MS/MS instead of on-line 2D-LC-MS/MS for profiling different areas of the ischemic brain and was able to identify only 39 unique proteins that include albumin and actin as the two most regulated candidates.²²⁰ Clearly, none of the above studies utilized the advantage of bottom-up or shotgun proteomics combined with MudPIT for unambiguous identification and isobaric labeling of peptides (i.e. by iTRAQ or TMT) for reliable quantification. However, this approach (i.e. iTRAQ or TMT with LC-MS/MS) has recently been used for profiling body fluids like CSF²²¹ or brain extracellular fluid²²² for identification and relative quantification of potential biomarkers of stroke.

Given the same strategy (iTRAQ-2D-LC-MS/MS) has already been validated in the previous studies with 4-plex iTRAQ experiments involving pre-clinical samples, in the current study, its application was extended to an 8-plex iTRAQ experiment for quantitative profiling of the chronic ischemic human brain specimens. Ischemic and age- location-matched control areas of the brains of seven female Japanese patients were used to generate the proteomic data set. Prior immunohistochemical evaluation (Braak staging) was undertaken to determine the presence of AD by grading the topographical distribution pattern of the neurofibrillary lesions and hyperphosphorylated tau protein. Mining the data set revealed the deregulation of several proteins novel in context of IS that will provide a better understanding about the pathology of chronic IS.

4.2 MATERIALS AND METHODS

4.2.1 Reagents

Unless indicated, all reagents were purchased from Sigma-Aldrich (WI).

4.2.2 Sample collection

4.2.2.1 Autopsy and sampling of brain tissues

Post-mortem brain samples of 9 patients (5 patients, 4 controls) were obtained from the brain bank of the Choji Medical Institute of Fukushima hospital (Toyohashi, Aichi, Japan). The protocols utilized were independently approved by the local ethics committees of the BF Research Institute and of the Fukushima hospital. The scientific use of human material was conducted in accordance with the Declaration of Helsinki, and informed consent was obtained from the guardians of the patients. Brains were dissected by an experienced neuropathologist (post-mortem interval (PMI), median: 3.5 h (min. 2 h – max. 36 h)); weighed at the time of autopsy and snap frozen with liquid nitrogen before storing at –80 °C. Two patients were excluded as clinical and pathological diagnosis showed the presence of SAH and old traumatic cortical contusion instead of an infarction. One (Identification number (ID-No) 1006) out of the remaining three ischemic patients had multiple infarctions. Two pieces of infarcted tissue one each from thalamus and parietal lobe were collected for the experiment.

Thus, total eight specimens of brain tissue (n=4 per group) were used for the 8-plex iTRAQ experiment.

4.2.2.2 Neuropathological Diagnosis

All patients and controls underwent neuropathologic characterization to document associated neurodegenerative disorders. Consortium to Establish a Registry for Alzheimer's disease (CERAD) criteria and the Braak staging were used to diagnose AD.^{223, 224} Pathological diagnosis of Alzheimer-type Dementia was carried out in accordance with the CERAD guidelines.²¹ The neurofibrillary pathology was confirmed by Braak staging. Briefly, the Braak staging is based on recognizing the topographical distribution pattern of the neurofibrillary pathology in specific cortical and subcortical regions and checks the sequence and evolution of AD-related neurofibrillary lesions. Neurofibrillary changes occurred in the form of neuritic plaques, neurofibrillary tangles and neuropil threads and follow a predictable pattern. There are six stages based on gradual increase in lesional density and can be subsumed under three more general units: I-II, III-IV and V-VI. The bulk of abnormal tau protein is detectable in the transentorhinal and entorhinal regions (stages I-II), in the limbic allocortex and adjoining neocortex (stages III-IV), or in the neocortex, including the secondary and primary fields (stages V-VI).^{223, 225} Screening for PD was performed according to the criteria described previously.²²⁶ For Vascular Dementia, the criteria, presented at the NINDA-AIREN International Workshop were used.^{227, 228}

The classification according to Braak staging was stage II or less for all subjects except patient ID-No: 0194, where it was Braak IV. According to pathological diagnosis, both ID-No: 0914 (test) and ID-No: 0406 (control) had AD, so they were compared.

4.2.3 Experimental Design

The experimental design is described in Figure 4.1 and Table 4.1. The groups were ethnicity (i.e. Japanese), age (control, 86 ± 4.7 ; stroke, 82.5 ± 0.1 yrs) and gender (sex, female) matched. The mean PMI was similar in both groups. As the brain samples were collected terminally during autopsy, a retrospective analysis is possible for the subjects. Thus, in addition to pathological diagnosis, other clinical or pathological data

were also collected for possible correlation of specific phenotypes to the deregulated proteome. Many neuronal disorders originate from specific locations of the brain (thalamus for schizophrenia or limbic system for epilepsy), thus making it easier to target defined region for proteomic analysis of the post-mortem tissue.

Figure 4.1 Representative photographs of the autopsied brain sections of five Japanese subjects with their respective IDs. The alphabets (i.e. G, I, J or H) are indicative of specific slice number (SL No.). Of note, ID-No: 1006 had two infarcts in slice J and H. **The marked** areas have been taken for the quantitative proteomic analysis. The three pairs (control vs. CIS) were matched by location as mentioned in the table. The control/test pairs were also matched by age, sex and ethnicity. Subject details can be found in Table 4.1. CIS is chronic IS.

Stroke being a circulatory complication, lacks this specificity and can affect any part of the brain. Thus, it is imperative to target more than one location to identify the perturbed proteome that is beyond the spatial influence. Hence, three functionally and structurally distinct regions (e.g., putamen, thalamus and parietal lobe) of the brain were chosen during patient selection. Human brain is structurally heterogeneous comprising of different cell types. Despite the relative abundance of neuronal and non-neuronal cells remains close to 1:1, the ratio of neuron/glia varies widely in different

parts of the brain. When human brain is roughly divided into cerebral cortex (represent about 82% of total brain mass), cerebellum (around 10% of total brain mass) and rest of the brain (containing basal ganglia, diencephalon and brain stem), the ratio of non-neuronal/ neuronal cell varies from 0.23 for cerebellum to 11.35 for 'rest of the brain'.⁵² Understandably, the resultant protein expression of specific part of the brain is a combined expression of all cell types present in that milieu. Thus, relative mass of different cell types should correlate better rather than the absolute number of each cell type. This becomes important as number wise, although it may indicate the predominance of neuronal proteins in the proteome of cerebellum as the absolute number of non-neuronal cells are much lesser; in practice, the low ratio (i.e., 0.23) is due to the presence of very large number of very small neurons.⁵² This clearly addresses the need to study different sub-structures of the brain to understand the general applicability of the proteome of interest in the context of the studied pathology.

Hence, considering the cellular heterogeneity, differences in cellular composition⁵² and differential susceptibility of specific substructures of the human brain to ischemic injury,²¹⁰ brain tissue for the control patients was collected from identical locations of the same hemisphere. Accordingly, 0914, 1006J, and 1006H were stroke samples from putamen, thalamus and parietal lobe respectively and 0406, 0003, 0917 were used as respective location-matched controls (Table 4.1). Putamen is a part of basal ganglia, whereas thalamus and parietal lobe are parts of diencephalon and cerebral cortex respectively. Thus, there were three pairs of location-matched samples (biological replicate =3) to obtain the list of perturbed proteins, regulated in a location-specific or - fashion. Additionally, the remaining two samples acted as a random internal control (ID-No: 9825, parietal occipital lobe) and test (ID-No: 1008, cerebellum cortex) that was used to validate the trends, obtained from three independent pairs of location-matched samples. IS is mostly heterogeneous having a scattered pattern of brain lesions (archipelago pattern) rather than a uniform pattern (fried egg pattern), where penumbra nicely covers the core tissue.¹⁸⁴ To address this, one patient (ID-No: 1006) with multiple infarcts was included with tissues from thalamus (slice J) and parietal lobe (slice H). This will provide with an estimate about the inherent spatial heterogeneity of clinical stroke at the level of protein expression. The samples from individual patients were not pooled keeping in mind the wide variation among human population, presence of different co-morbidities in both test and control patients and

due to the presence of small number of samples. Hence, the resulting data is expected to offer a rough estimate about the biological variation in a sex and race-specific manner in the same age-group. Extending the same strategy to larger cohort of patients could help to validate the trends to obtain a more generalized conclusion.

The LC-MS/MS analysis was performed thrice (technical replicate = 3) to measure the analytical variation of the MS instrument.^{162, 208}

4.2.4 Proteomics

4.2.4.1 Sample preparation

Frozen samples were homogenized with liquid nitrogen before lyses at 4 °C with ice-cold lyses buffer [2% sodium dodecyl sulfate; 1 M TEAB with Complete Protease Inhibitor Cocktail (COMPLETE, Roche, Mannheim, Germany)] by intermittent vortexing and sonication (amplitude, 30%; pulse: 3 s/9 s for 5 min) using a Vibra Cell high intensity ultrasonic processor (Jencon Scientific Ltd., Leighton Buzzard, Bedfordshire, U.K.). The crude extracts were centrifuged at 20 600g for 15 min at 4 °C. The supernatant was mixed with 4 volumes of ice cold 100% acetone by vortexing and kept at -20 °C overnight to precipitate the proteins. The lysates were centrifuged at 15 000g for 30 min to collect the protein pellet. The pellets were washed with 90% acetone by vortexing at -20 °C to remove remaining contaminants and collected by centrifugation at 15 000g for 30 min. The acetone precipitated proteins were redissolved at 4 °C in lyses buffer [6% SDS, 20 mM DTT, 100 mM Tris, pH 7.8] and stored in aliquots at -80 °C (longer term) or at -20 °C (shorter duration). Protein quantification was performed using 2-D Quant kit (Amersham Biosciences).

Table 4.1 Clinical Details of the Brain Samples and Isobaric Labels for the Proteomic Analysis^a

SI/No.	Patient ID-No	iTRAQ label	gender	age	Slice	Pathological Diagnosis of Brain	Infarct area	Post mortem interval (PMI)	Other clinical / pathological data
Location-matched control									
1	0406	114	F	89	G	1. Appearance of few Lewy body of α -synuclein positive in cerebral cortex and brain stem 2. Loss of dorsal nucleus of vagus nerve 3. SP CERAD 0, NFT Braak stage II 4. Angiopathic change is mild	putamen	4h	sudden death
2	0003		F	79	I	physiological aging		3h	hepatic cell carcinoma
3	0917	116	F	88	H	1. Small infarction in posterior inferior cerebellar artery territory 2. Senile plaque stage A, NFT stage I, Lewy body(-), Grain (-)	parietal lobe	36h	hyper tension
Location-matched test									
4	0914	117	F	82	G	AD plaque Braak C, PHF Braak IV, multiple small infarct and bleeding	putamen	20h	aortic aneurysm fracture
5	1006		F	83	J	Multiple infarct, gram disease (stage III), no plaque, NFT is Braak stage II		2h	heart failure
6	1006	119	F	83	H	Do	parietal lobe	2h	heart failure
Random control and test sample for internal validation									
7	9825 ^b	113	F	88	G	physiological aging	parietal Occipital lobe	3h	NA
8	1008	121	F	82	NA	NFT stage II, SP(-), Lewy: incidental, argyrophilic grain(-)	cerebellum cortex	9h	Diabetes mellitus-II, Heart failure, Binswanger type leukoencephalopathy

^aThere were three pairs of age, sex, location, and PMI-matched samples, color-coded for easier comparison.^b Patient ID-No: 9825, a patient died due to physiological aging acts as a random control. Patient ID-No: 1008, a patient of chronic ischemia acted as a random test; SP (-): Senile plaques, negative; NFT: Neurofibrillary tangles.

4.2.4.2 In-gel Tryptic Digestion and Isobaric Labeling

The samples (200 µg of protein/ condition) were subjected to a denaturing PAGE using a 4% - 6% - 25% gel following a similar procedure described previously.²⁰⁸ Briefly, proteins were migrated through the 4% into the 6% layer, but were retarded by the 25% layer, thus concentrating them in a narrow strip at the end of the stacking gel. The diced gel bands were then reduced (5 mM tris-(2-carboxyethyl) phosphine, 60 °C, 1 h) and alkylated (10 mM methyl methanethiosulfonate in isopropanol, room temperature, 45 min in dark) before being digested with 12.5 ng/µL of sequencing-grade modified trypsin (Promega, Madison, WI) in 50 mM TEAB, 2% ACN for overnight at 37 °C. The peptides were extracted with 50% ACN, 5% acetic acid and vacuum centrifuged to dryness. The dried peptides were reconstituted into 0.5 M TEAB and labeled with respective isobaric tags of 8-plex iTRAQ Reagent Multi-Plex kit (Applied Biosystems) as follows: 9825, 113; 0406, 114; 0003, 115; 0917, 116; 0914, 117; 1006J, 118; 1006H, 119; 1008, 121 for stroke patients (Figure 4.1). The labeling reaction was stopped by adding excess water after 2 h. The samples were combined and dried in a vacuum centrifuge.

4.2.4.3 Electrostatic Repulsion and Hydrophilic Interaction Chromatography (ERLIC)

The combined iTRAQ sample was desalted by Sep-Pak C18 SPE cartridges. A modified ERLIC using volatile salt-containing buffers was adopted.²²⁹ The dried iTRAQ-labeled peptide was reconstituted in 200 µl of Buffer A (10 mM HCOONH₄; 85% ACN, 0.1% FA) and fractionated using a PolyWAX LP column (200 × 4.6 mm; 5 µm; 300 Å) (PolyLC, Columbia, MD) on a Prominence HPLC system (Shimadzu, Kyoto, Japan) in a 65 min gradient with Buffer B (30% ACN, 0.1% FA). The HPLC gradient was composed of 100% buffer A for 10 min; 0-25% buffer B for 35 min; then 25-100% buffer B for 10 min; followed by 100% buffer B for 10 min. The column was further eluted with a 20 min gradient consisting of Buffer B and Buffer C (0.1% FA). The gradient was as follows: 100% buffer B for 5 min; 0-100% buffer C for 5 min; 100% buffer C for 5 min; and finally 100% buffer B for another 5 min. The chromatogram was recorded at 214 nm. Eluted fractions were collected in every 1 min.

and then pooled into 34 fractions depending on the peak intensities, before drying them in a vacuum centrifuge. They were stored at -20 °C till MS analysis.

4.2.4.4 LC-MS/MS Analysis using QSTAR

The iTRAQ-labeled peptides were reconstituted with 0.1% FA, 3% ACN and analyzed using a HPLC system (Shimadzu) coupled with QSTAR Elite Hybrid MS (Applied Biosystems/MDS-SCIEX) as described previously with minor modifications.²⁰⁸ Briefly, most of the LC parameters for a 90 min gradient including column configuration, gradient and flow rate were kept constant except the mobile phase A composition (0.1% FA in 3% ACN) and sample injection volume (15 µl of peptide solution/analysis). Regarding MS parameters, the precursors with a mass range of 300–1600 m/z and calculated charge of +1 to +5 were selected for fragmentation. The selected precursor ion was dynamically excluded for 20 s with a 50 mDa mass tolerance. All other parameters were kept identical with the previously reported study.²⁰⁸ The peak areas of the iTRAQ reporter ions reflect the relative abundance of the proteins in the samples.

4.2.4.5 Mass Spectrometric Raw Data Analysis

The spectral data acquisition was performed using the Analyst QS 2.0 software (Applied Biosystems). ProteinPilot Software 3.0, Revision Number: 114 732 (Applied Biosystems) was used for peak list generation, protein identification and quantification against the concatenated target-decoy Uniprot human database (total 191242 proteins) for calculating the FDR. Details of the analysis strategy have been described in the previous chapter.²⁰⁸

4.2.5 Bioinformatics Analysis

Enrichment analysis was performed by submitting Uniprot accession numbers of the list of proteins to several open-source software, namely WEB-based Gene Set AnaLysis Toolkit(WebGestalt),²³⁰ DAVID²³¹ and PANTHER 7.0.¹⁷¹ These provide a comprehensive set of functional annotation tools to understand biological meaning behind large list of genes. Various categories like Gene Ontology (GO), pathways, protein domains, interactions or diseases using separate resources such as, Kyoto

Encyclopedia of Genes and Genomes (KEGG)²³² pathway (for pathway analysis), MINT²³³ (for checking protein interactions), INTERPRO or SMART (for predicting the occurrence of functional domains) and OMIM (knowledgebase of human genes and genetic disorders)²³⁴ were used to identify particular trends that did not occur only by chance. For GO analysis, the uploaded list of genes was classified according to the molecular functions or biological processes or cellular components. A predefined reference or background data set and the final list of regulated proteins are compared during enrichment analysis to check the overrepresentation of certain phenotype (i.e. a group of proteins common to a specific pathway, class, location, function, disease or any other attribute) in the regulated list. A minimum of two proteins per group was used as the default option. I have used the complete list of confidently detected proteins from human brain samples as the reference data set to compare the final screened list of proteins obtained from the complete list as described later (Section 4.3.2). The Hypergeometric test was used in WebGestalt followed by multiple test adjustments (Benjamini-Hochberg method) to correct for biases.²³⁰ In DAVID, a modified Fisher's exact test is adopted to measure the significance of the gene-enrichment in annotation pathways.²³¹ Significances of overrepresentation were expressed by fold enrichment and p -value before and after corrections with Benjamini-Hochberg or modified Fisher's exact test. Any phenotype was considered enriched when p -value is less than 0.01 after corrections with both the above-mentioned methods. The PANTHER Classification System classifies the genes by their molecular functions, biological pathways, class or cellular component like GO analysis using published scientific experimental evidence and evolutionary relationships to predict the function even in the absence of direct experimental evidence.

Open-source Gene Pattern software (version, 3.3.3) was used for clustering the final list of regulated proteins. Clustering is a mathematical way of organizing the proteins or genes into different groups based on the similar patterns of expression. Hierarchical clustering algorithm was chosen from various modules in the software that is based closely on the pairwise average-linkage method.²³⁵ The iTRAQ ratios were converted to log (base 2) value to bring up- and down-regulated genes to the same scale. The list of proteins (row-wise) having log-transformed values for various pairs of control-test combinations (column-wise) were used as input data. The output is obtained as a tree structure, referred to as dendrogram with the protein expression values represented

graphically by differentially coloring each cell that is correlated with the magnitude of the expression. Pearson correlation was used for clustering the proteins (row) as well as the conditions (i.e. log of ratio of test-to-control or control-to-test) to determine the groups of conditions and proteins that follow similar patterns.

4.2.6 WB Analyses

WB was performed following a similar procedure as described in chapter 3 by probing with specific horseradish peroxidase (HRP)-conjugated primary antibodies directed against proteins at the indicated dilutions: VIM (1:4000; Millipore, Billerica, MA), ANXA2 (1:2000; Santa Cruz Biotech, Santa Cruz, CA)HSP70 (1:5000; Abcam Ltd.,Cambridge, UK), Ferritin (1:2000; Abcam Ltd.), VDAC1 (1:2000; Santa Cruz Biotech), GAPDH (1:5000; Santa Cruz Biotech) andactin (1:5000; Millipore, Billerica, MA).

4.3 RESULTS

4.3.1 Quality Control of iTRAQ Data Set

To minimize the false positive identification of proteins, an unused ProtScore ≥ 2 was used as the qualification criteria like previous data sets. With this criterion, 1520 proteins were identified with a FDR of 0.1% (<1%). The average number of unique peptides (having a confidence level of >95%) detected per protein was 6.96 and more than 30% of the proteins had >5 unique peptides which was similar to the result presented previously (Sections 2.3.3and 3.3.2.2).^{162, 208} The quality of the sample preparation was determined by plotting the distribution of the proteins according to their theoretical MW and theoretical pI values as depicted in Figure 4.2. The presence of proteins covering a wide MW spectrum from high (>240 kDa) to low (<10 kDa) and also a wide pI range from acidic (pI ~4) to basic (pI>12) character indicated an extensive representation of the whole proteome in the current data set. Notably, around 60% of the proteins having a pI value in the acidic range (4 - 6.8).

Figure 4.2 Distribution of identified proteins from the bias and background corrected iTRAQ data set (unused prot score >2) according to their MW (MW, KDa) and theoretical pI (pI).

