GENE EXPRESSION PROFILING IN HUMAN ACUTE ISCHEMIC STROKE

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Background: Gene expression profiling of human acute ischemic stroke (AIS) has the potential to identify a diagnostic panel for differential diagnosis of AIS early in the treatment phase.

Purpose: The objective of this dissertation was to identify peripheral blood biomarkers that could be further explored for use in differential diagnosis of AIS and the design of stroke therapeutics.

Methods: A prospective gene expression profiling study of 39 patients and 25 healthy controls was conducted. Peripheral blood samples were collected in Paxgene Blood RNA tubes from patients who were ≥18 years of age with MRI diagnosed AIS after differential diagnosis and controls who were Non-stroke neurologically healthy. In stroke patients, blood was redrawn 24 hours following onset of symptoms to determine changes in gene expression profiles over time. RNA was hybridized to Illumina humanRef-8v2 bead chips. Validation was performed using Taqman Gene expression polymerase chain reaction on significant targets.

Results: A nine gene profile has been identified for AIS. Five of these nine genes were identified in the previously published whole blood gene expression profiling study of stroke and therefore play a likely role in the response to AIS in humans. One of these nine genes (*s100A12*) was significantly associated with increasing age and therefore may be non-specific for stroke. Three genes were identified as the whole blood expression profile change over time (*LY96, IL8*, and *SDPR*). Pathway analysis revealed a robust innate immune response, with toll like receptor

(TLR) signaling as a highly significant pathway present in the peripheral whole blood of AIS patients.

Conclusion: The findings of this study support the claim that gene expression profiling of peripheral whole blood can be used to identify diagnostic markers of AIS. A plausible case for innate immunity through the activation of TLR4 as a mediator of response to AIS has been made from the results of this study. This study and those conducted by Moore et al (2005) and Tang et al (2006) provide the foundation of data that support the use of peripheral whole blood for future blood profiling studies of neurological disease; which significantly opens the door of opportunity.

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PREFACE

This dissertation has been quite a journey, both scientifically and personally, with its ups and downs and just about everything in between. A PhD is not a feat to be tackled alone, at least not successfully or sanely, and for me this dissertation represents the mentorship from my advisors and professors, the support of my friends, and the unconditional love from my family.

First and foremost I must thank my husband, Bobby, for giving me a beautiful family and the kind of love most dream about. This dissertation is equally your achievement. My children Brianna and Benjamin, for bringing so much happiness into my life and for helping me to understand what's most important; mommy loves you so much. I thank my parents, my sister Ashley, and my grandmother for helping me to realize my dreams and my extended family, I love you all.

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My graduate training was supported by a pre-doctoral intramural research training award through the National Institute of Nursing Research. The financial support provided by the NINR is what pulled me through my PhD. I am the first of my family to obtain a college degree, let alone receive a PhD, so this is not only a thank-you from me, but a thank-you from my entire family.

Lastly, I want to thank the patients and families that have come in and out of my life over the years. Although I have experienced joy and a sense of reward through nursing, I have also experienced death and the grief that accompanies it. These experiences have shaped my perspective on life and what it means to truly love and live. It is such a cliché to say that I became a nurse to help others; but it is true. Everything I do for science, I do for the sake of helping others and I will continue to strive to help make a difference in the lives of my patients; even if the difference is small.

People come into our lives each and every day and help to make us who we are. And I truly believe that each and every one of you was put into my life to help me attain my dream. Thank-you from the bottom of my heart.