Table 4.2 Quantitative Information of the Initial List of Significantly Regulated Proteins for the Three Location-matched Pairs, Obtained from the Bias and Background Corrected iTRAQ Data Sets along with the ‘Location-unmatched comparison’^{4d}

N	Unseq	r (cov 95)	Accession	Protein name	Peptides (95%)	Section I				Section II				Qualified										
						Location-matched pairs				Location-unmatched comparison for 117 (HD-No. 0914)		Location-unmatched comparison for 118 (HD-No. 1006)			Location-unmatched comparison for 119 (HD-No. 1006H)									
						PVal 117:114 (Putamen)	PVal 117:114 (Thalamus)	PVal 118:115 (Thalamus)	PVal 119:116 (Pancreatic obc)	PVal 117:115	PVal 117:116 (0914)	PVal 118:114	PVal 118:116		PVal 119:115	PVal 119:115	PVal 119:114	PVal 119:114						
4	127.5	16.5	Q15149	Plectin-1 (PLEC1)	96	1.28	0.00	2.09	0.00	9.04	0.00	1.24	0.04	3.84	0.00	2.21	0.00	6.37	0.00	3.08	0.00	3.25	0.00	0.00
8	97.1	67.6	P14136	Gliat fibrillary acidic protein (GFAP)	146	1.06	0.03	1.36	0.00	1.94	0.00	1.32	0.00	1.34	0.00	1.08	0.01	1.38	0.00	1.91	0.00	1.53	0.00	0.00
14	58.8	33.8	P07197	Neurofilament medium polypeptide (NEFL)	46	1.54	0.00	0.46	0.00	1.66	0.00	1.21	0.03	2.09	0.00	0.58	0.02	0.77	0.17	0.97	0.28	1.24	0.01	app. ok
15	58.7	62.2	P06576	ATP synthase subunit beta, mitochondrial (ATP5B)	71	0.51	0.00	0.85	0.50	0.46	0.00	0.77	0.21	0.69	0.05	0.56	0.00	0.76	0.17	0.52	0.02	0.34	0.00	ok
17	57.2	50.8	P09543	2,3-cyclic-nucleotide 3-phosphodiesterase (CNP)	59	3.66	0.00	0.33	0.00	1.21	0.13	1.22	0.05	1.36	0.02	1.00	0.98	0.37	0.00	1.09	0.32	3.25	0.00	app. ok
18	55.9	42.5	P02768	Serum albumin (ALB)	50	0.51	0.00	0.60	0.68	0.38	0.00	3.08	0.00	0.67	0.00	0.10	0.00	0.13	0.00	1.77	0.00	0.29	0.00	Special case
21	51.7	32.8	Q59FD4	Hexokinase 1 isoform HK1 variant (Hexosant)	43	0.43	0.00	0.96	0.70	0.39	0.00	0.44	0.00	0.35	0.00	0.95	0.45	0.76	0.05	0.49	0.00	0.49	0.00	ok

30	44.5	41.6	P08670	Vimentin (VIM)	32	1.09	0.46	14.72	0.00	29.11	0.00	7.73	0.00	13.55	0.03	2.27	0.00	23.99	0.01	19.41	0.00	3.05	0.00	ok
31	43.6	42.7	P25705	ATP synthase subunit alpha, mitochondrial (ATP5A1)	54	0.44	0.00	0.54	0.07	0.13	0.00	0.66	0.04	0.70	0.02	0.36	0.00	0.56	0.03	0.12	0.00	0.09	0.00	ok
36	39.8	42.2	O75746	Calcium-binding mitochondrial carrier protein Aralar1 (SLC25A12)	21	0.49	0.01	0.96	0.81	0.35	0.00	0.46	0.01	0.42	0.00	1.02	0.59	0.89	0.47	0.38	0.00	0.41	0.00	ok
45	33.9	50.2	Q6FHZ0	Malate dehydrogenase (MDH2)	40	0.52	0.00	0.88	0.35	0.37	0.00	0.67	0.05	0.56	0.01	0.67	0.02	0.73	0.06	0.43	0.00	0.33	0.00	ok
48	33.4	43.3	P00505	Aspartate aminotransferase, mitochondrial (GOT2)	28	0.35	0.00	1.15	0.99	0.46	0.01	0.54	0.03	0.39	0.01	0.74	0.30	0.85	0.37	0.63	0.05	0.41	0.01	ok
51	32.0	31.9	P02686	Myelin basic protein (MBP)	37	3.08	0.00	1.46	0.14	2.86	0.00	6.79	0.00	3.22	0.00	0.63	0.25	0.66	0.45	5.81	0.00	2.70	0.00	ok
61	29.1	31.9	B4DJAO	NADH-ubiquinone oxidoreductase 75 kDa subunit (NDUFS1)	16	0.63	0.01	0.79	0.04	0.60	0.01	0.73	0.07	0.70	0.05	0.67	0.01	0.76	0.03	0.63	0.01	0.53	0.00	ok
63	28.7	11.4	P75357	Contactin-associated protein 1 (CNTNAP1)	14	1.02	0.95	0.46	0.00	0.63	0.02	0.59	0.00	0.72	0.10	0.81	0.27	0.57	0.01	0.51	0.00	0.89	0.42	ok
64	28.5	21.6	Q93050	V-type proton ATPase 116 kDa subunit a isoform 1 (ATP6VA1)	18	0.35	0.00	1.05	0.56	0.29	0.00	0.36	0.00	0.30	0.00	1.02	0.95	0.86	0.82	0.35	0.00	0.34	0.00	ok
68	28.2	30.3	P17600	Synapsin-I (SYN1)	15	0.41	0.00	1.54	0.01	0.24	0.01	0.51	0.00	0.34	0.00	1.25	0.09	1.05	0.31	0.35	0.01	0.29	0.00	opp. ok
80	26.3	60.3	P16152	Carboxyl reductase [NADPH] 1 (CBR1)	15	1.60	0.02	0.66	0.16	2.25	0.00	1.17	0.26	2.21	0.00	0.89	0.98	1.22	0.23	1.20	0.28	1.63	0.03	ok
81	25.9	33.1	Q7LDD5	Calmodulin-dependent protein kinase II, alpha (CAMK2A)	17	2.38	0.04	0.83	0.51	0.47	0.02	0.33	0.00	0.38	0.00	5.65	0.00	0.97	0.95	0.41	0.01	2.88	0.00	opp. failed

86	24.7	24.2	A8K132	cDNA FLJ75476, highly similar to Homo sapiens glutamine tRNA	13	0.64	0.03	0.74	0.33	0.28	0.00	0.60	0.02	0.54	0.01	0.79	0.57	0.67	0.17	0.30	0.00	0.32	0.00	ok
88	24.3	51.9	P08758	Annexin A5 (ANXA5)	19	1.45	0.09	1.71	0.02	5.11	0.00	2.25	0.00	3.22	0.00	1.09	0.39	2.44	0.00	3.60	0.00	2.31	0.00	ok
89	24.2	12.5	O94856	Neurofascin (NF-ASC)	17	1.29	0.39	0.55	0.03	0.51	0.01	0.61	0.03	0.63	0.04	1.16	0.40	0.57	0.04	0.50	0.01	1.04	0.95	ok
93	23.3	27.8	P80404	4-aminobutyrate aminotransferase, mitochondrial (ABAT)	17	0.44	0.00	1.11	0.58	0.65	0.05	0.78	0.33	0.77	0.29	0.61	0.01	1.08	0.64	0.66	0.06	0.37	0.00	ok
97	23.1	5.4	P12111	Collagen alpha- 3(VI) chain (COL6A3)	11	0.29	0.00	0.46	0.00	9.12	0.00	3.80	0.09	1.14	0.61	0.88	0.31	3.44	0.01	21.48	0.00	2.49	0.00	opp. ok
101	22.6	38.6	P22695	Cytochrome b-c1 complex subunit 2, mitochondrial (CQRC2)	17	0.65	0.03	0.78	0.40	0.52	0.01	0.63	0.06	0.66	0.08	0.80	0.26	0.82	0.52	0.49	0.01	0.51	0.00	ok
105	22.2	32.6	O75390	Citrate synthase, mitochondrial (CS)	15	0.49	0.01	0.83	0.51	0.42	0.00	0.54	0.01	0.50	0.01	0.74	0.34	0.76	0.23	0.46	0.00	0.41	0.00	ok
109	21.2	54.4	Q0VAS5	Histone H4 (HIST1H4H)	19	2.49	0.00	1.53	0.05	2.68	0.00	1.69	0.02	2.47	0.00	2.27	0.00	2.23	0.00	1.84	0.01	2.70	0.00	ok
112	21.1	31.7	Q9UL12	V-type proton ATPase subunit H (ATP6V1H)	11	0.73	0.01	0.78	0.17	0.55	0.00	0.63	0.00	0.60	0.00	0.90	0.57	0.74	0.03	0.58	0.00	0.67	0.01	ok
113	21.1	25.8	Q8NCF7	cDNA FLJ90278 fls clone NT2RP1000325, highly similar to Phosphate carrier protein, mitochondrial precursor	12	0.46	0.00	0.89	0.43	0.39	0.00	0.53	0.01	0.49	0.00	0.75	0.12	0.81	0.23	0.43	0.00	0.37	0.00	ok
118	20.6	41.4	Q6LBH0	SUC25A11 protein (SUC25A11)	13	0.53	0.03	0.78	0.21	0.39	0.00	0.59	0.04	0.55	0.01	0.70	0.14	0.72	0.08	0.42	0.00	0.38	0.00	ok
128	19.9	40.7	Q5TZ9	ANXA1 protein (ANXA1)	12	2.31	0.00	5.70	0.00	19.59	0.00	4.57	0.00	8.55	0.00	2.88	0.00	10.67	0.00	11.17	0.00	5.65	0.00	ok

130	19.8	42.5	P45880	Voltage-dependent anion-selective channel protein 2 (VDAC2)	13	0.61	0.04	0.93	0.43	0.38	0.00	0.66	0.06	0.64	0.06	0.86	0.29	0.89	0.44	0.40	0.00	0.37	0.00	ok
136	18.8	18.6	P38646	Stress-70 protein, mitochondrial (HSPA9)	13	0.55	0.01	0.82	0.32	0.58	0.02	0.86	0.32	0.67	0.05	0.52	0.01	0.64	0.05	0.74	0.12	0.47	0.00	ok
145	18.2	32.4	P07355	Annexin A2 (ANXA2)	10	1.16	0.27	2.99	0.00	8.79	0.00	2.75	0.00	4.02	0.00	1.34	0.05	4.45	0.00	6.14	0.00	2.63	0.00	ok
151	17.9	22.9	B4DHQ3	Phosphoserine aminotransferase	11	1.87	0.00	0.08	0.73	2.68	0.00	1.19	0.29	2.03	0.00	1.54	0.04	1.67	0.03	1.58	0.02	2.47	0.00	ok
152	17.9	23.7	Q13228	Selenium-binding protein 1 (SELENBP1)	10	0.99	0.96	2.15	0.02	5.25	0.00	1.84	0.05	3.63	0.00	1.16	0.45	4.25	0.00	2.68	0.00	1.43	0.12	ok
160	17.4	26.9	Q59GM1	Synapsin II isoform IIb variant (Fragment)	12	0.47	0.01	0.90	1.00	0.52	0.02	0.70	0.14	0.72	0.11	0.61	0.06	0.93	0.88	0.51	0.03	0.35	0.00	ok
168	16.8	2.8	Q59FG9	Chondroitin sulfate proteoglycan 2 (Versican variant (Fragment))	12	3.80	0.00	0.57	0.01	1.07	0.73	1.00	0.87	1.25	0.23	2.17	0.01	0.70	0.07	0.86	0.29	3.25	0.00	app. failed
185	16.0	25.8	P54652	Heat shock-related 70kDa protein 2 (HSPA2)	20	1.94	0.03	0.59	0.07	2.29	0.01	1.18	0.44	2.21	0.01	0.97	0.92	1.12	0.75	1.22	0.26	2.01	0.01	ok
196	15.2	17.3	Q86Y15	Dihydropyrimidine S-acetyltransferase (DIAT)	9	0.64	0.05	0.86	0.49	0.45	0.01	0.63	0.07	0.57	0.02	0.87	0.36	0.78	0.17	0.50	0.02	0.51	0.01	ok
224	13.6	3.3	Q60FE6	Filamin A (FLNA)	8	0.59	0.16	2.40	0.05	4.92	0.00	1.49	0.26	1.49	0.26	0.97	0.80	2.44	0.05	4.83	0.00	1.98	0.04	ok
229	13.3	27.6	P11177	Pyruvate dehydrogenase E1 component subunit beta, mitochondrial (PDHB)	10	0.66	0.20	0.59	0.03	0.33	0.00	0.58	0.05	0.59	0.08	0.67	0.12	0.60	0.05	0.33	0.00	0.37	0.01	ok

234	12.8	15.4	Q5STMI	Myelin oligodendrocyte glycoprotein (MOG)	11	3.44	0.03	0.29	0.03	1.00	1.21	0.95	0.83	1.27	0.56	1.02	0.85	0.39	0.08	0.91	0.67	3.31	0.04	app- ok
252	12.4	18.8	Q8INJ6	NAD-dependent deacetylase sirinun-2 (SIRT2)	6	1.75	0.02	0.42	0.01	1.82	0.07	1.19	0.42	1.53	0.16	0.61	0.18	0.53	0.03	1.42	0.20	2.11	0.01	app- ok
298	10.8	28.6	Q86W19	Ferritin (FTL)	8	5.06	0.00	2.27	0.03	8.79	0.00	2.70	0.02	5.06	0.00	4.25	0.00	4.25	0.00	4.83	0.00	9.04	0.00	ok
358	9.5	21.1	Q6XS36	Ferritin (Fragmento (FTH))	5	3.16	0.00	1.10	0.43	2.78	0.00	1.17	0.31	2.11	0.01	2.88	0.00	1.98	0.02	1.54	0.07	4.06	0.00	ok

The denominators and numerators are stroke and control samples respectively. Note that the denominators are different and matched by locations with respective stroke samples (numerators). All ratios were compared from bias and background corrected data sets. The table was divided in two sections: I: location-matched and II: location-unmatched comparison. The 'accession' column provides the unique ID-No of the proteins from 'Uniprot human database'. The gene symbol (within bracket) is provided along with protein name. The **bold red** color indicates that the existence of a protein at the level of transcript (such as existence of cDNA(s), RT-PCR or Northern blots) only. This list contains 46 candidates qualified (out of 1521) through the initial filters (i.e., unused prot score >2.0 (confident identification), *p*-value <0.05 (significantly different from 1) for at least two ratios, among three location-matched pairs (117:114, putamen, 118:115, thalamus, 119:116, parietal lobe). Three different colors were used to indicate the ratios with significant *p*-value for three iTRAQ labels representing individual specimens (□ 117; □ 118; □ 119). Nine of the candidates were regulated significantly in all locations (putamen, thalamus, and parietal lobe) as indicated by the 'Protein name' in **bold**. Some others showed opposite regulation as mentioned in the final column (opp). This location-matched list was subjected to 'location-unmatched' comparison where denominators were from different locations (e.g., putamen/thalamus or putamen/parietal lobe). The proteins that showed consistent trends (i.e., significant change in the same direction or no change) with location-matched pairs were qualified ('ok' in the last column). Q59FG9 and Q7LDD5 were failed, whereas confidence of many proteins (e.g., CNP, SIRT2, VIM, and ANXA5) was increased. The gene name, if present is provided within bracket along with the protein name. (Of note, thalamus (label: 118) and parietal lobe (label: 119) were taken from the same patient (ID-No: 1006).

Table 4.3 Internal Validation of the Qualified List from Table 4.2 by Using One Random Control and One Random Test Sample^a

N	Unused	%(cov(95)	Accession	Protein name	Pepides(95%)		Location-matched pairs				Internal validation by using 113 as random control sample (Section I)				Internal validation by using 121 as random test sample (Section II)				Selected				
					117:114 (Putamen)	118:115 (Thalamus)	PVal 118:115	119:116 (Parietal lobe)	PVal 119:116	113:114	PVal 113:114	113:115	PVal 113:115	113:116	PVal 113:116	121:114	PVal 121:114	121:115	PVal 121:115	121:116	PVal 121:116		
4	127.5	16.5	Q15149	Plectin-1 (PLEC1)	66	1.28	0.00	2.09	0.00	9.04	0.00	0.54	0.00	0.52	0.00	1.67	0.02	1.33	0.00	1.27	0.06	3.91	0.00

8	97.1	67.6	P14136	Glial fibrillary acidic protein (GFAP)	146	1.06	0.03	1.36	0.00	1.94	0.00	0.75	0.00	0.95	0.00	0.96	0.01	0.98	0.42	1.22	0.00	1.25	0.00	yes
14	58.8	33.8	P07197	Neurofilament medium polypeptide (NEFM)	46	1.54	0.00	0.46	0.00	1.66	0.00	2.05	0.00	1.60	0.00	2.75	0.00	0.39	0.00	0.31	0.00	0.53	0.01	
15	58.7	62.2	P06576	ATP synthase subunit beta mitochondrial (ATP5B)	71	0.51	0.00	0.85	0.50	0.46	0.00	1.20	0.14	1.82	0.00	1.61	0.00	0.35	0.00	0.53	0.04	0.47	0.01	yes
17	57.2	50.8	P09543	2,3-cyclic-nucleotide 3-phosphodiesterase (CNP)	59	3.66	0.00	0.33	0.00	1.21	0.13	1.67	0.02	0.56	0.00	0.62	0.00	0.66	0.86	0.23	0.00	0.25	0.00	
21	51.7	32.8	Q59494	Hexokinase I isoform HKI variant (Fragment)	43	0.43	0.00	0.96	0.70	0.39	0.00	1.00	0.81	1.00	0.89	0.80	0.14	1.00	1.00	1.01	0.70	0.80	0.21	yes
30	44.5	41.6	P08670	Vimentin (VIM)	32	1.09	0.46	14.72	0.00	29.11	0.00	0.07	0.00	0.46	0.25	1.69	0.05	1.91	0.00	11.91	0.00	18.20	0.01	yes
31	43.6	42.7	P25705	ATP synthase subunit alpha mitochondrial (ATP5A1)	54	0.44	0.00	0.54	0.07	0.13	0.00	0.99	0.07	1.49	0.02	1.57	0.05	0.11	0.00	0.17	0.00	0.18	0.00	yes
36	39.8	42.2	O75746	Calcium-binding mitochondrial carrier protein Andrial (SLC25A12)	21	0.49	0.01	0.96	0.81	0.35	0.00	1.54	0.01	1.46	0.02	1.34	0.12	0.77	0.41	0.73	0.27	0.67	0.05	yes
45	33.9	59.2	Q6HH70	Malate dehydrogenase (MDH2)	40	0.52	0.00	0.88	0.35	0.37	0.00	1.15	0.61	1.49	0.04	1.25	0.29	0.53	0.00	0.69	0.10	0.58	0.01	yes
48	33.4	43.3	P00505	Aspartate aminotransferase, mitochondrial (AOT2)	28	0.35	0.00	1.15	0.99	0.46	0.01	1.16	0.38	1.80	0.07	1.32	0.31	0.58	0.08	0.91	0.41	0.66	0.10	yes
51	32.0	31.9	P02686	Myelin basic protein (MBP)	37	3.08	0.00	1.46	0.14	2.86	0.00	0.23	0.00	0.51	0.09	0.23	0.00	0.58	0.10	1.32	0.33	0.60	0.20	yes
61	29.1	31.9	B4DJX0	NADH-ubiquinone oxidoreductase 75 kDa subunit (NDUFS1)	16	0.63	0.01	0.79	0.04	0.60	0.01	1.11	0.38	1.31	0.09	1.25	0.12	0.60	0.01	0.70	0.07	0.67	0.05	yes
63	28.7	11.4	P78357	Contactin-associated protein 1 (CNTNAP1)	14	1.02	0.95	0.46	0.00	0.63	0.02	1.22	0.44	0.70	0.02	0.87	0.31	1.47	0.05	0.85	0.19	1.05	0.81	
64	28.5	21.6	Q93050	V-type proton ATPase 116 kDa subunit a isoform 1 (ATP6A0A1)	18	0.35	0.00	1.05	0.56	0.29	0.00	1.05	0.89	1.08	0.70	0.89	0.67	0.96	0.70	0.99	0.89	0.82	0.51	yes
68	28.2	30.3	P17600	Synapsin-1 (SYN1)	15	0.41	0.00	1.54	0.01	0.24	0.01	0.71	0.03	0.88	0.22	0.60	0.01	1.08	0.25	1.33	0.03	0.91	0.69	

80	26.3	60.3	P16152	Carbonyl reductase [NADPH] 1 (CBR1)	15	1.60	0.02	0.66	0.16	2.25	0.00	1.07	0.49	0.79	0.45	1.47	0.07	0.87	0.79	0.64	0.11	1.19	0.32	yes
86	24.7	24.2	A8K132	cDNA FLJ75476, highly similar to Homo sapiens glutaminase (GLS), mRNA	13	0.64	0.03	0.74	0.33	0.28	0.00	1.33	0.05	1.25	0.11	1.14	0.22	0.71	0.18	0.67	0.09	0.61	0.04	yes
88	24.3	51.9	P08758	Annexin A5 (ANXA5)	19	1.45	0.09	1.71	0.02	5.11	0.00	0.69	0.16	1.09	0.65	1.56	0.07	1.09	0.50	1.71	0.02	2.44	0.00	yes
89	24.2	12.5	O94856	Neurofascin (NFASC)	17	1.29	0.39	0.55	0.03	0.51	0.01	1.43	0.19	0.68	0.08	0.70	0.10	1.51	0.05	0.72	0.27	0.74	0.34	yes
93	23.3	27.8	P80404	4-aminobutyrate aminotransferase, mitochondrial (ABAT)	17	0.44	0.00	1.11	0.58	0.65	0.05	0.74	0.11	1.33	0.10	1.29	0.12	0.48	0.00	0.86	0.39	0.84	0.35	yes
97	23.1	5.4	P12111	Collagen alpha-3(VI) chain (COL6A3)	11	0.29	0.00	9.46	0.00	9.12	0.00	0.34	0.00	4.29	0.08	1.45	0.45	1.43	0.05	13.80	0.00	5.55	0.00	
101	22.6	38.6	P22695	Cytochrome b-c1 complex subunit 2, mitochondrial (UQCRC2)	17	0.65	0.03	0.78	0.40	0.52	0.01	1.46	0.07	1.42	0.04	1.50	0.03	0.63	0.05	0.61	0.08	0.64	0.11	yes
105	22.2	32.6	O75390	Citrate synthase, mitochondrial (CS)	15	0.49	0.01	0.83	0.51	0.42	0.00	1.31	0.14	1.46	0.09	1.33	0.22	0.65	0.13	0.74	0.21	0.67	0.08	yes
109	21.2	54.4	Q0VAS5	Histone H4 (HIST1H4H)	19	2.49	0.00	1.53	0.048	2.68	0.00	1.26	0.29	0.85	0.65	1.25	0.25	2.01	0.01	1.37	0.10	2.00	0.01	yes
112	21.1	31.7	Q9U112	V-type proton ATPase subunit H (ATP6V1H)	11	0.73	0.01	0.78	0.17	0.55	0.00	1.34	0.05	1.15	0.21	1.11	0.71	0.93	0.63	0.80	0.20	0.77	0.04	yes
113	21.1	25.8	Q8NCF7	cDNA FLJ90278 fis, clone NT2RP1000325, highly similar to Phosphate carrier protein, mitochondrialprecursor	12	0.46	0.00	0.89	0.43	0.39	0.00	1.31	0.16	1.53	0.04	1.39	0.08	0.63	0.03	0.73	0.14	0.67	0.07	yes
118	20.6	41.4	Q6IBH0	SLC25A11 protein (SLC25A11)	13	0.53	0.03	0.78	0.21	0.39	0.00	1.13	0.45	1.25	0.33	1.16	0.69	0.63	0.06	0.70	0.09	0.65	0.03	yes
128	19.9	40.7	Q5T7Z9	ANXA1 protein (ANXA1)	12	2.31	0.00	5.70	0.00	19.59	0.00	0.30	0.02	0.58	0.34	1.72	0.32	2.68	0.00	5.30	0.00	10.19	0.00	yes
130	19.8	42.5	P45880	Voltage-dependent anion-selective channel protein 2 (VDAC2)	13	0.61	0.04	0.93	0.43	0.38	0.00	1.01	0.91	1.10	0.86	1.05	0.85	0.69	0.08	0.74	0.13	0.71	0.13	yes

136	18.8	18.6	P38646	Stress-70 protein, mitochondrial (HSPA9)	13	0.55	0.01	0.82	0.32	0.58	0.02	0.87	0.48	1.36	0.13	1.07	0.64	0.57	0.01	0.90	0.46	0.70	0.08	yes
145	18.2	32.4	P07355	Annexin A2 (ANXA2)	10	1.16	0.27	2.99	0.00	8.79	0.00	0.35	0.00	0.86	0.60	1.41	0.12	1.72	0.14	3.84	0.01	5.60	0.01	yes
151	17.9	22.9	B4DHQ3	Phosphoserine aminotransferase	11	1.87	0.00	0.98	0.73	2.68	0.00	0.86	0.78	0.54	0.01	0.92	0.89	1.26	0.21	0.80	0.19	1.36	0.17	yes
152	17.9	23.7	Q13228	Selenium-binding protein 1 (SELENBP1)	10	0.99	0.96	2.15	0.02	5.25	0.00	0.30	0.02	0.54	0.57	1.13	0.27	0.20	0.06	0.34	0.95	0.82	0.10	yes
160	17.4	26.9	Q59GM1	Synapsin II isoform IIb variant (Fragment)	12	0.47	0.01	0.90	1.00	0.52	0.02	0.49	0.00	0.72	0.05	0.74	0.04	0.62	0.07	0.90	0.95	0.94	0.92	
185	16.0	25.8	P54652	Heat shock-related 70 kDa protein 2 (HSPA2)	20	1.94	0.03	0.59	0.07	2.29	0.01	1.04	0.79	0.63	0.13	1.18	0.51	1.06	0.66	0.64	0.17	1.21	0.40	yes
196	15.2	17.3	Q86YI5	Dihydrolipoamide S-acetyltransferase (DLAT)	9	0.64	0.05	0.86	0.49	0.45	0.01	1.22	0.33	1.20	0.23	1.10	0.62	0.77	0.18	0.76	0.26	0.69	0.09	yes
224	13.6	3.3	Q60FE6	Filamin A (FLNA)	8	0.59	0.16	2.40	0.05	4.92	0.00	0.25	0.01	0.59	0.32	0.61	0.31	0.96	0.87	2.38	0.04	2.44	0.05	
229	13.3	27.6	P11177	Pyruvate dehydrogenase E1 component subunit beta, mitochondrial (PDHB)	10	0.66	0.20	0.59	0.03	0.33	0.00	1.54	0.06	1.36	0.23	1.39	0.16	0.50	0.14	0.44	0.04	0.45	0.05	yes
234	12.8	15.4	Q5STM1	Myelin oligodendrocyte glycoprotein (MOG)	11	3.44	0.03	0.29	0.03	1.00	1.21	2.51	0.10	0.70	0.28	0.94	0.72	1.01	0.84	0.28	0.03	0.37	0.08	
252	12.4	18.8	Q8IX16	NAD-dependent deacetylase sirtuin-2 (SIRT2)	6	1.75	0.02	0.42	0.01	1.82	0.07	1.61	0.04	1.09	0.63	1.39	0.25	0.68	0.31	0.47	0.02	0.59	0.03	
298	10.8	28.6	Q86WJ9	Ferritin (FTL)	8	5.06	0.00	2.27	0.03	8.79	0.00	1.47	0.15	0.77	0.46	1.50	0.19	4.41	0.00	2.33	0.02	4.41	0.00	yes
358	9.5	21.1	Q6NS36	Ferritin (Fragment) (FTH1)	5	3.16	0.00	1.10	0.43	2.78	0.00	1.85	0.05	0.70	0.20	1.27	0.31	2.15	0.01	0.79	0.77	1.43	0.07	yes

^a This list contains 43 proteins qualified from Table 4.2. They were subjected to dual internal validation by comparing the protein expression in location-matched pairs with a random control (iTRAQ label: 113; section I) and a random test (iTRAQ label: 121, section II). Controls from all three locations were used as denominator for this comparison due to the absence of location-matched control. Ratios with significant *p*-value (<0.05) were considered real and were used for comparison (color coded). Unless the protein of interest showed a similar trend for 113 (section I) or an opposite trend for 121 (section II), the candidate was selected as indicated by 'yes' in the final column. For example, PLEC1 failed to qualify as it was up-regulated in 113/116 like three location-matched pairs. Thus, deregulation of PLEC1 is sensitive to ischemic changes (up-regulated in 121 also), but not completely specific. The candidates showing opposite regulation among the location-matched pairs (e.g., NEFM, CNP or MOG) were automatically excluded. The 'selected' (last column) proteins (33 out of 43) were taken for the functional analysis.

4.3.2 Selecting the Final list of Perturbed Proteins

The cutoff for up- or down-regulation was determined by using the p -value cut-off of 0.05 to obtain the list of proteins with significant ratios for each pair of samples (e.g., 117/114, 118/115, 119/116). Accordingly, 49, 34 and 141 proteins from 117 (ID-No: 0914), 118 (1006) and 119 (1006) respectively having overlap among each other showed significant perturbation in comparison with respective controls (i.e. 114, 115 and 116). The proteins that were significantly regulated in at least two of these three sample-pairs (i.e. at least in two locations from putamen, thalamus or parietal lobe) were included for further analysis. Thus, 46 candidates were short-listed (Table 4.2, Section I). Nine of them had significant p -values for all three pairs. Notably, 8 out of 46 proteins (~17%) showed opposite regulation (e.g., CNP >3.6 x up-regulated in putamen, whereas >3x down-regulated in thalamus) depending on the location. This could be an ideal example of the spatial pattern of differential response that could be related to the severity of lesion or the distance from the core or could account for an individual or non-specific variation. However, many of the molecular signals follow a phasic nature and may have typical temporal pattern depending on the onset of injury. As the agonal period (time from onset of ischemic episode to death) is long (i.e. death not due to acute ischemic symptoms, but a combination of ischemic and non-ischemic or age-related pathologies) and variable for different patients, proteins following an identical temporal pattern may apparently show differential regulation among the test samples only because the patients are not in the same stage of the disease. That may also contribute to the opposite regulation.

Presence of a non-ideal control (i.e., a control having an underlying pathology that causes a similar or an opposite regulation for certain proteins with respect to the ischemic samples) can potentially lead to under- or over-estimation of the ratio for specific proteins. Assuming such a situation may weaken the p -value, decrease the magnitude of regulation or may explain the opposite regulation, comparison of each test sample with the remaining two control samples were made to generate the list of ratios (e.g., for 117, 117/115 and 117/116). This comparison with alternative controls was named as 'location –unmatched comparison' (Table 4.2, Section II). Of particular interest was to cross-check those ratios from the 'location-matched' pairs that were not

significant (p -value >0.05), but the value do showed a consistent trend with the other two ratios with a significant p -value. For example, VIM and ANXA5 were not regulated for 117/114; although they were selected due to significant up-regulation in the other two location-matched pairs (i.e., 118/115, 119/116). The ratios of VIM and ANXA5 for 117/115 and 117/116 showed significant up-regulation with a good p -value. Conversely, CAMK2A and Versican, both of which were oppositely regulated in the location-matched pairs, showed erratic trends during location-unmatched comparison. The remaining six oppositely regulated candidates (i.e., NEFM, CNP, SYN1, COL6A3, Q5STM1, and Q8IXJ6) exhibited consistent trends irrespective of the denominator used. ALB, the only serum-derived candidate, represents a special case. Although perfusion status of the arteries is not known at the time of death of the donors, ALB was consistently down-regulated in two of the three location-matched pairs, indicating decreased tissue perfusion (reduced circulation volume) in diseased brain areas at the time of death. This is unlike what is observed in the affected hemisphere of rat model of transient MCAO²⁰⁸ or in the patients of acute stroke,²²⁰ where increased presence of signature acute phase proteins are expected due to the gradual and progressive opening of the BBB. This in turn implies permanently reduced circulation in the affected regions as expected from surviving chronic stroke patients. The conflicting trends of ALB for location-unmatched pairs are partly related to the differences in the micro-vascular network of the three anatomically distinct regions apart from the unpredictable status of perfusion as expected from the autopsied samples. Thus, this location-unmatched comparison helped to improve the confidence of many (e.g., CNP, SIRT2, VIM, ANXA5) while helped to eliminate (CAMK2A, Versican, ALB) a few proteins. Overall, most of the candidates (43 out of 46) exhibited consistent trends with 'location matched comparison' and were advanced to the next stage of analysis (Table 4.3).

Finally, the remaining two (location unmatched) samples (**control** from parietal occipital lobe, 113; and **test** from cerebellum cortex, 121) (Table 4.1) were introduced for internal validation of the selected 43 candidates (Table 4.3, Section I and II). Relative expression of these proteins was checked with respect to three control (e.g., 114, 115, and 116) brains used previously for location-matched comparison. Ideally, in comparison with location-matched pairs (117/114, 118/115, and 119/116), these 43 proteins should show no or **opposite** regulation in 113 (i.e., 113/114, 113/115, and

113/116) and no or **similar** trends in 121 (i.e., 121/113, 121/114 and 121/115) to be true positives. Remarkably, 33 out of 43 (~77%) candidates were qualified through this dual validation (Table 4.3, last column). Hence, this data set is a testimony of a response pattern, most of which are constant across different brain locations amid a few spatially distinct signatures. This final list of significantly regulated proteins (33 out of 1521; 2.2% of total hits) was advanced for the functional analysis to curate novel pathophysiological mechanisms (Table 4.5). This percentage is very close to the previous *in vivo* study (~2.7%) involving ischemic rodent hemisphere despite using entirely different selection criteria.²⁰⁸ Thus, the flexibility of the experimental design combined with the multiplicity of iTRAQ labeling technology allowed various comparisons to check the specificity and accuracy of the candidates. No cut-off based on the magnitude of the ratio was set like the previous two data sets (*in vitro* and *in vivo*) on the final list of proteins.

4.3.3 Bioinformatics Analysis of Age-Sex-Location-PMI matched proteome of human chronic ischemic brain

The final list of 33 proteins were compared with the complete data set containing 1521 proteins (FDR = 0.1%) to check the enrichment in various categories as mentioned previously. Uniprot accession numbers of 25 and 30 proteins (out of 33) were mapped in WebGestalt and DAVID respectively for GO analysis. In terms of cellular component, 'mitochondrion' was found to be highly enriched as around 50% of the annotated proteins (13 out of 25 in Webgestalt and 15 out of 30 in DAVID) were of mitochondrial origin or mitochondria-associated proteins. Of note, only 13 proteins of the mitochondrial proteome are being encoded by the mitochondrial genome and the rest must be imported from the cytosol.²³⁶ Thus, most mitochondrial proteins are made as larger precursors in the cytoplasm with a transit peptide at the N-terminal and are post-translationally incorporated into mitochondria. This was reflected by the 'SP_PIR_KEYWORDS' analysis, where the top two qualified and significantly enriched keywords was 'mitochondrion' and 'transit' having 15 and 11 (out of 33) proteins respectively (Table 4.4).

These 11 candidates were among the 15 mitochondria related proteins that are synthesized as preproteins and imported to mitochondria with the help of the transit

peptide that is cleaved later on. In the category of molecular function, calcium-dependent phospholipid binding, lipase or phospholipase inhibitor activity (ANXA2, ANXA1, and ANXA5) and eukaryotic cell surface binding (ATP5B, ATP5A1, and ANXA5) were featured as significantly enriched. The enrichment analysis with KEGG pathway showed that TCA cycle (MDH2, PDHB, DLAT, CS) and oxidative phosphorylation (ATP5B, ATP5A1, ATP6V1H, UQCRC2, ATP6V0A1) as the two significantly enriched pathways which are consistent with the enrichment of mitochondrial proteins. None of the enriched interactions or protein domains had significant *p*-value after correction, when INTERPRO, MINT or UCSC_TFBS module in DAVID was used. Searching the candidates for possible associations with genetic disorders in the OMIM knowledgebase did not retrieve any results.

Table 4.4 SP_PIR_KEYWORDS Analysis of the Final List of Selected Proteins by DAVID^a

Term	Count	%	Genes	Fold Enrichment	<i>p</i> -Value	Benjamini
Mitochondrion	15	50.0	Q6IBH0, P45880, P00505, Q8NCF7, P38646, P06576, P11177, Q6FHZ0, P80404, O75746, Q86YI5, Q59FD4, P25705, O75390, P22695	3.40	0.00	0.00
Transit peptide	11	36.7	P06576, Q6FHZ0, P11177, P80404, Q86YI5, P25705, P00505, O75390, Q8NCF7, P22695, P38646	4.01	0.00	0.01
Duplication	7	23.3	P07355, Q6IBH0, Q86YI5, Q59FD4, Q5TZZ9, P08758, Q8NCF7	5.95	0.00	0.03
Acetylated amino end	6	20.0	P07355, Q86WI9, P02686, Q5TZZ9, P08758, Q0VAS5	6.50	0.00	0.04
Annexin	3	10.0	P07355, Q5TZZ9, P08758	21.67	0.01	0.16
Endonexin fold	3	10.0	P07355, Q5TZZ9, P08758	21.67	0.01	0.16
Calcium/phospholipid binding	3	10.0	P07355, Q5TZZ9, P08758	21.67	0.01	0.16
Hydrogen ion transport	4	13.3	P06576, P25705, Q9UII2, Q93050	9.12	0.01	0.15
Mitochondrion inner membrane	6	20.0	P06576, O75746, Q6IBH0, P25705, Q8NCF7, P22695	4.41	0.01	0.14

^a Thirty out of 33 proteins were mapped. The background contains 1300 proteins confidently identified by iTRAQ analysis. The *p*-value cut-off before or after correction was set at 0.01 to consider the 'term'

as enriched. Among the top ten listed 'terms', only the top two (Mitochondrion and Transit peptide) were qualified.

Enrichment analysis was followed by hierarchical cluster analysis of the 33 selected proteins from Table 4.3 using Gene Pattern software where the quantitative information was used to group proteins based on expression patterns. The random control (Table 4.3, Section I) and test (Table 4.3, Section II) were also included (Figure 4.3). The result shows nice separation of the random control specimen (ID-No: 9825, iTRAQ label: 113) from the test samples (Figure 4.3C) thus justifying the reliability of the neuropathological and clinical diagnosis and the utility of multiple filters used to exclude the noise (proteins unrelated to the pathology of interest). Despite being parts of the same individual's brain (ID-No: 1006), the overall regulation pattern of ischemic tissue from thalamus (iTRAQ label: 118) and parietal lobe (iTRAQ label: 119) showed distinct pattern of differential expression in comparison to respective controls. Overall, the proteins were grouped in two main clusters (A: 19 proteins, 57.6% and B: 14 proteins, 42.4%) consisting of predominantly down-regulated (i.e. A) and up-regulated proteins (i.e. B) in test samples. Intriguingly, all mitochondria associated proteins were grouped in the cluster A (Fig 1A) and were related to energy metabolism (ATP synthesis). The enriched KEGG pathways (TCA cycle and oxidative phosphorylation) were also part of this cluster. Concordant down-regulation of all mitochondrial proteins in chronic ischemic brains compared to the control samples may be due to increased degradation or decreased synthesis of proteins and indicates a generalized failure of the mitochondrial machinery. The enriched functional category of calcium-dependent phospholipid binding proteins comprising of annexin family (ANXA1, ANXA2 and ANXA5) were coexpressed with GFAP and VIM despite having unrelated sequences. All of them were up-regulated to different extent in human ischemic brains, a response that is also partly observed in the cell line and animal models of cerebral ischemia in my study. Two isoforms of mitochondrial 'solute carrier' family of proteins (SLCA2511 and SLC25A12) were placed side by side as they were concomitantly downregulated. Noteworthy, the sequence similarity by multiple alignments was around 30% for three isoforms of annexins and a mere 11.6% for the two 'solute carrier' families of proteins.

Figure 4.3 Clustered display of the final list of regulated proteins from the three location-matched test samples (as indicated by the locations) and location-unmatched conditions taken for internal validation. Log-transformed ratios for all conditions (column) were represented by each protein (row). Pearson correlation was applied for measurement of row and column distance. Globally normalized view is presented here. The color scale of the heat map ranges from saturated green for -3.8 to saturated red for 4.1 in the natural logarithmic scale. The proteins were mainly clustered into two parts as shown by A and B. The columns were clustered into two sections, where the control (iTRAQ label: 113) was nicely separated from location-matched and -unmatched test samples (C). The protein names and accession numbers were taken from Uniprot protein database. The gene symbols are provided with in bracket along with the protein name, wherever available.