1.0 INTRODUCTION

1.1 ISCHEMIC STROKE

Stroke is the third leading cause of death in the United States (Thom, Haase et al. 2006) and accounts for 10% of deaths worldwide. (McKay, Mensah et al. 2005) Approximately 780,000 people experience a stroke each year in the US contributing to an overall financial burden of $65.5 billion per year. (Rosamond, Flegal et al. 2008) Stroke subtypes include ischemic stroke (87%), hemorrhagic stroke (10%) and subarachnoid hemorrhage (3%). (Thom, Haase et al. 2006; Rosamond, Flegal et al. 2008) Of the 88% of ischemic strokes that occur each year, 8-12% results in death within 30 days. (Thom, Haase et al. 2006) The risk of ischemic stroke is associated with various familial and environmental factors, such as the presence of hypertension, obesity, tobacco use, and a positive family history. (Rosamond, Flegal et al. 2008) Determinants of outcome include non-modifiable risk factors such as age, race, gender and genetic variation along with clinical phenotypes of severity, such as stroke scale score, the presence of fever and serologic blood markers. Advances in neuroimaging and acute clinical management have resulted in greater numbers of patients surviving the initial insult.(Kim and Liebeskind 2005) However, morbidity remains high secondary to complications following the primary event and initial misdiagnosis. The only Food and Drug Administration (FDA) approved treatment for ischemic stroke is recombinant tissue plasminogen activator (rtPA), alteplase. Multiple attempts

to identify other pharmacologic agents have resulted in negative findings; therefore a redirection of the science is necessary to understand the human variable response to ischemic stroke to provide alternative avenues for therapeutic treatment.

Since its commencement into the clinical arena in 1996, rtPA has proven to be a promising therapeutic treatment for ischemic stroke and is safe and effective for use in routine clinical practice. (1995; Wahlgren, Ahmed et al. 2007) However, its powerful effects are not seen without significant clinical complications. (1995) In addition, rtPA is only approved for use when patients present to the hospital within three hours from onset of symptoms. The downside is that the median time from stroke symptom onset to presentation to the emergency department is 3-6 hours.(Rosamond, Flegal et al. 2008) A recent study conducted by Hacke et al addressed the possibility of extending this limited therapeutic time window and the group was able to show that intravenous rtPA given between 3 and 4.5 hours after onset of symptoms significantly improves clinical outcomes following ischemic stroke compared to placebo. (Hacke, Kaste et al. 2008) This is promising given that the time window limit of 3 hours and a large list of contraindicating factors for thrombolytic therapy currently results in only 3-8% of stroke patients receiving rtPA. (Reeves, Arora et al. 2005)

The advancements of rtPA therapy aside, there is still a demand for alternative acute stroke therapies in clinical practice. Unfortunately, the results of recent clinical trials have demonstrated that there is still a gap in the understanding of the variable human response to ischemic stroke. Numerous promising pre-clinical therapeutics display insignificant clinical utility in human patients, which speaks to the difficulty of translating what we learn at the bench to the patient at the bedside. (Lees, Asplund et al. 2000; Ginsberg, Hill et al. 2006; Warach,

Kaufman et al. 2006; Diener, Lees et al. 2008; Montaner, Chacon et al. 2008) These negative findings may be due in part to the complexity of the human physiologic response to ischemic stroke, limited knowledge about the multiple pathways interacting in response to ischemic stroke and the implications of genomic variability on individual recovery from ischemic stroke. The difficulty may also be attributable to insufficient classification of ischemic stroke subtype. It is possible that gene expression profiling can help to identify subtypes of ischemic stroke, which has tremendous utility in designing therapeutic strategies for treatment. Only by studying the human genomic response to ischemic brain injury will we begin to understand how to clinically and pharmacologically treat the negative outcomes of ischemic stroke. A better understanding of stroke pathophysiology in humans and more appropriate stroke subtyping will ultimately provide the foundation needed to design patient appropriate clinical trials accounting for human variation and potentially unmask therapeutic effects of trial medications. To develop appropriate alternative treatments for ischemic stroke, the genomic process behind the events associated with cerebral ischemia in humans must be revealed.

1.1.1 The cerebral ischemic response

Ischemia and reperfusion related oxidative stress injury plays a critical role in ischemic stroke progression and recovery. (Kamada, Yu et al. 2007) Oxidative stress refers to an imbalance between antioxidant and pro-oxidant factors that favors tissue destruction and if left untreated ultimately leads to cell death. Reactive oxygen species (ROS) are produced in the healthy human body as a by-product of metabolism. However, during episodes of ischemia the number of ROS generated dramatically increases. Since the brain does not store glucose and oxygen like other parts of the body, ROS are produced very quickly and in large amounts. Low antioxidant

capacity and low oxygen reserve in the brain establishes an environment normally on the brink of tissue ischemia. Therefore small perturbations of cerebral blood flow can have significant negative repercussions.