Concordant expression and clustering of seemingly unrelated proteins in the ischemic brains may or may not be based on biological necessity, but they do impart an opportunism to explore new interactions or functions of a known protein from a different perspective. However, the presence of a larger data set would have helped to visualize the collective cellular response in a better way to bring out the interesting trends by this unsupervised clustering method. Finally, guided by the above trends, the selected list of proteins were manually classified and curated to understand the regulation in context of chronic IS.

Table 4.5 Final List of Selected Regulated Proteins from the Location-matched Chronic Ischemic Human Brain Samples along with Quantitative Expression Data from Random Control and Test Samples^a

N		Unused		%Cov(95)		Accession		Protein name		Peptides (95%)		Location-matched pairs			121 as random test			113 as Random control		
												117:114 (Putamen)	118:115 (Thalamus)	119:116 (Parietal lobe)	121:114	121:115	121:116	113:114	113:115	113:116
Energy Metabolism																				
Glycolysis																				
21	51.7	32.8	Q59FD4	Hexokinase I isoform HK1 variant (Fragment)	43	0.43	0.96	0.39							1.00	1.01	0.80	1.00	1.00	0.80
Pyruvate dehydrogenase complex (PDC)																				
196	15.2	17.3	Q86Y15	Dihydrolipoamide S-acetyltransferase (DLAT)	9	0.64	0.86	0.45							0.77	0.76	0.69	1.22	1.20	1.10
229	13.3	27.6	P11177	Pyruvate dehydrogenase E1 component subunit beta, mitochondrial (PDHB)	10	0.66	0.59	0.33							0.50	0.44	0.45	1.54	1.36	1.39
TCA cycle																				
36	39.8	42.2	O75746	Calcium-binding mitochondrial carrier protein Aralar1 (SLC25A12)	21	0.49	0.96	0.35							0.77	0.73	0.67	1.54	1.46	1.34
45	33.9	59.2	Q6FHZ0	Malate dehydrogenase (MDH2)	40	0.52	0.88	0.37							0.53	0.69	0.58	1.15	1.49	1.25
48	33.4	43.3	P00505	Aspartate aminotransferase, mitochondrial (GOT2)	28	0.35	1.15	0.46							0.58	0.91	0.66	1.16	1.80	1.32
86	24.7	24.2	A8K132	cDNA FLJ75476, highly similar to Homo sapiens glutaminase (GLS), mRNA	13	0.64	0.74	0.28							0.71	0.67	0.61	1.33	1.25	1.14
118	20.6	41.4	Q6IBH0	SLC25A11 protein (SLC25A11)	13	0.53	0.78	0.39							0.63	0.70	0.65	1.13	1.25	1.16
105	22.2	32.6	O75390	Citrate synthase, mitochondrial (CS)	15	0.49	0.83	0.42							0.65	0.74	0.67	1.31	1.46	1.33
Oxidative phosphorylation																				

15	58.7	62.2	P06576	ATP synthase subunit beta, mitochondrial (ATP5B)	71	0.51	0.85	0.46	0.35	0.53	0.47	1.20	1.82	1.61
31	43.6	42.7	P25705	ATP synthase subunit alpha, mitochondrial (ATP5A1)	54	0.44	0.54	0.13	0.11	0.17	0.18	0.99	1.49	1.57
61	29.1	31.9	B4DJA0	NADH-ubiquinone oxidoreductase 75 kDa subunit (NDUFS1)	16	0.63	0.79	0.60	0.60	0.70	0.67	1.11	1.31	1.25
101	22.6	38.6	P22695	Cytochrome b-c1 complex subunit 2, mitochondrial (UQCRC2)	17	0.65	0.78	0.52	0.63	0.61	0.64	1.46	1.42	1.50
Reactive Gliosis														
8	97.1	67.6	P14136	Glial fibrillary acidic protein (GFAP)	146	1.06	1.36	1.94	0.98	1.22	1.25	0.75	0.95	0.96
30	44.5	41.6	P08670	Vimentin (VIM)*	32	1.09	14.72	29.11	1.91	11.91	18.20	0.07	0.46	1.69
Anti-inflammatory Proteins														
128	19.9	40.7	Q5TZZ9	ANXA1 protein (ANXA1)	12	2.31	5.70	19.59	2.68	5.30	10.19	0.30	0.58	1.72
145	18.2	32.4	P07355	Annexin A2 (ANXA2) *	10	1.16	2.99	8.79	1.72	3.84	5.60	0.35	0.86	1.41
88	24.3	51.9	P08758	Annexin A5 (ANXA5)	19	1.45	1.71	5.11	1.09	1.71	2.44	0.69	1.09	1.56
Chaperonic Proteins														
136	18.8	18.6	P38646	Stress-70 protein, mitochondrial (HSPA9) *	13	0.55	0.82	0.58	0.57	0.90	0.70	0.87	1.36	1.07
185	16.0	25.8	P54652	Heat shock-related 70 kDa protein 2 (HSPA2)	20	1.94	0.59	2.29	1.06	0.64	1.21	1.04	0.63	1.18
Iron Storage Proteins														
298	10.8	28.6	Q86W19	Ferritin (FTL) *	8	5.06	2.27	8.79	4.41	2.33	4.41	1.47	0.77	1.50
358	9.5	21.1	Q6NS36	Ferritin (Fragment) (FTH1)	5	3.16	1.10	2.78	2.15	0.79	1.43	1.85	0.70	1.27
Others														
51	32.0	31.9	P02686	Myelin basic protein (MBP)	37	3.08	1.46	2.86	0.58	1.32	0.60	0.23	0.51	0.23
64	28.5	21.6	Q93050	V-type proton ATPase 116 kDa subunit a isoform 1 (ATP6V0A1)	18	0.35	1.05	0.29	0.96	0.99	0.82	1.05	1.08	0.89
80	26.3	60.3	P16152	Carbonyl reductase [NADPH] 1 (CBR1)	15	1.60	0.66	2.25	0.87	0.64	1.19	1.07	0.79	1.47
89	24.2	12.5	O94856	Neurofascin (NFASC)	17	1.29	0.55	0.51	1.51	0.72	0.74	1.43	0.68	0.70

93	23.3	27.8	P80404	4-aminobutyrate aminotransferase, mitochondrial (ABAT)	17	0.44	1.11	0.65	0.48	0.86	0.84	0.74	1.33	1.29
109	21.2	54.4	Q0VAS5	Histone H4 (HIST1H4H)	19	2.49	1.53	2.68	2.01	1.37	2.00	1.26	0.85	1.25
112	21.1	31.7	Q9UII2	V-type proton ATPase subunit H (ATP6V1H)	11	0.73	0.78	0.55	0.93	0.80	0.77	1.34	1.15	1.11
113	21.1	25.8	Q8NCF7	cDNA FLJ90278 fis. clone NT2RP1000325, highly similar to Phosphate carrier protein, mitochondrialprecursor	12	0.46	0.89	0.39	0.63	0.73	0.67	1.31	1.53	1.39
130	19.8	42.5	P45880	Voltage-dependent anion-selective channel protein 2 (VDAC2)	13	0.61	0.93	0.38	0.69	0.74	0.71	1.01	1.10	1.05
151	17.9	22.9	B4DHQ3	Phosphoserine aminotransferase	11	1.87	0.98	2.68	1.26	0.80	1.36	0.86	0.54	0.92
152	17.9	23.7	Q13228	Selenium-binding protein 1 (SELENBP1)	10	0.99	2.15	5.25	0.20	0.34	0.82	0.30	0.54	1.13

"The list of proteins along with the quantitative values that was used for cluster analysis. The *p*-value column is not shown. The colored cells have significant *p*-value (<0.05). The proteins have been classified based on their respective primary function (in bold). The 'energy metabolism' group is sub-divided into three groups. The proteins of energy metabolism were downregulated, whereas reactive gliosis and anti-inflammatory response were activated. Proteins participating in iron storage were also highly expressed. Chaperonic and other proteins exhibited a mixed trend. *Proteins validated by WB.

4.3.4 Postproteomic Validation by WB Analysis

Four proteins (i.e. VIM, ANXA2, HSP70, and ferritin) out of the 33 filtered candidates were selected for the validation of iTRAQ data set (Figure 4.4). VDAC1 was also included that showed a similar decrease like VDAC2, which was one of the filtered candidates. Each of the selected proteins represents distinct aspects of ischemic stroke pathology as mentioned in the Table 4.5. GAPDH and actin were included as they are considered as traditional loading controls. However, our result shows persistent down regulation of both of these housekeeping proteins in the infarcted areas of the brain which is consistent with a previously published report.²²⁰ Thus, an external reference was included as loading control to measure and normalize inter-gel variability between multiple WB experiments for the same protein.

Figure 4.4 (A) Postproteomic validations of the selected proteins from the location matched/unmatched control and infarcted brain tissue as indicated in the chart. Equal amount of protein was loaded as measured by the 2D Quant kit. Each of the blots has one external reference (e.g. band 5 from left for the ferritin blot) that was used to normalize inter-gel variation (B) Graphical representation of the three pairs of ratios matched by their locations and calculated by densitometric analysis. The unmatched control (band 1) and test (band 8) were not included for analysis. The straight line is indicative of no change in the relative expression of proteins in the infarct.

Traditionally used loading controls (e.g. actin and GAPDH) showed reduced level in the infarcts irrespective of their locations necessitating the inclusion of an external control to normalize the inter-gel variation. VIM, ANXA2, ferritin were upregulated whereas HSP70, VDAC1 were down-regulated in the infarcts compared to the control samples.

4.4 DISCUSSION

I report for the first time an overview of the quantitative ischemic proteome obtained from three pairs (biological replicate = 3) of ethnicity, age, sex, location and PMI-matched human specimens of chronic cerebral ischemia. Location-unmatched comparison (Table 4.2) and internal validation with one random control and one chronic ischemic sample (Table 4.3) were used to exclude the biological and technical noise. The final list of perturbed proteome was subjected to enrichment (Table 4.4) and modified hierarchical clustering analysis (Figure 4.3) highlighting the expected yet crucial role of mitochondria in the pathogenesis of IS (Table 4.5).

4.4.1 De-regulation of the Malate- Aspartate shuttle (MAS) and pyruvate dehydrogenase complex (PDC)

Glucose metabolism is compartment-specific and tightly regulated among cytosol and mitochondria of both neurons and astrocytes. Presence of several trans-mitochondrial or trans-cellular transporters in neuron and glia having distinct localization pattern and variable cellular or subcellular specificity helps to perform their respective functions. The astrocyte-neuron lactate shuttle, Glu-Gln shuttle in the glutaminergic synapses or MAS between cytosol and mitochondria are examples of these neurometabolic coupling. My *in vivo* study already reported the probable deregulation of astrocyte-neuron lactate shuttle and Glu-Gln shuttle during the acute and subacute phase of cerebral I/R injury.²⁰⁸

Role of MAS has been described in heart development during fetal and perinatal growth, in the physiology of pancreatic β -cells by influencing glucose-induced activation of mitochondrial metabolism and insulin secretion and in pathological conditions like autism.²³⁷ Here my data indicates for the first time the failure of MAS as seen by the concomitant down-regulation of all participating proteins (SLCA2511, SLCA2512, GOT2 and MDH2) in human brain mitochondria following chronic IS (Table 4.5). SLC25A12 or aralar is a brain-specific aspartate/Glu carrier (AGC1), expressed predominantly in neurons. It is an electrogenic, Ca^{2+} -dependent and unidirectional carrier that transports a protonated Glu into the mitochondria in exchange of an aspartate anion, so the flow is “pushed” by the mitochondrial

membrane electrochemical gradient. AGC1 requires the presence of mitochondrial aspartate aminotransferase (AATM or GOT2) to generate aspartate from oxaloacetate (OAA) that is coupled with the generation of α -KG from glutamic acid (protonated Glu). GDH although can generate α -KG using Glu, doesn't support AGC1 because it cannot supplement aspartate. SLCA2511 (oxoglutarate carrier, OGC), which also localizes to the IMM like AGC1, mediates the electroneutral exchange of α -KG for malate. This malate is converted to OAA by mitochondrial malate dehydrogenase (MDH2) to provide substrate for AATM. In this way, AGC1 and AATM work in concert with OGC to complete the MAS that drives the reduction equivalents (NADH) to polarized mitochondria in an irreversible manner under physiological condition to sustain cytosolic glycolysis and mitochondrial TCA cycle and oxidative phosphorylation (Figure 4.5).²³⁸

Figure 4.5 Schematic diagram showing the complex interplay of glycolysis, TCA cycle, MAS and oxidative phosphorylation (OxPhos) in the ischemic human brain. Blue arrows designate glycolysis. Green and brown arrows are for TCA cycle and MAS respectively. The product of glycolysis, pyruvate is generated in the cytosol and enters the mitochondria and is converted to acetyl Co-A by enzymes of PDC (e.g. E1, E2). Mitochondrial MDH (MDH2) is an enzyme used by both TCA cycle and MAS. OGC and AGC1 are two transporters present on mitochondrial membrane. The proteins in red color (OGC, AGC1, AATM, MDH, E1, E2 and CS) are quantified by the iTRAQ experiment with acceptable levels of confidence (p -value<0.05). All of them were down-regulated as well as the proteins of OxPhos. Aspartate is generated in the neuronal mitochondria and transported through MAS to the cytosol. The red arrows indicate the transaxonal transfer of aspartate to support the synthesis of myelin lipids in the oligodendrocyte. The failure of energy metabolism and neuron-oligodendrocyte cross-talk is apparent in the human brain following chronic IS. 1,3- BPG is 1,3-bisphosphoglycerate.

This data set also detected two components (i.e. PDHB or E1 and DLAT or E2) of the mitochondrial PDC, both of which were down-regulated (Table 4.5). PDC catalyzes

the oxidative decarboxylation of pyruvate to form acetyl Co-A and NADH using one molecule of NAD^+ , linking glycolysis to the TCA cycle and fatty acid synthesis (Figure 4.5). Regulation of PDC activity is crucial under stress and its inactivation has been suggested as an adaptive response under conditions of glucose deprivation.²³⁹ Pyruvate dehydrogenase deficiency due to mutations of E1 or E2 has been associated with lactic acidosis and neurological dysfunction like ataxia and hypotonia.²⁴⁰ This indicates that the presence of similar pathological (i.e. lactic acidosis) or neurological (i.e. ataxia) features in stroke patients may be related to PDC deficiency.²⁴¹

Translocation of protoporphyrin IX and coproporphyrin III through the down-regulated OGC constitute an important mechanism for the synthesis of heme in the mitochondrial matrix, where it acts as a precursor for several heme proteins including the cytochromes.²⁴² This also justifies the significant down-regulation of UQCRC2 (mitochondrial Cytochrome b-c1 complex subunit).

Mitochondria being the primary site for oxygen consumption are liable to oxidative stress due to the generation of reactive oxygen species. Oxidative stress can inactivate large number of enzymes including dehydrogenases (e.g. NDUFS1) and transport ATPases due to the oxidation of the critical sulfhydryl groups that must be maintained in the reduced form for proper functioning. Glutathione (GSH), a low MWthiol supplies the vital sulfhydryl group and is indispensable for upholding of the redox status of mitochondria. Depletion of intracellular GSH has been associated with increased infarction following MCAO model of IS.^{243, 244} Studies have established OGC as one of the major transporters of GSH from cytosol to mitochondria.²⁴⁵ Thus, decreased expression of NDUFS1 (an enzyme having NADH dehydrogenase activity) may be related to the alteration of the mitochondrial GSH level that corroborates the down-regulation of OGC.

4.4.2 MAS shuttle failure induced hypomyelination and Glu-Gln shuttle deregulation

AGC1 deficiency in human has been associated with severe, early postnatal psychomotor developmental arrest, hypotonia and seizures. A dominating feature of the syndrome is global cerebral hypomyelination.²⁴⁶ Mitochondrion is the major site of

aspartate production in the brain. Under physiological condition, passage of aspartate through AGC1 initiates the production of N-acetyl aspartate (NAA) by aspartate-N-acetyl-transferase in neuronal cytosol and microsomes. Neuronal N-acetyl aspartate subsequently undergoes transaxonal transfer to oligodendrocytes, where it supplies acetyl groups to acetyl-CoA for the synthesis of myelin lipids including galactocerebrosides (Figure 4.5). Accordingly, knockout of UDP-galactose:ceramide galactosyl-transferase (CGT), the enzyme responsible for the synthesis of galactocerebrosides exhibited comparable phenotype with the AGC1-knockout mice. However, despite similarity in terms of hypomyelination between AGC1-knockout and CGT-knockout mouse, the overall phenotype was much more severe for AGC1 (-/-) mice indicating additional roles of AGC1 in the brain.²⁴⁷ In the mice lacking AGC1, apart from the drastic fall in brain aspartate and NAA levels, reduction of myelin associated proteins, loss of neurofilaments in cerebral cortex and gray matter of striatum and astrogliosis as exemplified by the elevated staining for GFAP have been observed.^{247, 248} Hence, the increased expression of GFAP in all and decreased level of NEFM (Neurofilament medium polypeptide) (Table 4.3) in at least two of the specimens in my data set may be related to the AGC1 down-regulation. In contrast, albeit unexpectedly MBP, an oligodendrocyte specific protein showed unregulation in the stroke affected specimen. The failure of MAS could also lead to the gradual failure of Gln-Glutathione as seen by the down-regulation of neuron-specific glutaminase in the current data set.²⁴⁷

Hence, my study shows that concordant down regulation of MAS, PDC along with enzymes of oxidative phosphorylation are correlated and may contribute to the failure of energy metabolism, hypomyelination, oxidative stress and cell death at the molecular level and explains the manifestation of some of the neurological dysfunctions seen in stroke patients at the clinic. On the other hand, my data explains chronic IS as materialization of mitochondrial dysfunction, a sub-cellular molecular signature shared by chronic neurological disorder like AD.²⁴⁹

4.4.3 Deregulation of gamma-aminobutyric acid (GABA) metabolism

Down-regulated cytosolic protein 4-aminobutyrate aminotransferase (ABAT) or GABA-transaminase (GABA-T) is responsible for catabolism as well as synthesis of

GABA. GABA-T metabolizes GABA into succinic semialdehyde only in presence of α -KG that acts as the receiver of the amino group (Figure 4.6).

Figure 4.6 Role of GABA-T in GABA metabolism.

On the other hand, GABA-T transaminates α -KG into Glutamate which is converted to GABA in presence of neuronal glutamic acid decarboxylase enzyme in the GABAergic neurons. Glu and GABA are respectively the major excitatory and inhibitory neurotransmitter of the brain. Acute and excess release of Glu and persistent depolarization of the post-synaptic neuron causes Glu excitotoxicity that is one of the primary mechanisms of cell death in the post-ischemic brain. Considering the counterbalancing function of glutamatergic and GABAergic transmissions under physiological condition, enhancing the GABAergic transmission by increasing the endogenous synthesis and release of GABA may be beneficial. In fact, according to recent reports increase in GABAergic synaptic transmission by blocking GABA-T with γ -vinyl GABA or anti-epileptic drug vigabatrin displayed neuroprotective effects respectively in gerbil model of repetitive forebrain ischemia²⁵⁰ or primary corticostriatal slice culture model of *in vitro* ischemia.²⁵¹ Thus, in the current study decreased level of GABA-T in absence of any neuroleptic drugs could indicate an adaptive response of the ischemic brain to maintain the level of GABA in the ischemic area. The failure of MAS components and probable paucity of α -KG should also retard the degradation of GABA by GABA-T. Interestingly, congenital deficiency of GABA-T is a rare disorder of GABA catabolism that causes severe psychomotor retardation and recurrent episodic lethargy with intractable seizures.²⁵² Given the inhibitory nature of GABA activity in the CNS, the paradoxical neurological phenomenon associated with seizures in cases of excess GABA is poorly understood. Reports have linked GABA-T with several disorders that include Gastro-esophageal reflux disease,²⁵³ neuroleptic-induced tardive dyskinesia²⁵⁴ and autism²⁵⁵ based on preclinical or clinical case studies.