1.1.2 The cerebral genomic response

Animal modeling has allowed the assessment of the cerebral genomic response under various conditions of ischemia and reperfusion. (Millan and Arenillas 2006) Cerebral ischemia stimulates expression of genes involved in excitotoxity, inflammation, apoptosis and neuroprotection and can result in both cell death and cell recovery.(Buttner, Cordes et al. 2009) In rat brain following middle cerebral artery occlusion (MCAO) there appears to be a delayed peak in differentially expressed genes which supports the previous hypothesis that the majority of damage produced by ischemia is the result of delayed damaging processes, rather than the primary ischemic insult. (Kim, Piao et al. 2004; Buttner, Cordes et al. 2009) However, there is evidence that the genomic response in the rat brain does not entirely reflect the process of stroke evolution in humans, secondary to species specific responses and pathophysiologic differences between human stroke and induced stroke in animals.(Mitsios, Saka et al. 2007) Even so, the animal models and human brain investigations have identified episodes of ischemia result in severe reductions in messenger RNA (mRNA) expression and protein synthesis within the ischemic core. The penumbra receives some blood flow and therefore this area can maintain some fundamental cellular homeostatic functions and is therefore salvageable within a certain time frame. Given the results of studies at present, it seems logical that the most fruitful areas of investigation will be involved in identification of treatments to augment the delayed secondary responses to ischemia and rescue of the ischemic penumbra.

1.2 HYPERINTENSE ACUTE REPERFUSION INJURY MARKER

One goal of Healthy People 2010 is to decrease death and disability in stroke patients by 20%, from 60 to 48 per 100,000 of the population. To achieve this goal, attention must be made to the cause of death and disability following ischemic stroke. In the first month after ischemic stroke, 51% of deaths can be attributed to secondary complications of the primary ischemic event, such as oxidative stress injury followed by cerebral swelling and hemorrhage.(Vernino, Brown et al. 2003) There are no treatments for the secondary sequela of cerebral ischemia and oxidative stress. While reperfusion at times reduces infarct volume and helps to improve clinical symptomatology in some patients, in others it has been shown to exacerbate ischemia and produce additional cerebral reperfusion injury.(Aronowski, Strong et al. 1997) Ischemia and reperfusion injury contribute to free radical production, an increase in proteolysis, and activation of the inflammatory/immune response, resulting in impairment of cerebral vessels and disruption of tight junction proteins. Disruption of tight junction proteins can lead to cerebral swelling, hemorrhagic transformation and blood brain barrier (BBB) disruption.

Up to 33% of ischemic stroke patients display evidence of reperfusion injury as BBB disruption on magnetic resonance imaging (MRI). (Latour, Kang et al. 2004; Warach and Latour 2004) BBB disruption can be observed as delayed gadolinium (Gd-DTPA) enhancement of the cerebrospinal fluid (CSF) space on fluid-attenuated inversion recovery (FLAIR) MRI. The presence of this enhancement has been termed *H*yperintense *A*cute *R*eperfusion injury *M*arker (HARM). Treatment with rtPA is associated with an increased prevalence of BBB disruption with subsequent hemorrhagic transformation (HT) and worse clinical outcome.(Kidwell, Latour et al. 2008) Therefore, it is of primary importance to determine which patients are more

susceptible to BBB disruption and the detrimental effects of thrombolytic therapy. By doing so, the therapeutic effects of rtPA can be maximized.

1.3 WHOLE GENOME EXPRESSION

A novel approach to the study of cerebral ischemia in humans is gene expression profiling using microarray technology.(Baird 2006; Sharp, Xu et al. 2006; Baird 2007; Sharp, Xu et al. 2007) In the last 5-10 years there has been an incredible increase in the knowledge of genetic and genomic mechanisms that contribute to simple and complex diseases (http://www.genome.gov/gwastudies/ ; http://www.ncbi.nlm.nih.gov/sites/entrez?db=omim)

Ischemic stroke is a multi-factorial complex neurologic insult that is mediated by a specific interplay between genes and gene products and their interactions with other genes and the environment. For such a complex disorder it is difficult to use candidate gene approaches and directed hypotheses to identify significant biomarkers associated with prognosis and outcome. The nature of the disease is such that it requires a platform, such as microarray, that can assess various genes and molecular pathways simultaneously and integrate that knowledge into information that can be interpreted with clinical relevance.