4.5 LIMITATIONS

The patients were aged and presence of co-morbidities was expected. The complete retrospective information of these and the medications was not available. These patients are a surviving population following a non-fatal attack of stroke thus represents cases of subacute or chronic ischemia. The duration between the onset of stroke symptoms and death is variable and not controlled. Due to the seemingly long agonal period, presence of an overlapping or predisposing pathology of neurological (e.g. AD) or non-neurological origin (e.g. diabetes) may affect the pattern or magnitude of proteins' expression. The patency of the arteries in the affected region was also not known at the time of death. Thus, the tissue may be permanently or temporarily ischemic which could potentially affect the protein expression profile. Even if all the above-mentioned issues are taken into account and controlled accordingly, the brain samples always experience a global hypoxia or ischemia depending on the time-delay for autopsy. Nonetheless, most of these limitations will be common to any similar studies done on human autopsied brain due to logistics, technical or availability related constraints.²²⁰ Hopefully, quality of post-mortem human brain based on mRNA stability and pH has been found to be at least at par with surgical biopsy tissue having a freezing delay of maximum 1 h.²⁵⁶ This indicates that the variability in PMI may not be a critically limiting factor. In addition, the strategy of multiple comparisons (location-matched, unmatched or internal validation with random test or control) should help to eliminate some of the background noise or patient-specific artifacts. This is reflected by clustering of the three pairs of location-matched conditions from the random control and by the consistent trends of certain regulations found with the *in vitro* or *in vivo* models. I assume that the knowledge generated from the current study will complement the small repertoire of proteomic studies involving clinical ischemic brain specimens and stimulate further interest in this emerging area. Hence, keeping in mind the limited sample size, similar studies are needed using larger cohort of patients to confirm the regulation pattern of the enlisted proteins found in these Japanese patients and to extend the general applicability of the understanding gained from the current study.

4.6 CONCLUDING REMARKS

My study provides the first demonstration of a comprehensive iTRAQ-2D-LC-MS/MS based quantitative proteomic profiling of autopsied human brain samples from the patients of chronic IS. The flexibility of this multiplex strategy to compare individual samples in different combinations allowed for screening of the data set to generate the perturbed proteome with a minimum of sample-specific artifacts. Failure of mitochondrial energy metabolism is apparent through the deregulation of MAS, PDC complex or enzymes of oxidative phosphorylation that is characteristic of a chronic pathology. Deregulation of GABA metabolism also emphasizes the failure of Glutamate homeostasis. The uniform pattern of differential regulation for majority of the proteome between various locations of human brain is striking and emphasizes the need for similar studies to disentangle the intricate pathology of human IS. In addition, consistent pattern of some proteins or pathways with cell line or animal models indicates the utility of the translational design of the project to fish out generic molecular signatures for detailed investigation. In conclusion, this study will provide a new insight in the understanding of stroke pathophysiology while the data set will act as a reference for future studies.

5. Quantitative Proteomics for the Discovery of Potential Diagnostic Biomarkers of Lacunar Infarction

5.1 INTRODUCTION

LACI is one of the subtypes of IS that accounts for approximately a quarter of all IS cases.¹⁰ It is defined as a small cerebral infarct with 3-20 mm in diameter resulting from the occlusion of a single perforating artery lying in the deep white or grey matter or pons and is associated with specific neurological syndromes.¹⁰

Current stroke guidelines do not differentiate between lacunar and nonlacunar strokes (e.g. large vessel stroke or cardioembolic) with respect to treatment, risk factor modification or long-term outcome.^{10, 257} Similarly, many of the major secondary stroke prevention trials have not distinguished between the different types of IS, which is important for determining the differential protective influence of various therapeutic approaches (e.g., antiplatelet drugs or thrombolysis) depending on the incidental stroke subtypes.²⁵⁸ However, mounting evidence indicates at the difference of the underlying LACI pathology in comparison with the non-lacunar strokes. Taken together, LACI remains a poorly understood area among acute IS whose etiology, pathophysiology, and more importantly, the diagnosis and treatment largely remains in the shadow of the non-lacunar subtypes despite its relatively higher prevalence among the Asian population.^{6, 259}

Lack of well-defined diagnostic strategies and risk-factor based classifications are some of the major reasons for the above-mentioned shortcomings.¹⁰ Routinely used brain imaging techniques are often not sufficient to produce high resolution images for patients with LACI affecting small arterioles (0.2 – 0.8 mm) and having smaller sized infarcts than the larger territorial infarct seen in non-lacunar strokes. CT has a low sensitivity to detect small infarcts in certain locations (posterior fossa, cortex) and in the acute stage and cannot differentiate between acute and old lesions.^{36, 260} The diagnostic dilemma causes misclassification of approximately 20% of lacunar stroke as cortical stroke or vice-versa.¹⁰ In addition, there are situations where MRI is contraindicated, such as in medically unstable patients or those with an MRI-incompatible implanted device, body metal, or severe claustrophobia.²⁶¹ Hence, an unmet need exists to complement and improve the effectiveness of the existing symptomatic and diagnostic protocols for LACI in the acute settings.

The short-term prognosis of ischemic cerebral small-vessel disease, including LACI is more favorable, with almost negligible early mortality, absence of neuropsychological impairment and excellent neurological recovery. However, LACI causes an increase in the mid- or long-term risk of recurrent vascular disorder and of different types of vascular dementia or neuropsychological abnormalities. It has been shown recently that the proportion of dementia caused by small-vessel disease ranges from 36 to 67%.¹¹ Therefore, apart from early diagnosis of LACI, identifying the patient cohorts that are at mid- or long-term risk for recurrent vascular events or secondary complications such as vascular dementia or neuropsychological abnormalities will individualize treatment and recovery paradigms.

Considering the research gaps stated above, I hypothesize that blood-based biomarkers can serve as an alternative tool to complement and improve the effectiveness of the existing protocols that relied on clinical features and neuroimaging. Conversely, the biomarkers can also be used as surrogate markers in LACI related clinical trials to monitor the consequences of therapeutic interventions as suggested in the recent STAIR recommendations.³² The detailed profiling of the plasma samples of LACI patients will facilitate a better understanding of the underlying pathology and stimulate research interest on individual candidates because it is impossible to acquire comparable data set from autopsied LACI affected brain samples.

Biomarker for diagnosis or prognosis of IS is a relatively new concept compared to other therapeutic areas such as cancer. Blood, CSF²⁶² or brain extracellular fluid²²² has been used as starting materials for biomarker discovery in stroke. Although several studies had been performed to validate protein biomarkers from blood,^{94, 95, 261} only a few of them were directed specifically to SVD.²⁶³⁻²⁶⁶ In addition, most of them tried to validate one or a few candidates⁹³ and although suggested,⁹⁵ a shotgun proteomics guided biomarker discovery approach has never been utilized to discover a panel of candidate biomarkers. Finally, no single or panel of plasma biomarkers has been validated by large clinical trials for acute IS. This unbiased systematic approach is superior to the traditional reductionist approach of biased selection and validation of a single or few proteins.

Accordingly, I propose to apply iTRAQ-2D-LC-MS/MS strategy for the discovery of potential plasma biomarker of LACI by quantitative profiling of groups of LACI patients

and healthy age-matched controls. The LACI patients were monitored for up to 5 yrs to confirm no recurrence of any vascular event or no decline in cognitive functions during this period before being enrolled for this study. Plasma samples of 36 subjects were divided in two groups (Control, 17 patients; LACI, 19 patients) and pooled plasma from each group was divided in three sub-fractions by differential centrifugation for iTRAQ experiments. Comparison of three iTRAQ data sets was used for internal validation to filter the significantly regulated proteins that show identical trends in all data sets. Given that blood collection is a simple and cheap procedure; these candidates once validated in larger cohort of LACI patients may have additive value over the existing imaging, clinical or neurobehavioral modalities used in the clinic.

5.2 MATERIALS AND METHODS

5.2.1 Reagents

Unless indicated, all reagents were purchased from Sigma-Aldrich (WI).

5.2.2 Sample Collection

The plasma was obtained from the LACI patients with recent TIAs or nondisabling IS who were treated in the Singapore General Hospital between 1999 and 2005 for the cognitive sub-study of European Australasian Stroke Prevention in Reversible Ischemia Trial (ESPRIT-cog). Detailed methodology of ESPRIT and ESPRIT-cog has been reported previously.^{267, 268} Briefly, for ESPRIT, patients were eligible if they were within 6 months of a TIA (including transient monocular blindness) or a nondisabling IS (grade ≤ 3 on the modified Rankin scale [mRS])²⁶⁹ of presumed arterial origin. The exclusion criteria were: a possible cardiac source of embolism, high-grade carotid stenosis for which carotid endarterectomy or endovascular treatment was planned, moderate to severe leukoaraiosis on brain imaging (for randomization into anticoagulation), any blood coagulation disorder, any contraindication for aspirin or dipyridamole, and a limited life expectancy. The additional exclusion criteria for ESPRIT-cog are confusion, severe expressive or receptive aphasia, major psychosis diagnosed according to the Diagnostic and Statistical Manual of Mental Disorders, 4th Edition criteria,²⁷⁰ or dominant upper-limb paralysis. In the ESPRIT and ESPRIT-cog trial all patients were randomized to

either aspirin (100 mg/day) or aspirin combined with dipyridamole (75-450 mg/day). The control plasma was collected at the same site during 2004-2006. EDTA was used as the anti-coagulant for both groups.

5.2.3 Standard Protocol Approvals, Registrations, and Patient Consents

The study protocol was approved by Singapore General Hospital's and Nanyang Technological University's Institutional Review Board and Ethics Committee. Written informed consent was obtained from all patients or legal guardians. The ESPRIT trial was registered under <http://clinicaltrials.gov> with the identifier NCT00161070.

5.2.4 Neuropsychological Test Battery

The cognitive status of the patients was determined using standard neuropsychological test battery that has been validated for use in Singapore. Details of the procedure has been described previously.²⁶⁸ The LACI patients were classified in three groups namely, cognitive impairment no dementia (CIND) –mild, CIND –moderate and no cognitive impairment (NCI) as per baseline cognitive assessment.

5.2.5 Outcome Measures

The LACI patients were followed up annually for up to 5 years. During this period, they underwent full neuropsychological assessment and monitored for occurrence of any vascular event. Strokes, peripheral artery disease, intracranial bleeds, and any cardiac ischemia (stable and unstable angina, myocardial infarctions) or deaths from any of the above were considered to be a recurrent vascular event. Patients who did not suffer a recurrence or cognitive decline during this period were selected for the current study and were named as 'LACI, no adverse outcome'.

5.2.6 Sample Preparation

5.2.6.1 Separation of Plasma Membrane Derived Vesicles (PMV) and Exosome by Differential Centrifugation

Frozen individual plasma samples were thawed on ice and pooled in a group-wise manner to obtain two tubes containing about 5 ml of LACI and healthy plasma specimens. The

samples were subjected to differential centrifugation to isolate the plasma membrane derived vesicles and exosomes using a modified protocol as described previously.²⁷¹ Briefly, sonicated plasma (5 X 1 min) was centrifuged at 4000g twice for 30 min to collect the pellets (P1). The supernatant for each sample was centrifuged at 12 000g for 30 min to collect another pellet (P2). The resulting supernatant was subsequently diluted approx. five times with ice-cold 1X PBS before doing ultra-centrifugation at 30 000g for 2 h to collect the pellet of PMVs (P3). The supernatant was finally ultra-centrifuged again at 200 000g for 2 h 15 min to collect the exosome pellet (P4) (Figure 5.2). The supernatant was stored at -20 °C. All pellets were washed at least twice with 1X PBS at respective centrifugal force. For PMV and exosome, the final wash was performed in smaller tubes (5.2 ml, SW 55 Ti rotor) for ease of collection. The supernatants after washing were thrown. All pellets (P1-4) were lyophilized and kept at -20 °C until protein extraction.

5.2.6.2 Protein Extraction and Quantification

The lyophilisate was dissolved at 4 °C using 50-100 µl of ice-cold dissolution buffer [6% sodium dodecyl sulfate; 20 mM DTT, 100 mM tris-HCl with Complete Protease Inhibitor Cocktail (COMPLETE, Roche), pH 7.75] by brief vortexing. Sequential heating and vortexing was used for P1 and P2, initially at 60 °C for 10 min, followed by at 95 °C for 3-5 min to dissolve most of the visible lumps. P1 and P2 were centrifuged at 20 600g to collect the supernatant for protein content estimation. Protein quantification was performed using 2-D Quant kit for all pellets. At least 100 µg proteins are needed per condition in an iTRAQ experiment. Thus, P2 and P3 were mixed together to collate sufficient amount of protein for iTRAQ labeling as individually the protein yield in P2 and P3 was less than 100 µg. Finally P1, P2+P3 (hereon will be referred as P2-3), P4 from two samples were taken for three parallel iTRAQ experiments.

5.2.7 Proteomics and Bioinformatics

5.2.7.1 In-Gel Tryptic Digestion and Isobaric Labeling

The samples (500 µg / condition for P1 and P4 and 100 µg/ condition for P2-3) were subjected to denaturing PAGE using a 4% - 6% - 25% gel following an identical procedure as described in the chapter 4.²⁰⁸ Briefly, after overnight in-gel digestion and

extraction of peptides, the dried peptides were reconstituted into 0.5 M TEAB and labeled with respective isobaric tags of 4-plex iTRAQ Reagent Multi-Plex kit (Applied Biosystems) as follows: Control, 114; LACI, 115 (Figure 5.1). Two labels of three separate 4-plex iTRAQ kits were used for labeling P1, P2-3 and P4 pellet of control and LACI group.

5.2.7.2 ERLIC and Reverse Phase LC-MS/MS Analysis

Parameters for off-line ERLIC and online reverse phase LC were identical with the procedure used for the fractionation of ischemic human brain samples (Refer to chapter 4). Most of the MS parameters were also kept similar except minor modifications. The maximum accumulation time was set at 1.0 s instead of 2.0 s that was used previously.²⁰⁸

5.2.7.3 Mass Spectrometric Raw Data and Bioinformatics Analysis

The spectral data acquisition and database searching parameters using ProteinPilot software were identical as mentioned previously (Section 3.2.5.7).²⁰⁸ The bioinformatics analysis was performed using different attributes such as GO, pathway, protein interaction, keywords or protein domains using DAVID to extract out hidden trends and enrichment of certain groups of proteins. Details of the procedure can be found in chapter 4.

5.2.8 Statistical Analysis

GM instead of arithmetic mean was calculated for the iTRAQ ratios (115/114 or test/control) of the same protein detected in at least two of the three experiments. Percent co-efficient of variation (%CV) was used to determine the reproducibility or precision among iTRAQ experiments.

Figure 5.1 Schematic representation of the experimental design.

5.3 RESULTS

A total of 20 LACI patients who were recruited for ESPRIT-cog and had been prospectively followed for up to 5 yrs, participated in the current study. One patient was excluded due to detectable hemolysis of the plasma sample. Plasma of seventeen healthy

age-matched controls that never had a stroke or cancer and were free from cognitive problems was included. Most of the (35 out of 37) participants were Chinese. The demographic characteristics of the study population stratified by single LACI and control are summarized in Table 1. The incidence of CIND (mild or moderate) was observed in 7 out of 19 LACI patients (37%) during baseline cognitive classification, as majority (68%) did not have any cognitive impairment at the initiation of the study. None of them have previous peripheral artery disease or stroke. Older and male patients were more likely have LACI. The median delay for blood draws was slightly more than a month (32.5 days) from the occurrence of single LACI event.

Table 5.1 Demographic Characteristics of the Patient Population Stratified by Difference in Adverse Outcome

Characteristic	Healthy control	LACI, No adverse outcome
N (%)	(N = 17)	(N = 19)
Age, Mean (SD)	56 (9)	61 (9)
Sex, Male	4 (26)	17 (90)
Diabetes mellitus	6 (35)	7 (37)
Hypertension	10 (59)	11 (58)
Previous stroke	None	0 (0)
Hyperlipidemia	10 (59)	8 (42)
Ever smoker	1 (6)	5 (26)
Previous ischemic heart disease	3 (18)	2 (11)
Previous myocardial infarction		0 (0)
Previous angina		2 (11)
Previous peripheral artery disease	None	0 (0)
Baseline cognitive classification		
NCI	None	13 (68)
CIND-mild		4 (21)
CIND-moderate		3 (16)
Delay to blood draw, Days (Median)	NA	32.5

5.3.1 Proteomics

The proteomics approach for biomarker discovery from crude plasma or serum is limited by its complexity and extreme dynamic range ($>10^{10}$) resulting in poor sensitivity for detecting the low abundant proteins.²⁷² To overcome this challenge, multiple approaches have been described for simplifying the whole plasma that includes biophysical

fractionation, enrichment of target sub-proteome and immunodepletion of the most abundant interfering proteins. However, none of them were able to significantly outclass the other techniques.^{273, 274} Till date, the largest confidently identified plasma proteome contains only 889 proteins that also generated by the multi-laboratory initiative of Human Proteome Organization.²⁷⁵

Figure 5.2 Pictures of a representative exosome pellet (P4) against different backgrounds. The loose orange-reddish hollow at the bottom of the tubes (shown by arrow) was obtained by ultracentrifugation of PMV depleted and PBS diluted plasma at 200 000g for 2 h 15 min.

Here, an alternative approach of isolation of microvesicles by differential centrifugation was coupled with isobaric labeling and MudPIT to improve the depth of the identification and quantification of the plasma proteome. Microvesicles are membrane-bound vesicles, ranging in size from 0.04-1 μ m and secreted from endothelial cells, platelets, leukocytes and erythrocytes.²⁷⁶ These include PMV and exosome. Having a varied but defined morphology, I speculated that isolating the microvesicles will help to reduce the interference of high abundant plasma proteins during the bottom-up quantitative profiling. Recently, they are increasingly being recognized as reservoirs of potential biomarkers. The involvement in cell-to-cell communication (short or long distance paracrine signaling), antigen presentation and in the pathogenesis of thrombosis, diabetes, inflammation, atherosclerosis and vascular cell proliferation inspired myself to use these enigmatic entity for the current study.^{277, 278}

Hence, I isolated the microvesicles (i.e. PMV and exosome) by differential ultracentrifugation for independent iTRAQ experiments (Expt. 2 and Expt. 3 in Figure 5.1 and Figure 5.2). However, due to insufficient amount of proteins from PMV (P3), P2 was combined with P3 for the iTRAQ experiment (Expt. 2 in Figure 5.1). In addition, the low speed fractions (P1) from both samples were also quantified by another independent iTRAQ experiment (Expt. 1 in Figure 5.1). Thus, each of the two pooled groups (LACI and control) of plasma specimen was compared by three independent and parallel iTRAQ experiments (Expt. 1: P1, Expt. 2: P2-3 and Expt. 3: P4) (Figure 5.1). Of note, each of the

three experiments has three MS replicates indicating that nine independent run have been collated to generate the final data.

5.3.2 Quality Control and Filtering of iTRAQ Data Set

Similar quality control parameters for confident identification ($\text{FDR} \leq 1\%$ and unused prot score >2.0) were used like previous Proteinpilot 3.0 software-generated data sets.¹⁶² The three data sets (Expt. 1, 2 and 3) identified 407, 741 and 288 proteins respectively having an unused score more than 2.0. All of them had an unacceptably high FDR ($>10\%$) unlike the previous studies in chapter 2-4. This is likely due to the presence of the reverse sequence of plasma proteins in the decoy database some of which are identical to normal peptides from a small set of other proteins. Similar observation is however been reported by a recent study dealing with shotgun proteomics of plasma samples.²⁷⁹ Increasing the unused score cut-off to 4.0 improved the FDR to $<3\%$ for the resulting data sets, which is reported as an acceptable filtering criteria in the proteomic study of plasma samples.²⁷⁹ Accordingly, the final data set contained 227, 341 and 161 proteins from Expt. 1, Expt. 2 and Expt. 3 respectively having a %FDR of 2.6, 1.8 and 1.2. This result is either comparable²⁸⁰ or even better^{273, 279} than many published reports in the recent past. Moreover, all these studies address technical issues only and none of them tried to quantify the proteome. The current approach not only provided quantitative information of these proteins but also used simpler protocol to achieve this.

In total, 483 unique proteins were identified after combining the three iTRAQ data sets (Figure 3). Some of the proteins were commonly identified in a couple of iTRAQ experiments, whereas 70 proteins (14.5%) were detected in each of the pellets. However, majority (307 out of 483 or 64%) of them was unique and exclusively identified in only one of the pellets (78, 177 and 52 proteins from Expt. 1, 2 and 3 respectively). Thus, differential centrifugation could be a simple and fast alternative to separate plasma proteins that improved the depth of overall proteome coverage in the current study. Of note, this separation is not absolute rather relative, because the unidentified proteins might be present in a quantity that is below the lowest detection limit of the mass spectrometer. The maximum number of protein counts as well as the highest percentage of unique proteins (177 out of 341 or 51.9%) in Expt. 2 could be related to the combination of two pellets (P2 and P3) used for this iTRAQ experiment.

Figure 5.3 Comparison of protein counts from the three iTRAQ experiments. 227, 341 and 161 proteins were identified (FDR <3%, unused prot score ≥ 4.0) from Expt. 1, 2 and 3 respectively. A total of 483 unique proteins were detected by combining the three data sets.

Next, traditionally used cut-off of p -value <0.05 was used for filtering the proteins with significant ratios from each experiment. There were 98 proteins for which ratio (i.e. LACI/control) with acceptable p -value have been found in at least one of the three iTRAQ experiments. Based on the statistical evaluation of ProteinPilot software, these values were considered as real and not due to random variation. Individually, Expt. 1, 2 and 3 had 31, 83 and 20 ratios with acceptable p -value having overlap in between experiments. A protein that has been detected in more than one experiment with an acceptable ratio offers an opportunity to cross-verify the regulation. After excluding three isoforms of keratins, 25 proteins were identified that can be subjected to an internal validation. The significant ratios were compared across the experiments for estimating the precision of quantification. GM and %CV were calculated for each of these proteins having at least two significant ratios. The median %CV of the distribution was 17.6 (range of %CV 0.7 – 84.5), while it was 31.4 when all 98 proteins were taken into account. A recent study reported similar run-to-run variability (i.e. %CV = 30.9) in a label-free approach where immunodepletion has been used on plasma samples.²⁸⁰ Another report also showed variation between replicates in the range of 11-22% when multiple MS runs were compared.²⁷⁴ In the current study, starting samples are different as they represent different pellets generated from the pooled plasma. That could have contributed to a wider range of %CV and higher variability. Ideally, PMV and exosomes

being distinct and independent entity derived from the pooled plasma, these pellets are not experimental replicates. Thus it may not be surprising to observe different regulation of the same protein in individual pellets for LACI group compared to normal plasma. This on the other hand shows that a protein in a particular plasma sample does not follow an identical pattern under specific centrifugal force and fractionates in a sample-specific manner even when the samples are processed at the same time. The preferential partition-mediated depletion or enrichment of certain proteins in pellet or microvesicles'-specific manner should be due to biological reasons as suggested recently.²⁸¹ The differences in the presence of interacting proteins or other non-protein entities could also account for this behavior. Increase in sample handling steps may affect their stability and can introduce artifacts. Hence, this exposes the inextricable and contradictory objectives of proteomics based biomarker discovery where increase in the depth of coverage is needed with minimum of sample handling to avoid quantification artifacts.

Most of the 25 candidates were consistent among the three iTRAQ experiments when the trends of expression rather than the magnitudes were considered. Seven out of 25 showed upregulation in LACI group whereas 16 proteins were downregulated. Only two (A2MG and VWF) showed significant yet opposite regulation among different pellets –a situation discussed in the preceding paragraph. A2MG or Alpha-2-macroglobulin is an inhibitor of coagulation, whereas VWF or von Willebrand factor is a platelet membrane glycoprotein facilitating coagulation. It should be remembered that iTRAQ is a good tool to generate a comprehensive overview of the trends of regulation from complex biological specimens, but may not be equally reliable when it comes about precisely estimating the magnitudes. In fact, iTRAQ tends to underestimate the regulation as the ratios are shrunk towards 1 and the error increases when fold changes cross 2 orders of magnitude.^{282, 283} The major objective of the study is to discover novel signature of plasma proteins in the LACI group that could be validated on priority basis using larger cohorts of individual patients rather than targeting the known candidates for validation. Hence, these shortlisted proteins from the three parallel iTRAQ experiments as well as other potential candidates from the complete list of 483 proteins will provide the right initiative for future studies. In the next section, the selected 23 and the complete list of 483 proteins were considered for bioinformatics analysis to retrieve useful biological trends.

5.3.3 Bioinformatics Analysis of perturbed plasma proteome from LACI group by DAVID

DAVID uses modular enrichment analysis where the term-term/gene-gene relationships are considered for enrichment p -value calculation. It calculates the probability of the number of genes in the list that hit a given biology class as compared to pure random chance. It can be performed with the aid of Fisher's exact test.²⁸⁴ To check the enrichment, p -value ≤ 0.05 and FDR $< 1\%$ was used as an initial cut-off. It should be mentioned that even though annotation terms may be associated with significant enrichment p -values, the final selection is purely based on a priori biological knowledge of the pathology of interest. It is not uncommon to ignore specific group of proteins even with extremely good p -value. The enriched list of annotations only facilitates the rational exploration. Uniprot accession numbers of the filtered and the complete list of proteins was uploaded in DAVID to compare them separately with the human proteome containing 19235 protein entries as background. 127 proteins from the list of 483 proteins remained unmapped. These are orphan terms or genes and lack strong relationship with the neighboring genes or terms.

Blood is the only body fluid that is so intimately connected to all the organs and is generally considered as a window to the physical condition of the patient. Brain being one of the most perfused organs in the body is expected to leave a signature following any pathological condition that affects its normal functioning. The chances are higher for IS or LACI per se, which originates in the brain microvasculature itself. Alteration of BBB permeability or endothelial dysfunction is known to be involved in the pathology LACI or IS as a whole.²⁸⁵ Thus, the primary curiosity was to identify the brain-specific candidates from the whole data set and to search for the enrichment of such categories by bioinformatics analysis. Having a brain-specific origin will also have higher chance to be able to sense the progression of the disease and subsequently the outcome, an attribute necessary for higher accuracy and specificity of the candidate biomarker.

Hence, the tissue specificity and enrichment of each category were checked using the 'UP_TISSUE' module. The 483 proteins were assigned to at least 27 different tissues or body fluids that include major organs like liver, lung, heart, bile or even saliva. As expected, 'plasma' and 'platelet' secured the top two slots, whereas liver has maximum

representation with 131 proteins. Interestingly, out of the top five enriched categories, 'Cajal-Retzius cell' and 'Fetal brain cortex' were also featured with 43 and 39 candidates having an overlap in between them. Cajal-Retzius cell is reelin-producing neurons of the human embryonic marginal zone. Similarly, using 'GOTERM_CC_FAT' module where GO analysis based on cellular component was performed, few of the proteins were annotated to 'axon' and 'axon part'. Searching for individual proteins found candidates of potential interest such as DBN1 (Drebrin), MBP (Myelin basic protein) or SPTBN4 (Spectrin, non-erythroid beta chain 3). Drebrin is known as Developmentally-regulated brain protein and might play a role in the cell migration, extension of the neuronal processes and in the plasticity of dendrites. MBP isoforms are mainly expressed in both the central and the peripheral nervous system, with strong immunoreactivity in nerve fibers and in nucleus of glial cells. SPTBN4 is abundantly expressed in brain and also in pancreatic islets. It participates in cytoskeletal anchoring at plasma membrane and in vesicle-mediated transport process. Unfortunately, none of them have confident quantification information (i.e. p -value >0.05). The GO based classification points towards enrichment of categories that is related to 'platelet', 'immune system', 'coagulation' or 'complement pathway' among others. There are other interesting categories related to adhesion (cell, biological, focal or leukocyte), vesicles or granules (membrane-bounded, cytoplasmic or secretory) and regulation of apoptosis, dendrite development, cell death or cellular response to reactive oxygen species. Searching for enriched pathways according to KEGG or BIOCARTA showed 'Focal adhesion', 'ECM-receptor interaction' and 'Rho cell motility signaling pathway' as significantly over-represented. Increased endothelial cell adhesiveness and recruitment of leukocyte and procoagulant exosomes to the area of thrombosis are known to amplify thrombogenesis via tissue factor and the extrinsic coagulation pathway.²⁸⁶ Considering major part of the data is being generated from plasma microvesicles that are mostly derived from activated endothelial cells, leukocytes and platelets having proteins related to their respective cell of origin, this is an expected finding.

Next, the same enrichment analysis was repeated with the filtered list of proteins using the same background of whole human proteome. The GO or pathway analysis produced similar results with emphasis on adhesion or coagulation. REACTOME_PATHWAY retrieved 'Integrin cell surface interactions' as one of the enriched pathway. However, regarding enrichment of proteins from specific tissue using 'UP_TISSUE' module, none

of the CNS-related matches were enriched. The small size of the data set (only 23 proteins) could be responsible for this.

Many low abundant proteins typically not detectable were also identified that includes CD36, CD81, CD44 and many kinases (e.g. ILK, B4DRT3, SKAP2, and ROCK2). Thus, the current differential centrifugation approach has been able to identify the part of the proteome that generally remains inaccessible in most of the proteomic studies. The filtered data set was finally classified manually guided by the above bioinformatics analysis.

5.4 DISCUSSION

No approved biomarker exists for stroke as a whole. LACI being relatively chronic in nature compared to non-lacunar IS and due to its lower case fatality, has longer and realistic window of opportunity for diagnosis and prognosis. The diagnosis of LACI is critically dependent on the clinical symptoms, risk factors and the imaging techniques, which is often not sufficient to detect these cases. None of the approaches are quantitative in nature and may vary considerably among clinicians depending on their experience. Here, I report significantly altered plasma proteome from a group of LACI patients by comparing them with healthy age-matched group of control patients in an effort to discover candidate biomarkers.

5.4.1 Downregulation of Integrin Signaling – Anti-platelet Effect of Aspirin

Several candidates from the integrin signaling pathway (e.g. ITGA2B, URP2 or FERMT3, FLNA and TLN1) are down-regulated in the LACI patients. ITGA2B or Integrin alpha-IIb/beta-3 or CD41 is a platelet membrane glycoprotein and receptor for diverse ligands including fibronectin, fibrinogen, plasminogen, prothrombin, and thrombospondin. It is a heterodimeric protein having α and β subunits that couple extracellular ligands to the cytoskeleton and intracellular signaling networks through various adaptors or signaling proteins. Activated ITGA2B mediates platelet spreading and aggregation on vascular surfaces during hemostasis and thrombosis. URP2 or KIND3 belongs to a family of intracellular proteins, expressed by various endothelial cells that link integrin signaling to

the actin cytoskeleton. The C-terminal FERM domain of URP2 are most closely related to the FERM domain of TLN1 (talin), which is also involved in the regulation of integrin signaling.²⁸⁷ However, talin and kindlins bind to unique sites on the cytoplasmic tail of β integrin. It has been shown that TLN1 can independently activate integrin.²⁸⁸ Filamin (FLNA) on the other hand can compete with TLN1 for binding to integrins, thereby blocking its activation under certain circumstances.²⁸⁹ In a recent study, involvement of TLN1-dependent activation of ITGA2B or Rac1 in platelets has been demonstrated for late phase stability of thrombus on undisturbed endothelial cells.²⁹⁰ Thus overall suppression of integrin signaling is complementary to the downregulation of fibronectin, fibrinogen or Thrombospondin 1 variant which are ligands for ITGA2B. Down-regulated GPIBA is another platelet surface membrane glycoprotein that functions as a receptor for VWF. It also mediates platelet adhesion to vascular subendothelium after vascular injury, and initiates signaling events within the platelet that lead to enhanced platelet activation, thrombosis, and hemostasis.

Considering the fact that all LACI patients were on aspirin and/or dipyridamole, downregulation of the above-mentioned platelet proteins and more specifically suppression of integrin signaling pathway in platelet is expected to produce the desired anti-thrombotic effect. Noteworthy, apart from blocking the classical arachidonic acid pathway by irreversibly inhibiting cyclooxygenase-1 enzyme, aspirin is also reported to partially inhibit the inside-out ITGA2B signaling.²⁹¹ Platelet being a non-nucleated cell, the synthesis of downstream proaggregatory thromboxane A₂ (TXA₂) or integrin-mediated focal adhesion will not recover until the replacement of the affected cohort of platelet by fresh ones – a process that may take 7-10 days. The production of protacyclin (PGI₂) by vascular endothelium will be affected to a much lesser extent. This shifts the balance between PGI₂ and TXA₂ in the circulation and is responsible for the anticoagulant action. Thus, my study provides first demonstration that aspirin and/or dipyridamole induced down-regulation of several mediators in the plasma microvesicles related to focal adhesion or coagulation pathway may be partly responsible for a better outcome in patients with non-disabling stroke or TIA.

5.4.2 Upregulation of Erythrocytic Proteins – Presence of a Hemolytic Response

The presence of few erythrocytic proteins (ANK1, SPTA1, Q59FP5, and B3AT) in the plasma is unexpected. ANK1 or ankyrin-1 is membrane protein found in different cell types with most abundant expression in erythrocytes. SPTA1 or Spectrin is the major cytoskeletal protein that associates with band 4.1 and actin to form the cytoskeletal superstructure of the erythrocyte plasma membrane. B3AT or band 3 anion transport protein is the major integral glycoprotein of the erythrocyte membrane. Their combined regulation could indicate a hemolytic condition resulting from handling artifacts. But when observed carefully, it was found that the relative expression is consistently higher in LACI group only, pointing towards an underlying pathological link. Incidentally, this group of proteins were depleted in exosome (P4 or Expt. 3) and enriched in P2-3. Searching the complete data sets did show the presence of hemoglobin in all the experiments with higher expression in LACI group (GM = 1.95, %CV = 20.2). However, it did not appear in the final list as it failed to qualify through the *p*-value cut-off. Literature mining revealed that serum free hemoglobin has been suggested as a potential biomarker for acute IS. In that study, no correlation was found between the presence of hemoglobin and the subtypes of IS according to TOAST classification. However, the study did not provide any subtype specific information although it recruited 47 patients within 3 days from the onset of stroke.¹⁹³ Here, the median delay for blood draw is more than one month (32.5 days) that could have contributed to the normalization of the hemolytic condition partially. The data generated from the animal model in chapter 3 also indicated at prevailing hemolytic condition in the circulation possibly due to localized dysfunction of endothelium and free radical stress resulting in hemolysis. Increased presence of hemoglobin in the perfused ischemic hemisphere of rat was observed during the first 24 h of reperfusion injury following 2 h of focal cerebral ischemia.²⁰⁸ Given that MACO model better mimics large artery stroke rather than LACI and absence of specific correlation in between hemoglobin with particular subtypes of stroke, it appears that a hemolytic signature is a complementary rather than a decisive trend to predict LACI.

Table 5.2 The List of Qualified and Regulated Plasma Proteins from LACI patients^a

Name	Uniprot accession	Gene symbol	Unused prot score			% Coverage			Peptides(95%)			Expt. 1, P1	Expt. 2, P2-3	Expt. 4, P4		GM (115:114)	STD	%CV		
			Ex1, P1	Ex2, P2-3	Ex4, P4	Ex1, P1	Ex2, P2-3	Ex4, P4	Ex1, P1	Ex2, P2-3	Ex4, P4	115:114	PVal 115:114	115:114	PVal 115:114					
Coagulation cascade																				
Coagulation factor XIII A chain Fibrinogen beta chain Platelet glycoprotein Ib alpha chain	P00488	F13A1	40.3	18.5	10.4	27.1	14.5	11.2	26	13	6	0.53	0.01	0.52	0.01	1.05X	0.86	0.53	0.00	0.7
	P02675	FGB	425.2	110.8	124.2	89.4	81.9	78.0	1241	114	209	0.17X	0.54	0.64	0.03	0.29	0.00	0.43	0.25	56.8
	P07359	GP1BA	19.2	23.0	0.5	14.2	15.3	0.0	9	10	0	0.64	0.02	0.46	0.00	0.86X	0.81	0.54	0.12	22.9
Focal adhesion / integrin signaling																				
Integrin alpha-Ib	P08514	ITGA2B	57.2	74.8	13.7	29.1	35.2	8.0	49	72	6	0.64	0.01	0.63	0.00	1.12X	0.85	0.63	0.01	2.0
Fermitin family homolog 3	Q86UX7	FERMT3	13.3	41.9	NA	15.0	41.1	NA	7	23	NA	0.33	0.01	0.54	0.00	NA	NA	0.42	0.16	36.9
Talin-1	Q9Y490	TLN1	155.5	270.8	38.6	44.4	64.7	11.0	127	258	19	0.65	0.00	0.89	0.00	0.65X	0.11	0.76	0.17	22.2
Filamin-A	P21333	FLNA	172.8	302.4	39.8	42.8	61.9	11.1	125	240	21	0.84	0.01	0.88	0.00	0.88X	0.42	0.86	0.03	3.3
Fibronectin	P02751	FN1	760.0	102.5	217.4	72.1	28.5	52.9	1489	67	229	0.22	0.00	0.45	0.00	NA	NA	0.31	0.17	52.6
Thrombospondin 1 variant (Fragment)	Q59E99	NA	41.6	71.4	18.2	19.7	29.5	8.7	24	50	8	0.80X	0.35	0.59	0.00	0.55	0.01	0.57	0.02	3.9
Galectin-3-binding protein	Q08380	LGALS3BP	NA	34.6	41.9	NA	34.4	34.5	NA	23	44	NA	NA	0.39	0.00	0.30	0.00	0.34	0.06	17.6
Immune system																				
CD5 antigen-like	O43866	CD5L	10.1	23.0	35.2	12.7	36.6	47.3	3	14	28	0.87X	0.86	0.56	0.01	0.46	0.00	0.51	0.07	14.4
Complement C3	P01024	C3	192.2	199.2	257.0	55.8	56.2	68.8	150	165	291	0.73X	0.18	1.91	0.00	1.54	0.00	1.71	0.26	15.0

Erythrocyte proteins																				
Band 3 anion transport protein	P02730	SLC4A1	37.8	93.7	2.1	27.2	41.8	1.4	34	98	1	1.24	0.03	1.96	0.00	0.82X	0.70	1.56	0.51	32.9
	P16157	ANK1	54.1	141.5	0.2	22.8	45.8	0.0	26	103	0	1.47	0.03	1.84	0.00	NA	NA	1.64	0.26	15.7
	P02549	SPTA1	50.1	228.5	0.2	13.3	56.3	0.0	23	137	0	1.94	0.00	1.51	0.00	NA	NA	1.71	0.30	17.6
	erythrocyte Spectrin, beta, erythrocytic (Includes spherocytosis, clinical type I)	Q59FP5	NA	67.8	201.2	2.0	19.0	49.8	0.4	35	127	1	1.66	0.00	1.57	0.00	1.02X	0.96	1.61	0.06
Other proteins																				
Tubulin beta-1 chain	Q9H4B7	TUBB1	38.6	30.9	4.8	56.8	60.5	5.5	30	33	3	0.42	0.00	0.37	0.00	0.56X	0.23	0.40	0.04	9.1
Inter-alpha (Globulin) inhibitor H4 (Plasma Kallikrein-sensitive glycoprotein)	B2RMS9	ITI1H4	NA	36.1	24.0	NA	22.5	15.0	NA	17	14	NA	NA	1.92	0.01	1.69	0.03	1.80	0.16	9.1
Vinculin	P18206	VCL	14.1	73.1	NA	6.6	37.0	NA	6	45	NA	0.36	0.02	0.41	0.00	NA	NA	0.38	0.04	10.4
Clathrin heavy chain 1	Q00610	CLTC	20.5	40.2	0.5	8.2	15.5	0.0	11	20	0	0.45	0.01	0.58	0.00	0.80X	0.64	0.51	0.09	18.3
Histone H4	Q0VAS5	HIST1H4H	14.4	6.9	NA	49.5	26.2	NA	11	3	NA	0.38	0.00	0.17	0.02	NA	NA	0.25	0.15	59.6
Lipoprotein, Lp(A)	Q1HP67	LPA	59.1	46.7	21.3	36.7	23.0	14.7	48	35	14	1.37	0.03	2.25	0.00	2.81	0.02	2.05	0.72	35.3
Non-muscle myosin heavy polypeptide 9	Q60FE2	MYH9	214.7	302.3	41.0	52.8	63.4	14.0	181	333	25	0.53	0.00	0.88	0.00	0.49X	0.10	0.69	0.24	35.5

^aThe list contains quantitative information of the selected proteins from the three (i.e. Expt. 1, Expt. 2, Expt. 3) bias and background corrected iTRAQ data sets. The denominators and numerators are LACI and control samples respectively. Unused score and %coverage are parameters for confident identification. This list contains 23 candidates qualified (out of 483) through the initial filters [i.e., unused prot score >4.0 in at least 2 out of 3 iTRAQ experiments and FDR <3% for each data set (confident <0.05 (significantly different from 1) for at least two ratios)]. The remaining ratios with insignificant *p*-value (>0.05) were indicated with 'X' and not identified for calculating the GM and %CV. The proteins that are not detected in certain experiment are marked with 'NA' in respective columns. Proteins related to coagulation cascade and focal adhesion were downregulated, whereas proteins of erythrocytic origin were upregulated.