Gene expression profiling allows for the simultaneous assessment of the approximately 25,000 genes of the human genome and is a powerful and effective approach to either identify genes correlated with a phenotype or find a classifier for predicting a phenotype. The best tissue for gene expression profiling in ischemic stroke is brain; however this is not feasible in most clinical situations, being only accessible at post-mortem. Proof of principle gene expression profiling studies have been performed on ribonucleic acid (RNA) extracted from whole blood in

ischemic stroke.(Moore, Li et al. 2005; Tang, Xu et al. 2006; Grond-Ginsbach, Hummel et al. 2008) These studies support the potential for genomic profiling of whole blood RNA to transform neurologic disease research in the post-genome era.(Moore, Li et al. 2005; Du, Tang et al. 2006; Tang, Xu et al. 2006) Identifying patterns of gene expression associated with ischemic stroke has the potential to pave the way for advanced diagnostics and therapeutic strategies aimed at the complex biological pathways associated with the human response to ischemic stroke.

1.4 PURPOSE

The purpose of this project was to elucidate the clinical variability of gene expression in acute ischemic stroke (AIS) patients through the use of gene expression profiling using microarray to identify a panel of markers for the diagnosis of acute ischemic stroke. This data can be used to further study the candidate genes identified and inflammatory/immune pathways for their relationship with response to ischemic stroke and BBB disruption.

1.5 SPECIFIC AIMS

1. Determine which expressed transcripts are under- and over-expressed in acute ischemic stroke patients compared to neurologically healthy age-matched control subjects.

2. Determine the changes in gene expression that occur in the first 24-48 hours following acute ischemic stroke.

3. Determine whether acute ischemic stroke patients with BBB disruption have a specific blood gene expression profile compared to AIS patients without BBB disruption.

1.6 RESEARCH QUESTIONS/HYPOTHESES

Question 1: Is there a specific gene expression profile in blood associated with AIS that can be used to identify candidates for diagnostic biomarkers of acute ischemic stroke?

Hypothesis 1: AIS patients will display a specific gene expression profile in blood that will aid in identifying candidate biomarkers for ischemic stroke diagnosis and in identifying molecular pathways to target treatment.

Question 2: How will blood gene expression profiles in blood change between the acute phase of ischemic stroke (0-24 hours) and 24-48 hours following onset of symptoms?

Hypothesis 2: Gene expression patterns in blood will change over time reflecting individual responses to cerebral ischemia and may aid in identifying molecular pathways involved in the delayed neuronal response to ischemia.

Question 3: Is there a specific gene expression profile in blood associated with the development of BBB disruption as HARM on MRI after AIS?

Hypothesis 3: A specific gene expression profile in blood will be associated with BBB disruption in acute ischemic stroke patients that will serve as a readily accessible marker of HARM and may help elucidate the mechanisms behind BBB disruption in this population

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1.7 DEFINITION OF TERMS

1.7.1 Independent variables

1.7.1.1 Acute ischemic stroke

Cases have been classified by acute ischemic cerebrovascular syndrome (AICS) diagnostic criteria. (Kidwell and Warach 2003) This classification scheme for ischemic stroke defines four categories ranging from "Definite AICS" to "Not AICS", based on evidenced-based neuroimaging diagnostic certainty. Only patients with a diagnosis of Definite AICS were recruited for the aims of the study. See Appendix A for AICS diagnostic criteria.

1.7.1.2 Hyperintense acute reperfusion injury marker

Baseline (0-20 hours) and follow-up (20-40 hours) MRI images were assessed for presence (e.g., sulcal, bilateral, ventricles, vitreous) and level (none, mild, moderate, severe) of HARM. See Appendix B for NINDS Stroke Team-HARM Rating Scale.

1.7.2 Dependent variables

1.7.2.1 Whole genome expression

For its ease of use in the clinical setting, and its ability to maintain the specific biological signatures (i.e. stabilization of RNA) at the time of the blood draw,

Paxgene whole blood RNA tubes were used to collect 2.5ml of whole blood from acute ischemic stroke patients at baseline (0-20 hours) and follow-up (20-40 hours) time points and control subjects at one time point.

1.7.3 Covariates

1.7.3.1 Environment

Previous history of ischemic stroke, history of hypertension, and medication history were obtained from the patient or the patient's medical record during the initial clinical assessment. Age and gender were used as covariates in the modified k-prototypes algorithm. There were only four African American stroke patients recruited at this study site and given the small number they were not included in this analysis.

1.8 CONCEPTUAL FRAMEWORK

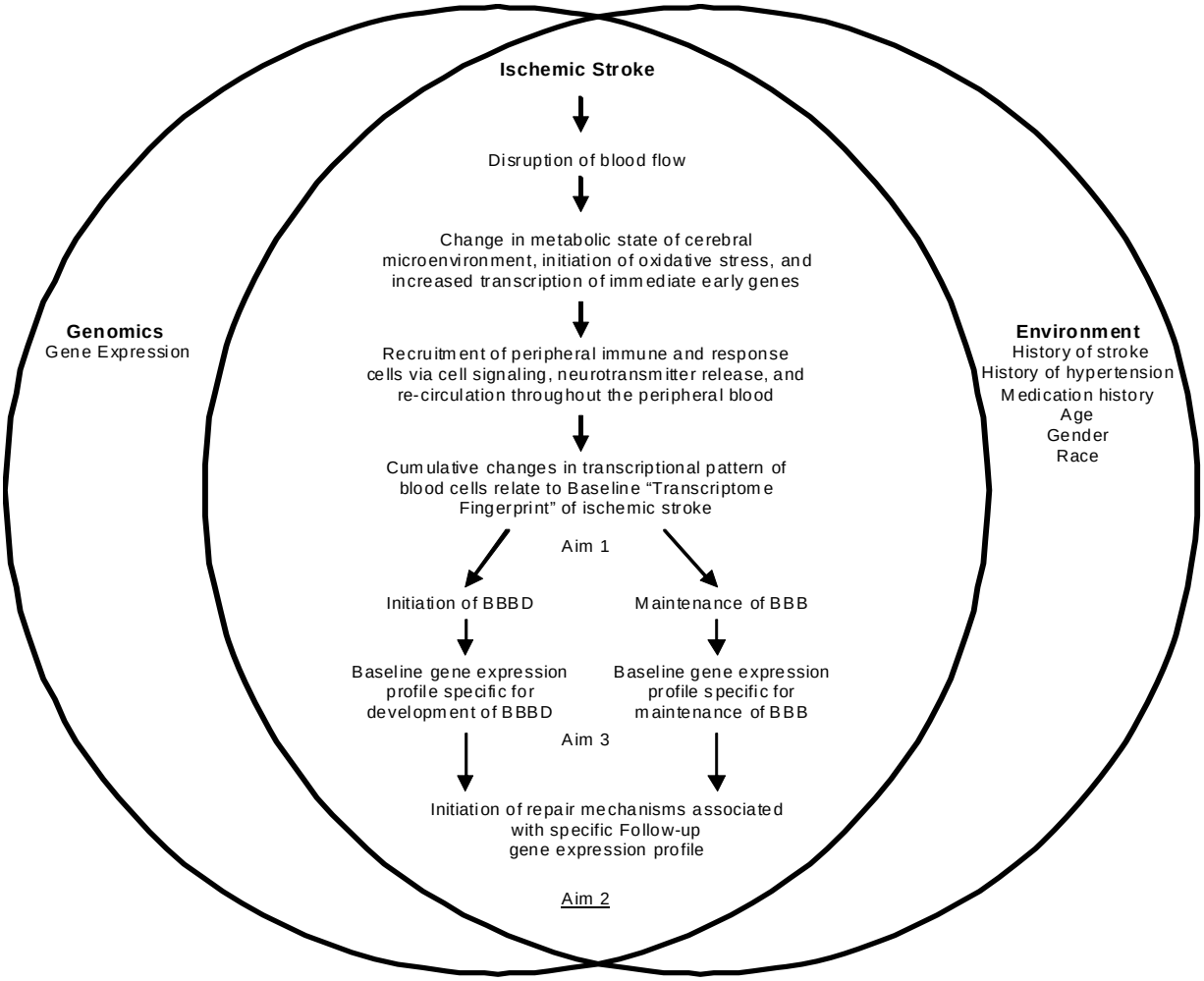


Figure 1. Conceptual Framework

2.0 BACKGROUND AND SIGNIFICANCE

2.1 ISCHEMIC STROKE

Ischemic stroke occurs when there is a decrease or loss of blood flow to an area of the brain resulting in tissue damage or destruction. It is the largest subtype of stroke pathologies and therefore accounts for the majority of the death and disability associated with stroke.

2.1.1 Pathophysiology of Ischemic Stroke

There are numerous scenarios that contribute to compromised cerebral perfusion; combine this with the multifactorial effects of the environment and individual genomic responses secondary to DNA variation and epigenetic DNA modification and the result is variability of patient presentation and recovery. This complexity makes ischemic stroke very difficult to treat, both medically and pharmacologically. Most cerebral ischemic pathologic conditions involve alterations in cerebrovascular reactivity (Cipolla and Curry 2002; Dohmen, Bosche et al. 2007; Gur, Gucuyener et al. 2007; Kunz, Park et al. 2007) and clot formation.(Kuge, Yokota et al. 2001; Smith, Bis et al. 2008) Ischemia is the consequence of one or more of the following causes: thrombosis, embolism or decreased systemic circulation. Thrombosis is a localized obstruction of blood flow in one or more blood vessels, most commonly caused by atherosclerosis.(Badimon and Vilahur 2007) Embolic stroke refers to a blockage of the blood