5.4.3 Differential Presence of Selected Proteins in Specific Sub-fractions – Utility of Differential centrifugation

The number of detected peptides having a confidence level of 95% or more was compared among the three iTRAQ experiments for each of the filtered list of 23 proteins.

Three proteins (LG3BP, CD5L and C3) were found to be enriched in exosome specifically. LG3BP or Galectin-3-binding protein promotes integrin-mediated cell adhesion. In the plasma samples of deep venous thrombosis, LG3BP was overexpressed in the microvesicles or exosomes as observed by iTRAQ based proteomic analysis.²⁸¹ This protein is crucial for galectin mediated biological processes and also plays a role in cell apoptosis and shedding. Binding of galectin-1 with LG3BP up-regulated P-selectin that may play important role in thrombogenesis.²⁸⁶ CD5L and C3 are components of immune system. Plasma level of C3 was elevated in cryptogenic and LVD and is associated with unfavorable outcome.²⁹² However, my study shows that despite elevated plasma level of C3 in LACI patients, the patients had a positive outcome for at least next 5 yrs. No studies report the downregulation of either LG3BP or CD5L protein in a similar context. However, downregulation of LG3BP may be beneficial by negatively affecting galectin-1 activation or integrin-mediated cell adhesion.

Proteins with higher peptide counts such as FIBB' (fibrinogen beta chain) or FINC (fibronectin) were enriched in low gravity fraction (i.e. P1) indicating their presence in higher density particles in the plasma. Fibronectin is secreted by hepatocytes, whereas cellular fibronectin (c-Fn) is an endothelium derived protein deposited as fibrils in the extracellular matrix. Higher Serum level of c-Fn at baseline is predictive of HT and parenchymal hematoma after treatment with tPA in patients with acute IS.²⁹³ In non-anticoagulated IS patients, higher level of c-Fn was associated with polyvascular atherothrombotic disease with higher rates of vascular recurrence.²⁹⁴ In a separate study, high concentration of plasma c-Fn was associated with the development of malignant MCA infarction when massive MCA infarction was used as control.²⁹⁵ However, none of the reported studies specify any correlation with LACI or SVD. My study shows significant down-regulation of plasma fibronectin in the LACI group

which is not contradictory with the above studies considering there were no adverse vascular events in the studied cohort. In cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) patients, immunohistochemical analysis of the deceased brains did not show major leakage of plasma fibrinogen or fibronectin indicating the intactness of BBB.²⁹⁶ CADASIL is a hereditary small arteriolar disease that may have certain degree of pathologic similarity with LACI.²⁸⁵ Progression of lacunar stroke during acute phase with worsening of neurological deficits has been associated with higher FIBB concentration in plasma that is also predictive of poor outcome after 3 months.²⁹⁷ Thus, decreased level of both of this acute phase parameters (c-Fn and FIBB) is consistent with the better outcome as seen by absence of recurrent vascular event or cognitive deterioration in the studied group of LACI patients.

5.5 LIMITATIONS

The blood was not collected in the acute phase (i.e. within hours) rather it was during the sub-acute or chronic phase of the ischemic injury. The patients were on anti-platelet therapy throughout this variable duration. Both of this can cause normalization of the acute proteomic response in the plasma. Future studies should try to concentrate on early collections or inclusion of patients who did not receive any medication to detect and compare the unbiased response of the brain. Despite the identification of a reasonably good number of proteins, the poor *p*-value led to the exclusion of many interesting proteins (~80%). However, these proteins are amenable to validation by an independent multiple reaction monitoring based targeted proteomic approach. A small fraction (~5%) of proteins having consistent trends among the three pellets has been discussed. Conversely, inconsistent trends among the pellets may itself be a signature of LACI for certain proteins considering their preferential enrichment into microvesicles is a part of the systemic response. However, rigorous validation using complementary techniques or independent biological replicates is needed to prove this hypothesis for such candidates. The small amount of the starting plasma (~ 5 ml) and paucity of the resulting microvesicles did not allow validation in the current set of study population. Hence, fresh plasma from a different group of patients can be used by carefully selecting some of these candidates for extensive validation of the candidate biomarkers. The priority should be in the following order; the top 5% (23

proteins discussed here), then the 15% proteins $[(98-23) = 75, \sim 15\%]$ detected and regulated in at least one pellet or fraction and finally the remaining candidates.

Although isolated, due to insufficient yield of PMV and P2 (pellet obtained at 12 000g), they were pooled for the iTRAQ experiment thereby losing the PMV or P2 specific information. The plasma derived microvesicles may include microvesicles originated from any organ apart from majority of platelet derived microvesicles. Targeting specifically the population of microvesicles generated from CNS would have significantly increased the detection of brain-specific proteins having higher potential as a biomarker for a disease like LACI.

5.6 CONCLUDING REMARKS

The current study provides the biggest proteomic imprint ever generated from clinical samples of lacunar stroke. The therapeutic importance of plasma microvesicles and technical advantage of quantitative shotgun proteomics are addressed in a unique experimental design. A systemic understanding of the LACI pathology as reflected in the plasma provides some novel indications to discover potential biomarkers. Downregulation of most of the platelet related proteins including the proteins of integrin pathway and coagulation cascade due to an anti-platelet therapy is related to a favorable outcome in LACI patients. Thus, other related pathways and the plasma proteome on a whole appears to be altered resulting in masking of the actual systemic response due to LACI. However, this paradoxically proves that the current experimental strategy or the perturbed candidates per se can also be used to measure the efficacy of drug induced changes or to monitor the effect of therapy on the long-term functional outcome of the disease. Probable presence of a hemolytic condition constitutes a general response of IS and may not be specific to LACI.

On the technical front, this study establishes differential centrifugation based microvesicles' isolation as one of the viable alternatives for the discovery of plasma based biomarkers by maintaining a nice balance between minimum sample handling and a reasonably good coverage of the identified proteome. Successful identification of nearly 500 proteins (FDR <3%), where majority (~64%) is fraction-specific is a testimony to the above fact.

Given the fact that around 20% of all stroke cases are of LACI subtype and the burden of risk factors (e.g., diabetes, hypertension) among an increasingly aging population is escalating, having a complementary diagnostic tool that is quantitative in nature will be a significant clinical achievement. This study could pave way for large-scale validation of LACI biomarkers that will reduce diagnostic errors and stratify patients on priority for hospital admission, treatment or rehabilitation. My study can also stimulate interest among fellow researchers to try the proposed panel of validated plasma biomarkers for predicting the risk of LACI or other SVD. This is nevertheless another important proposition considering the fivefold higher prevalence of silent infarct than the clinical stroke in the general population.³⁶ In conclusion this study will foster the emerging area of quantitative neuromproteomics as a viable tool for the discovery of novel biomarkers for IS.

6. DISCUSSION AND CONCLUSION

6.1 DISCUSSION

In this dissertation, I have applied an integrative forward proteomics strategy by mixing the ‘molecular’ and ‘systems’ approach of target discovery⁸² in the area of IS. iTRAQ-based MudPIT guided quantitative profiling approach was adopted for all four studies. The overarching aim of the first three studies using preclinical IS models and clinical chronic IS specimens was to discover potential therapeutic targets for IS and to understand their involvement in the evolution of the disorder. As target discovery includes discovery of biomarkers apart from therapeutic targets which are mutually supportive, LACI, an inadequately diagnosed subtype of IS was chosen for biomarker discovery. Several proteomic studies have targeted more than one type of samples related to the pathology of interest for performing comparative or subtractive analysis to select candidate proteins with higher confidence either as probable therapeutic target or biomarker.^{222, 298} Hence, the first three iTRAQ data sets were compared to identify model or sample-specific and generic (consensus) molecular signatures while the fourth one (biomarker data set) was kept as an external reference (Figure 6.1).

Despite the use of identical proteomic technique for analysis, these data sets are not directly comparable since they represent ischemic samples from different levels. However, there are a number of important features in terms of similarities and differences that render a comparative approach useful. Direct validation of some preclinical regulations in the clinical samples is possible by overcoming the technical noise or model-specific artifacts while short-listing protein(s) of higher confidence. The translational confidence of the prospective therapeutic strategy targeting these consensus candidates should be better, provided the target patient cohorts in clinical trials are chosen carefully based on the pathological similarity. However, the dissimilarity or model-specific regulation of a protein could also indicate subtle variations of the underlying pathology representing only subpopulation of patients at certain juncture during the evolution of IS. My study already exhibited that even in the same IS patient (ID-No: 1006), there are areas of brain showing opposite pattern of regulation for a single protein at a specific moment indicating each of these areas evolve differently with time and may follow a patient specific pattern (Section 4.3.2). Hence a careful and selective rather than a thorough combinatorial approach was taken from bench to bedside or bedside back-to bench. Proteins from the same functional

class or pathway were correlated across the models to extract out converging or diverging trends.

6.1.1 Comparison of Cell line, Animal and Human iTRAQ Data Sets - A Translational Perspective

The B104 cells were exposed to a controlled and sub-lethal ischemic or hypoxic stress that may be close to an early penumbral condition in the ischemic brain. Thus, the initial time points (2+0 h or 2+4 h) in the rat I/R model of MCAO, which is characterized by the presence of large penumbral tissue, could be comparable with the hypoxic and ischemic condition of the *in vitro* data set. Given the net response from intact brain is a function of individual cell-type specific responses and the result of multiple intercellular interactions, most of the systems-level properties (e.g. BBB damage, astrocyte-neuron or neuron-oligodendrocyte cross-talk) of the neurovascular unit discussed previously will be absent in a cell line model. However, sub-cellular responses should be partially comparable especially when neuronal response or basic biochemical pathways are concerned. There should be no compatibility problem as a rat cell line (i.e. B104 cell line) and whole rat were chosen consciously for better comparison. Conversely, the ischemic human brain data set represents cases of chronic ischemia with mixed pathology that includes AD or age-related complications. The etiology is least likely to be LVD on which the animal data is based on. Noteworthy, the fourth iTRAQ data set was generated using the plasma of LACI, which is one subtype of the SVD. Keeping in mind these differences, careful comparisons are made mainly between intracellular events across the models.

6.1.1.1 Energy Metabolism

The relatively higher abundance of proteins related to energy metabolism helped to identify them in all three data sets (*in vitro*, *in vivo* and human brain) in varying proportions (Tables 2.4, 3.6, 4.5 and Figure 6.1). Both *in vitro* and *in vivo* models showed the up-regulation in expression for most of these candidates unlike the human ischemic brain data set where a general down-regulation was observed. Considering the preclinical models are acute in comparison to clinical cases, an increased expression could indicate an adaptive and survival response. This also shows better

translational confidence from cell line to animal models compared to pre-clinical and clinical samples. Notably, it can be speculated that a particular therapeutic strategy based on modulating the energy metabolism in the above models is unlikely to succeed in IS patients who are pathologically similar to the group of chronic IS patients used for this study.

Figure 6.1 Schematic representation of the comparative analysis of *in vitro*, *in vivo* and clinical human brain iTRAQ data sets showing generic and consensus patterns of protein regulation. Proteins of energy metabolism and IF (i.e. vimentin) were quantified in all experiments. GDH showing similar trend of regulation in B104 cell line and rat model was selected for detailed discussion.

6.1.1.2 Anti-inflammatory Proteins

The annexin family of proteins was up-regulated in isolated neuronal model (ANXA1 and ANXA2; Table 2.3, Figure 2.8) as well as in the autopsied human brain (ANXA1, ANXA2 and ANXA5; Table 4.5). In the rat ischemic hemisphere, ANXA6, ANXA5 and ANXA11 were detected with an upward trend although they failed to qualify due to a poor *p*-value. Annexins are markers of post ischemic anti-inflammatory response. Overall, irrespective of starting samples, up-regulation of this family of proteins indicates a generalized tendency to inhibit the activity of PLA₂ and downstream generation of various mediators of arachidonic acid pathway (Figure 2.10). There are several reports to suggest protective role of annexins in cardiovascular and cerebrovascular complications. In healthy adult brain, ANXA1 is expressed in endependymal, glial and neuronal cells and in vascular tissue. It is a potent inhibitor of

neutrophil extravasation *in vivo*.²⁹⁹ ANXA1, its small peptide mimetics and receptors have been suggested as new therapeutic targets in the management of a wide range of neuroinflammatory diseases, including stroke and neurodegenerative conditions.³⁰⁰ ANXA2 is an endothelial cell surface receptor for plasminogen and tPA. Recently it has been recognized as a key cell surface entity to modulate vascular physiology by regulating fibrin balance and angiogenesis. Soluble recombinant ANXA2 reduces thrombus formation in rat carotid arteries and MCA *in vivo*. Combination of ANXA2 and low-dose tPA has been found to significantly enhance fibrinolysis when compared with high-dose tPA alone in a rat model of focal embolic stroke.³⁰¹ Overall, my study recommends detailed future studies to exploit the protective influence of annexin family of proteins for therapeutic benefits in the area of IS.

6.1.1.3 Proteins of Reactive Gliosis

The cytoskeleton is formed by interaction of intermediate filaments (IFs) with actin filaments and microtubules. In human, 65 different IF proteins have been identified.³⁰² GFAP and VIM are the main components of IF. Up-regulation of both of these proteins in post-ischemic animal brain during sub-acute phase (Figures 3.8, 3.11, 6.1 and Table 3.6) and in human brain (Tables 4.5 and 6.1) is indicative of reactive gliosis. GFAP is predominantly expressed by astrocytes and can form IF on its own unlike VIM. The level of VIM in astrocytes varies from intermediate to low depending on the subpopulation of astrocytes.¹⁹⁸ Although significant up-regulation of VIM was observed in the *in vitro* data set, GFAP failed to make it to the shortlist due to the neuronal origin of B104 cell line. However, searching the complete data set was able to identify 6 peptides (unused score 3.25) that were assigned to GFAP indicating very low levels of expression in the B104 cells.

IF up-regulation and reactive gliosis can happen due to acute or chronic CNS trauma, around growing tumors and even due to physiological aging apart from IS as seen in the current study. The exact role of reactive gliosis could be either positive or negative depending on the time, location, character and extent of damage. It may facilitate post-traumatic healing by controlling the migration of astrocytes to the site of injury, by forming the glial scar or by regenerating the BBB. Conversely it can amplify cell damage by inhibiting axonal regeneration, by causing reverse Glut transport and by

producing and releasing arachidonic acid metabolites or reactive oxygen species.⁶¹ Combining, my study provided evidences of IF up-regulation in neuron and glial cells during early (i.e. 4 h in B104 cell line), intermediate (i.e. 24 h in rat) and chronic (i.e. human brain) phase of an ischemic injury.

6.1.2 GDH – A Potential multi-modal therapeutic target for IS

Mitochondrion remained the most represented organelle in all three data sets. Keeping the importance of mitochondria in IS pathology in mind, GDH was selected as a consensus candidate for detailed discussion as it was up-regulated in both cell line and animal model (Figure 6.1).

This mitochondrial enzyme GDH is present in almost all living organisms and catalyzes the reversible oxidative deamination of L-Glu to 2-oxoglutarate using NAD⁺ and/or NADP⁺ as coenzyme.²¹⁶ It accounts for 10% of total protein in the mitochondrial matrix in astrocyte processes of the brain.³⁰³ Despite the basic biochemistry of GDH has long been known, its regulation under pathological condition that affects cellular carbohydrate or nitrogen metabolism are still poorly understood. Recent studies have also linked GDH with lipid metabolism. However, as a multifunctional mediator of several important biochemical pathways, tight and organ-specific allosteric control of GDH activity is expected during normal or altered state of cellular homeostasis.^{304, 305} The role of GDH in mitochondrial dysfunction complements the accumulating knowledge about its involvement during IS or in IS-induced chronic complications.

6.1.2.1 Manipulation of Systemic Responses during Acute IS –Probable Role of GDH

In addition to brain, high GDH enzymatic activity have been observed in pancreatic β -cells, kidney and heart, all of which have specific involvements in the pathology that predisposes to IS and in its progression. Of note, GDH is well-known to be involved in insulin secretion in pancreatic β -cells and in Gln or ammonia metabolism in kidney. Pancreas is the affected organ in diabetes, whereas diabetic complications independently or along with renal dysfunction and hypertension may increase the risk of stroke. Accordingly, I hypothesized that during IS, apart from the deregulation of

neuronal or brain GDH found in the current study, aberrant regulation of GDH may also happen in peripheral organs, like pancreas or kidney. Unraveling these multi-organ responses could pave way for better management of acute IS therapy.

Currently, the management of IS in clinic depends heavily on the use intravenous thrombolysis or aspirin or hemicraniectomy⁴⁶ apart from maintaining the function of other crucial organs and systemic parameters (e.g. electrolyte balance, euglycemia) within a stroke unit. Thus, understanding the participation of far-off organs through the bidirectional communication of neuroendocrine and immune systems is becoming important. This will help to optimize the supportive therapies and to draw correlations in between the functions of peripheral organs or systemic parameters with the prognosis of IS. Demonstration of alterations of the proteome of pituitary, adrenal gland and splenic lymphocytes following 24 h of MCAO in rats is a testimony of the IS induced systemic responses.⁶²

Figure 6.2 Schematic representation of organ-specific role of GDH following IS induced systemic complication. Individual organs are denoted by separate rectangles. Unidirectional and bidirectional arrows are indicative of known relations. ‘??’ represents unknown but probable interactions. Purple ovals indicate potential or approved therapeutic intervention strategies to tackle stroke or stroke-induced systemic complications. Up-regulation of GDH has been observed in B104 neuroblastoma cell line and rat brain following IS in the current study highlighted by the red arrow.

6.1.2.1.1 IS Induced Perturbation of Glycemic Status of Circulation - Glucose Paradox and Probable Role of GDH

Post-stroke hyperglycemia occurs in up to 60% of acute stroke patients. Diabetes mellitus accounts for around one fifth of all enrolled patients in large-scale randomized trials for acute IS therapies. Both Diabetes and acute post-stroke hyperglycemia are predictors of mortality and poor outcome.^{306, 307} Thus, glucose although is the main source of energy in brain, pre- or post-stroke acute or chronic hyperglycemia adversely affects the outcome in numerous clinical or animal studies (Figure 6.2). This phenomenon has been termed as ‘glucose paradox’ to describe the apparent contradictory effect of the sole energy source of brain. Considering glucose as a culprit, use of insulin and strict control of plasma glucose were advocated. Surprisingly, moderate diabetes is associated with smaller infarcts despite greater edema and HT in some animal studies.³⁰⁸ The results are conflicting and heterogeneous following meta-analysis on the correlation of infarct size and hyperglycemia and the effect of insulin to reduce infarct size in MCAO model.³⁰⁹ In the clinical setting, in spite of many studies the results are inconclusive till date to determine the role of insulin alone or in combination with potassium or glucose in the management of glycemia to improve functional outcome or to reduce infarct growth for acute post-stroke hyperglycemia.^{307, 310} Clearly, due to the failure of insulin per se, the underlying pathology of glucose paradox remains debatable. No consensus has been achieved regarding the optimal management of occult diabetes or non-diabetic hyperglycemia in the post-stroke scenario, despite accumulating data from randomized clinical trials. However, it indicates that the underlying pathology of hyperglycemia induced brain damage is more complex than expected.

Hyperinsulinism/hyperammonemia (HI/HA) syndrome, the most common form of congenital hyperinsulinism, provides an ideal example of how deregulated GDH can manipulate the glycemic status of the circulation. Gain of function mutations in GDH causes an increase in insulin secretion and recurrent episodes of symptomatic hypoglycemia in combination with a persistent mild hyperammonemia.³¹¹ Thus, increasing pancreatic GDH activity may be useful to counteract acute post-stroke hyperglycemia. The recent discovery of post-translational inhibition of pancreatic GDH through SIRT4-mediated ADP-ribosylation provides another level of regulation

for GDH to control the mitochondrial metabolism (Figure 6.2).¹⁸⁰ This regulation seems tissue and function specific making SIRT4 a potential target for stroke induced hyperglycemia. In addition, SIRT4 binds GDH in brain and liver. Given the higher level of expression of SIRT4 in brain, kidney, heart and liver compared to pancreas, further studies to explore its role in regulating GDH activity under physiological or ischemic condition are needed to better integrate the response at the cellular and organ level.