vessel not caused by a localized process, but rather material originating from outside of the cerebral circulation, most commonly the heart.(Weir 2008) Decreased systemic perfusion results in a decrease in cerebral perfusion pressure, and ultimately cerebral blood flow secondary to cardiac pump failure or systemic hypotension.(Murkin 2002) Although each scenario has a different origin, the result is either a temporary or permanent decrease or loss of cerebral blood flow. Permanent loss of cerebral blood flow to an area of the brain resulting in cell death is termed infarction. The penumbra is the area of the brain receiving less than optimal blood flow and is damaged but "salvageable", not yet infarcted. In all cases of ischemic stroke, the intent of therapy is to rescue this penumbral tissue and if therapy or reperfusion occurs quickly, this tissue can be rescued. (Lo 2008) The extent of tissue damage depends on the location and duration of the infarction or lack of blood flow and the extent to which collateral vessels can supply oxygen and nutrients to compromised areas. The cerebral ischemic response is complex and involves a decrease in oxygen and glucose delivery but also an accumulation of detrimental metabolic waste products. Therefore reactive oxygen species (ROS) and inflammatory mediators play a critical role in the events following ischemia.

Immediately following ischemic brain injury a cascade of events occurs in response to loss of blood flow (either trickle flow or complete obstruction of flow). Alterations at the cell membrane result in release of glutamate, activation of N-methyl-D-aspartic acid (NMDA) receptors and release of calcium (Ca+2) into the extracellular space. This process ultimately leads to the activation of Immediate Early Genes (IGE's), such as c-fos and c-jun. IGE's propagate the physiologic response by participating in transcription of neurotrophic factors (endogenous neuroprotection), heat shock proteins (general stress response), cytokines and immune mediated complexes (inflammatory and immune activation), and nitric oxide synthase

(NOS) activation (neuronal stimulation). Pathway specific responses are mediated by non-modifiable factors (DNA variation, age, gender, and severity of injury), modifiable factors (diet, physical activity, temperature, and environmental stress) and the interaction of signaling molecules within the pathways themselves. The collective response, secondary to human genetic variation, results in either propagation of injurious mechanisms and cell death or initiation of repair mechanisms and neuronal sustainment.

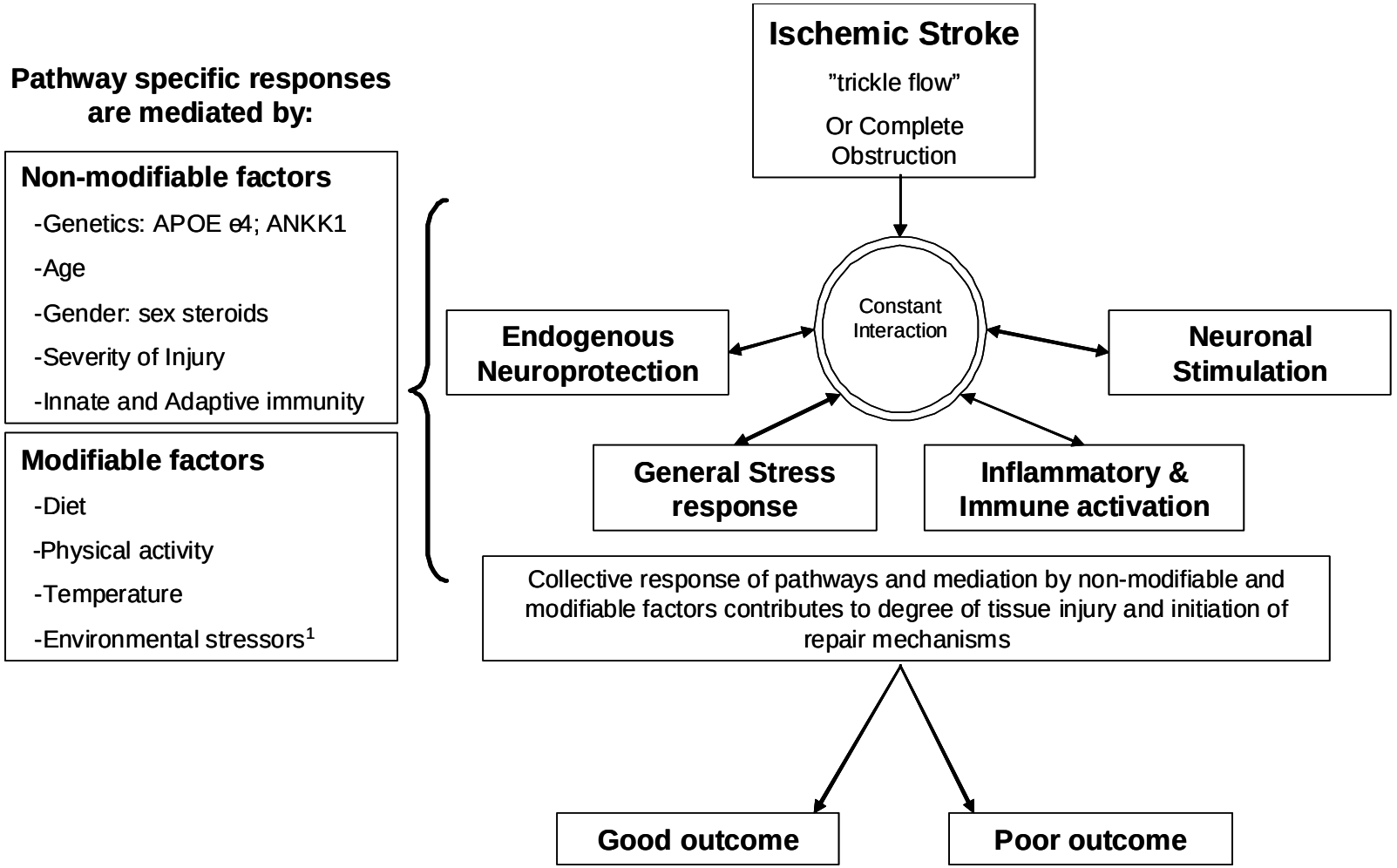


Figure 2. Multifactorial response to ischemia

2.1.1.1 Reactive oxygen species

Since the brain does not store oxygen or glucose, cellular energy production fails to maintain normal metabolism within minutes following compromised cerebral blood flow. Within the mitochondria, the electron transport chain removes electrons from an electron donor and passes them onto oxygen to form water through a series of redox reactions. These reactions create a proton gradient across the mitochondrial membrane that drives production of adenosine triphosphate (ATP). ATP then enters the Krebs cycle (citric acid cycle) to become part of a metabolic pathway that converts carbohydrates, fats, and proteins into usable forms of energy (e.g. carbon dioxide and water). When oxygen is unavailable the electron transport chain can no longer accept electrons; a proton gradient is not produced, ATP production ceases, and pyruvate becomes the final acceptor of electrons in the chain. This switch from oxygen dependent aerobic metabolism to anaerobic energy production results in an accumulation of lactic acid and ionic pump failures.(Adam-Vizi 2005)