6.1.2.1.2 Insulin or Hypothermia Induced Hypokalemia – Probable Role of GDH

Intravenous insulin when used for controlling acute post-stroke hyperglycemia, has lead to hypokalemia in around one fifth of IS patients according to a recent report.³¹² Intriguingly, hypothermia which remains one of the most tested neuroprotective strategies for IS also induces hypokalemia and metabolic acidosis in experimental and clinical studies.^{313, 314} The underlying mechanism is not clear, but could be related to the redistribution of plasma potassium as urinary excretion of potassium was lower in hypothermic hypokalemic patients.³¹³ Recent literature has related systemic hypokalemia with increased Gln catabolism and ammoniogenesis mediated by GDH in the kidney in both human and experimental animals (Figure 6.2).³¹⁵

6.1.2.1.3 Probable Involvement of GDH in Glucose Paradox

Noteworthy, most of the *in vitro* studies failed to observe glucose paradox indicting the involvement of unknown systemic parameters like corticosteroids that may exacerbate ischemic damage.¹⁴⁸ In contrast, my *in vitro* model of sub-lethal ischemia indicates the presence of a relative glucose paradox, as described earlier in section 2.3.7. The expected trend of synergistic toxicity of oxygen and glucose deprivation in OGD compared to HYP become apparent in functional assays only when the duration of stress was increased to 6 or 8 h (Figures 2.3 and 2.4).¹⁶² The presence of high glucose (25 mM) in the absence of oxygen (in HYP condition) may promote injury through increased accumulation of lactate and intracellular acidosis. A MRI-based observational study has described correlation between blood glucose and infarct growth, potentially mediated via elevated brain lactate in anaerobically metabolizing ischemic brain tissue.³¹⁶ Recent findings suggest acidosis can activate ASIC or NMDA-type Glu receptor to cause calcium overload through independent pathways

and failure of cellular antioxidant machinery leading to the demise of neurons following IS (Figure 6.2).¹⁶ Given the presence of excess L-Gln in the culture media used for the *in vitro* experiment and significant up-regulation of GDH with parallel down-regulation of most of the antioxidant proteins, Gln catabolism induced ammoniogenesis may oppose the deleterious effects of lactic acidosis in my *in vitro* model of OGD (Table 2.4, Figure 2.8). B104 cells in absence of glucose will be dependent on Gln as an alternative source of energy at least for shorter interval (i.e. 4 h) as used in the experiment. Thus, GDH induced Gln catabolism and subsequent generation of ammonia and α -KG (2-KG or 2-ketoglutarate) will provide an apparent protection under OGD albeit for a short time. In turn, formation of lactic acid will be higher under anaerobic condition in HYP due to the abundance of glucose. This explains the lack of additive toxic effect of glucose and oxygen deprivation in the *in vitro* model of OGD. Accordingly, increased GDH activity and subsequent ammoniogenesis may directly antagonize the lactic acidosis or indirectly by blocking several acidosis induced deleterious mechanisms (like ASIC or NMDA activation) (Figure 6.2). Unknown interactions may also exist between GDH and ASICs in the ischemic brain considering metabolic or lactic acidosis activates both of them. Recently, high doses of aspirin and salicylate have been found to protect the cortical neurons from acidosis-induced neuronal injury by inhibiting ASIC.³¹⁷ This may also contribute to beneficial effect of aspirin in the clinic apart from the known modulation of circulatory environment.

Taken together, the above discussion points toward probable positive influence of higher levels of GDH during stroke-induced lactic acidosis in the CNS and in a couple of systemic responses like, post-stroke hyperglycemia and insulin- or hypothermia-induced hypokalemia or metabolic acidosis that critically affects recovery following IS (Figure 6.3).

6.1.2.2 Involvement of GDH in IS-induced Chronic Complications

Fall in intracellular pH and subsequent acidosis has been considered as a hallmark of several neurological disorders including IS and non-fatal stroke induced chronic complications such as vascular dementia, or AD.³¹⁸ Aging, which is a non-modifiable risk factor for stroke causes selective loss of glutamatergic synapses and neurons. The baseline extracellular levels of Glu in brain, as well as the increases in Glu release

following depolarization of neurons, are 75 to 100% greater in the brains of aged rodents as compared with those of young organisms.^{319, 320} Given the vital role of GDH in releasing Glu from neurons¹⁸⁶ and in metabolic or lactic acidosis, it may not be surprising that perturbation of GDH activity and regulation have been reported in a number of conditions that lead to neurodegeneration.¹⁸⁶ Although my study involves acute changes in GDH expression in case of *in vitro* or *in vivo* models of IS, its aberrant expression and activity in a longer timeframe may affect the pathogenesis of chronic neurodegenerative disorders through acidosis-mediated pathways. It is worth mentioning that in the autopsied human brain samples of chronic ischemia with degenerative pathology, although GDH did not appear in the shortlisted data set, down-regulation of mitochondrial aspartate aminotransferase (GOT2) or GABA-T shows a failure of brain Gln metabolism (Section 4.4.3, Figure 4.5).

Figure 6.3 Simplified schematic representation of the involvement of GDH in diverse aspects of pre- or post-stroke pathology in brain-specific and multi-organ level. Brain specific GDH deregulation is observed in the preclinical models of IS in the current study. Indirect evidences support previously unappreciated role of GDH in IS induced systemic complications and probably in post stroke neurodegeneration.

6.1.2.3 Targeting GDH or metabolic acidosis for pharmacological intervention in IS

GDH is a mitochondrial enzyme and a member of the important ‘dehydrogenase’ family of proteins. Screening of the successful and research targets indicate that ‘enzyme’ and ‘short-chain dehydrogenase’ is featured among the enriched biochemical

class and protein families. In a report published on 2006, as much as 50% of the successful drug targets are found to be enzymes.⁸⁷

GDH has been implicated as a target for HI/HA syndrome, where its pharmacological inhibition may be of potential interest.²¹⁶ Importance of acidosis and tissue pH has also been recognized to complement conventional MRI-based diagnostic techniques in stroke. Endogenous pH-weighted MRI, by sensitizing to local tissue pH, offers a promising tool for providing a surrogate imaging marker of lactic acidosis and altered tissue metabolism.³²¹

EGCG, a polyphenol found in green tea, acts as an inhibitor of both mutant and wild-type GDH.³²² A few studies using EGCG as a probable neuroprotective for the treatment of acute IS have recently been reported.³²³⁻³²⁶ The hypothesis was that the potent antioxidant effect or a reduction of MMP9 activity by this dietary polyphenol could rescue brain cells and reduce infarct volume following ischemia. Although most of them provide positive findings on infarct volume during acute (i.e. 24 h)^{323, 325} and subacute phase of I/R injury,³²⁴ the beneficial effect is not significant in the sub-acute phase (i.e. 2 weeks) according to a separate study.³²⁶ Moreover, one of them demonstrated increased risk of intracerebral hemorrhage associated with EGCG treatment in the same dose that is claimed to reduce infarct volume or improve functional outcome.³²⁴ Compiling, the results are at best limited and inconclusive to suggest EGCG alone as a potential treatment strategy for IS. Surprisingly, none of these studies tried to take into account the potent inhibitory influence of EGCG on GDH activity and resulting ammoniagenesis in the ischemic brain and its probable implication on the functional outcome. Recapitulation of a similar condition in simplified cell line systems indicate that micromolar concentrations (5-50 μ M) of EGCG significantly decreases cell viability in MTT assay under glucose deprived condition in rat (i.e. B104) or mouse (i.e. N2a) neuroblastoma cell line when exposed for short (i.e. 4 h) or long intervals (i.e. 8 h) (Data not shown). Although checking the enzymatic activity rather than the absolute expression of this enzyme in brain or any other peripheral organs was outside the scope of the current study, the observed up-regulation of GDH may well be a protective response as discussed in the previous section. Hence, my study presents a case to consider the GDH inhibitory effect of EGCG, which is unrelated to its antioxidant activity²¹⁶ during future IS related efficacy

studies. Evidence of modulation of insulin secretion by EGCG through GDH inhibition³²⁷ in pancreas may also contribute adversely in the setting of post-stroke hyperglycemia or diabetes.

Rat cell line and whole rat have been used for the data generation and interpretation in my work. The above discussion collates experimental evidences obtained from several *in vitro*, animal or clinical studies. Although human has a brain and testis specific isoform of GDH (i.e. GLUD2) with 97% sequence homology with the ubiquitous GLUD1, rat has only one isoform (i.e. Glud1) for GDH.³⁰⁴ Thus, inferences drawn from the experience in rat models should be extrapolated with care especially for brain. No study has been performed to show the species-specific or organ-specific spatiotemporal differences in the regulation of these isoforms in course of development and during pathological conditions like IS. Similarly, due to the ubiquitous presence of GDH in different cell types of brain and the damaging role of Glu excitotoxicity on neuronal and glial cells, GDH might be an important multi-cellular mediator.

In short, my *in vitro* and *in vivo* model of IS revealed that pharmacological or genetic modulation of GDH directly or indirectly through SIRT4 would likely to have a multi-modal effect not only on brain but also by modifying systemic complications and function of peripheral organs in the post-stroke scenario. Exhaustive proof of concept studies using knock-down or over-expressed animals are needed to understand the function of GDH in the brain and to unravel its systemic consequences in an organ-specific manner following cerebral ischemia. In retrospect, many failed treatment strategy has solely considered the target in neuron of CNS, often ignoring the modulation of the same target in glial cells or peripheral organs that may also contribute to the adverse effects.

6.2 LIMITATIONS

Thorough knowledge about the possible limitations of the current study is important to exploit its advantages and to avoid overt extrapolation of the findings.

The comparative potential of the findings across the studies is limited due to various reasons. The autopsied brains belong to patients who did not die primarily due to IS as

the agonal period is variable with the presence of co-morbidities. Being a retrospective evaluation, most of the parameters are not controlled like the pre-clinical models. This is in contrary with MCAO model, where the I/R injury is in acute or sub-acute phase as the termination time is maximum 26 h from the onset of IS. The animal brain and LACI plasma data sets cannot be directly correlated either as MCAO is a model of LVD unlike LACI, which affects small arteries or arterioles. However, it should be mentioned that there is no appropriate or widely accepted animal model for LACI.⁴² At the same time, biomarker discovery using the plasma of LVD patients doesn't represent a clinical priority as it is for LACI, where imaging modalities are insufficient unlike non-lacunar strokes.

Pooling strategy has been used in the experimental design for the *in vitro* and *in vivo* studies as well as for the discovery of biomarkers from the patients' plasma. Although biological and experimental replicates were pooled due to specific reasons mentioned in the respective chapters, increasing time and escalating cost of proteomics experiment are the underlying and common reason. Thus, it should be noted that estimates of central tendency and variance are based on mainly technical replicates and not on biological replicates. Although the trends of protein expression will remain similar, the magnitude of differences may vary if biological variations are estimated using parallel and separate set of iTRAQ experiments.

On the technical end, although iTRAQ has emerged as an effective discovery tool in the recent times because of its multiplex nature and flexibility that can effectively normalize technical and experimental variations when combined with a foolproof experimental design, it should be kept in mind that proteins' function are often governed by the PTMs e.g. phosphorylation or glycosylation, which cannot be traced by the experimental design adopted in the present study. The stoichiometry of the PTM rather than the absolute level of these regulatory proteins changes under stress. Thus, I may have lost some vital information as a result of filtering out many crucial proteins without significant change in their relative expressions among different conditions. Alternative techniques like pre or post labeling enrichment of phosphopeptides or glycopeptides should be performed with established techniques using ERLIC, SCX-IMAC or TiO₂ to capture and map the differential change in PTM

in a quantitative manner in order to get clearer idea about the intricacies of the signaling pathways.

The proteomic techniques inherently under-represent membrane proteins including ion channels and G-protein coupled receptors which are targets for around 70% of the currently available drugs.³²⁸ A similar situation is encountered for proteins with extreme molecular weights or isoelectric pH. Owing to the lack of amplification step like polymerase chain reaction in genomics, detection of low abundance proteins remains an unsolved problem. Although I have observed a fairly wide distribution of proteins in the MW *vs* pI maps (Figure 2.6 and 4.2), no commercial standard with a known spread of proteome was spiked during sample preparation to directly compare the actual and expected spread of proteome for the current experiments. The enrichment of mitochondrial proteins in all the three data sets using cell line or tissues can be partially attributed to this relative bias towards soluble intracellular proteins. This along with limitations of databases (e.g. presence of many putative uncharacterized proteins) and bioinformatics tools may lead to a subjective prioritization during shortlisting potential therapeutic targets from each of the studies. However, the inclusion of multiple experimental models for comparative and subtractive analysis could help to partially overcome this problem. Thus, the regulated candidates derived from this study should be considered as initial targets only or as molecular signatures that will lead to the true drug targets, when additional proof-of-concept studies are used to consolidate the hypotheses.

This study does not provide any roadmap to select specific candidate(s) ahead of others from the jeopardized protein network as realistic therapeutic target or biomarker.⁸² The problem becomes tangible, when expensive and time-consuming proof-of-concept studies are attempted by selecting one or a few proteins, as real causative proteins cannot be distinguished from the effects or bystanders in the complex molecular network. However, this represents a problem of the next phase in the target discovery pipeline, where transitioning of the putative therapeutic or biomarker target into a *bona fide* drug or biomarker target takes place.³²⁸ By analyzing the common characteristics of clinically successful and research targets, a set of standard rules has been proposed to prioritize the potential therapeutic targets based on the information about proteins' family, sequence, pathway involvement or organ

specificity among others. Standard statistical learning method has also been applied to predict druggable proteins.⁸⁷ However, these methods are only a guide for rational selection and not a replacement of experimental validation. Detailed understanding of the pathophysiology and the role of selected proteins still holds the key for the success of the proposed hypothesis. Subsequently, RNAi could offer a fast and relatively cost-effective solution that seems to hold reasonable promise to exploit the utility of quantitative proteomics as an upstream tool for therapeutic target discovery. For biomarkers, ‘multiple reaction monitoring’ based targeted proteomics could be the method of choice for multiplex validation in the large population of patients.

6.3 FUTURE PERSPECTIVE

Quantitative Neuroproteomics – A Way Forward to an Encouraging Future from the Translational and Academic Standpoint

In the present work, I was able to generate a pathological signature of proteins following cerebral ischemia from all possible types of samples (except CSF) to provide a comparative overview of the complex disease that is beyond the capacity of a traditional reductionist approach of targeting a single model, pathway or protein.

To extend the current strategy, a series of closely related *in vitro* models with changes in any one of the controllable parameters (e.g. type of cell line – neuronal, glial or astrocytic, composition of ambient media) as a function of time can simulate ischemic injury at specific regions of the brain in a spatiotemporal fashion. Taking advantage of the multiplicity of 8-plex iTRAQ experiment, inclusion of cell lines of differing origins (i.e. combination of neuronal with astrocytic or microglial) can also be planned in the same experiment to get an idea about how the common or unique proteome of individual cellular origin behaves in presence of identical levels of stress. Suitable controls should be included to normalize and exclude the endogenous signals from housekeeping proteins to obtain true changes in expression of pathologically critical proteins. This will help to simplify and effectively understand the currently available *in vivo* models that are technically challenging and biologically more complex due to the heterogeneous nature of brain tissue.

In the next level, specific pathological questions can be addressed by modifying the animal models to include various wild-type or genetically modified species; by introducing co-morbidities; by using aged animals or by changing the type and duration of ischemia or reperfusion injury. Regarding retrospective study with clinical specimens, the problem of availability of IS subtype specific samples with complete patients' information (e.g. documentation about agonal period and medication) is the limiting factor. A prospective clinical study as performed with the plasma of LACI patient is always preferable, but may not be feasible with brain tissue. Close collaboration of well-established stroke-specific brain, CSF or plasma banks and proteomics laboratory is essential to obtain high quality data sets from clinical samples.

Taken together, the application of translational bioinformatics³²⁹ for handling and comparing these quantitative proteomic data sets generated across different levels as exemplified by the current study along with supporting information by other complementary techniques like RNAi or immunohistochemistry could identify and prioritize suitable panel of candidate therapeutic targets or potential biomarkers. The time has come to kick-start a multi-pronged neuroproteomic approach to pave the way for personalizing the therapeutic or diagnostic strategy of IS by better case selection during preclinical or clinical trials, treatment and rehabilitation. Finally, this strategy should be expanded two dimensionally by multicenter collaboration to include various other ‘-omics’ technology to screen models or clinical samples at every level to generate a detailed repository of perturbed transcriptome, proteome, lipidome and metabolome of IS.

6.4 CONCLUDING REMARKS

This dissertation is the first of its kind to address basic, translational and clinical questions related to the discovery of therapeutic targets and biomarkers for cerebral ischemia using a high-throughput iTRAQ-based quantitative proteomic platform. I capture the 1) early stages of neuronal response to cerebral hypoxic and ischemic stress in a validated *in vitro* neuronal hypoxia-ischemia model; 2) global-temporal protein expression profile of the affected hemisphere in a validated *in vivo* model of transient focal IS; 3) perturbed proteome from a group of individual IS patients and ethnicity, age, sex and location-matched control patients; to better understand neuron's and brain's response following IS.

I show the presence of a dynamic equilibrium between mutually competing protective and deleterious pathways under sub-lethal hypoxic or ischemic stress in the neuronal penumbra. This could provide a therapeutic window to rescue the stressed cells by promoting the protective and suppressing the deleterious pathways. I detected several trans-cellular mechanisms (e.g., neuron-astrocyte cross-talk or damage of BBB) in the neurovascular unit without separating its individual components by combining this unique technical platform with well-validated animal model and ischemic human brain samples. The similarity and differences between these models and clinical brain specimens at the level of perturbed proteome present an ideal example in the pre-clinical setting to justify the need for studying several available models and clinical samples to propose and prioritize hypotheses or potential therapeutic targets.

At the bedside, successful application of iTRAQ-2D-LC-MS/MS by comparing the proteome of prospectively studied cohorts of LACI and healthy control patients shortlisted a panel of potential biomarkers. In doing so, I address an important question by proposing an alternative diagnostic approach of trying to complement the imaging techniques for LACI, one of the most poorly detected and misclassified subtypes of IS.

Identification and relative quantification of many previously unreported candidates from every data set demonstrated the utility of this discovery approach as a screening tool in the translational or clinical research. Up-regulation of GDH in both *in vitro* and *in vivo* models of cerebral ischemia and its probable participation in the central (e.g.

lactic acidosis) or peripheral events' (e.g. acute hyperglycemia) mediated brain injury opens up an opportunity for detailed proof-of-concept study by traditional molecular biological techniques. This also calls for expansion of the present concept of brain-specific therapy to multi-organ therapy with due emphasis on the temporal perturbation of the proteins. This and other regulated novel proteins can be exploited for their validity as pharmacodynamic markers for checking the consequence of drug-target interactions or for reporting the efficacy of the prospective therapeutic candidates on the progression of ischemic injury or as diagnostic or prognostic biomarkers.⁴⁵

In conclusion, this study establishes quantitative proteomics as a mechanistic and translational tool for the discovery of potential therapeutic targets and biomarkers in the area of IS.

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APPENDIX

LIST OF PUBLICATIONS

1. **Datta, A.**; Park, J. E.; Li, X.; Zhang, H.; Ho, Z. S.; Heese, K.; Lim, S. K.; Tam, J. P.; Sze, S. K., Phenotyping of an in vitro model of ischemic penumbra by iTRAQ-based shotgun quantitative proteomics. *Journal of Proteome Research* **2010**, 9 (1), 472-484.
2. Zhang, H.; Guo, T.; Li, X.; **Datta, A.**; Park, J. E.; Yang, J.; Lim, S. K.; Tam, J. P.; Sze, S. K., Simultaneous characterization of glyco- and phosphoproteomes of mouse brain membrane proteome with electrostatic repulsion hydrophilic interaction chromatography. *Molecular and Cellular Proteomics* **2010**, 9 (4), 635-647.
3. **Datta, A.**; Jingru, Q.; Khor, T. H.; Teo, M. T.; Heese, K.; Sze, S. K., Quantitative neuroproteomics of an in vivo rodent model of focal cerebral ischemia/reperfusion injury reveals a temporal regulation of novel pathophysiological molecular markers. *Journal of Proteome Research* **2011**, 10 (11), 5199-213.

LIST OF CONFERENCE PRESENTATIONS

1. HUPO 9th World Congress in Sydney, Australia from 19-23 September, **2010**.
Title: Phenotyping of an *In Vitro* Model of Ischemic Penumbra by iTRAQ-Based Shotgun Quantitative Proteomics
2. Annual Conference of SSMS, Biopolis, Singapore from 22-23 September, **2011**.
Title: Temporal Signature of Cerebral Ischemia-Reperfusion Injury by iTRAQ-Based Quantitative Proteomics