Sodium potassium (Na+/K+) pumps are highly dependent upon ATP energy production and begin to fail within minutes of anaerobic metabolism. Na+, water and calcium (Ca+2) begin to pass from the extracellular space to the intracellular space and cerebral cells begin to swell, resulting in cytotoxic edema. Capillary endothelial cells begin to function abnormally and the tight junctions between them loose their integrity, leading to BBB disruption. Intravascular fluid leaks into the extravascular space and spreads easily throughout the white matter, resulting in vasogenic edema. Additionally, excess intracellular $Ca^{+}2$ triggers free radical production along with free radicals produced during anaerobic metabolism contributing to protease and lipase activation. Superoxide and peroxynitrite (free radicals) production increases beyond the cells capacity to quench them, which in turn activates the production of other detrimental

ROS.(Chrissobolis and Faraci 2008) Eventually the swollen cells enter cell death pathways through necrotic or apoptotic processes dependent upon the cell type, severity of injury and the level of available ATP. Unfortunately, reoxygenation through reperfusion also acts as a substrate for enzymatic reactions that produce ROS. Cells attempt to minimize damage caused by ischemia by rebalancing energy supply and demand. This early neuroprotective response results in an overall suppression of non-essential energy consumption. (Barr and Conley 2007)

All of these events together result in BBB permeability, loss of cell ion homeostasis, and excitotoxicity, (Wong and Crack 2008) resulting in a modulation of gene and protein expression. The molecular imprint of these processes is visible within all cells that migrate and circulate throughout the area of cerebral injury. These cells then circulate out of the central nervous system and throughout the body making it possible to obtain an indirect measurement of the cerebral microenvironment by sampling the peripheral blood.(Emsley, Smith et al. 2003)

2.1.1.2 Neuro-inflammatory and immune responses

Inflammation following ischemic stroke is initiated at the blood endothelial interface and is propagated over a period of time following the initial infarct. Polymorphonuclear leukocytes rapidly enter the brain tissue at the site of ischemia followed by mononuclear cell invasion. Vascular and non-vascular cells generate cytokines and chemokines which activate inflammatory mediators which may be both detrimental and beneficial.(Hallenbeck 1996) An inflammatory response begins in the microcirculation of the cerebral vessels following microglia release of the pro-inflammatory cytokines, interleukin-1beta (*IL1B*) and tumor necrosis factor-alpha (TNFα). Microglial activation parallels the induction of cellular apoptosis and correlates with severity of ischemia.(Rupalla, Allegrini et al. 1998) This activation is early and contributes to a secondary response mediated by *IL6* and *IL8*. (Rodriguez-Yanez and Castillo 2008) These cytokines trigger

the release of cell adhesion molecules (e.g. selectins, immunoglobulins, and integrins). Leukocytes begin to adhere to the endothelial cell wall and aggregate contributing to a pro-coagulant environment within the luminal endothelium, resulting in microvascular occlusion. Leukocytes activate matrix metalloproteinases (MMPs) through the release of nuclear factor-κB (*NF-κB*) which degrades the extracellular matrix, basil lamina, and tight junction proteins allowing leukocyte entry past the endothelium. (Rosenberg, Estrada et al. 1998; Rosenberg 2002; Castellanos, Leira et al. 2003; Montaner, Molina et al. 2003; Rosenberg 2009) MMP activity, specifically *MMP9* (e.g. gelatinase B) increases the permeability of the BBB contributing to edema and subsequent hemorrhagic transformation.

Evidence suggests that mediators of post-ischemic inflammation have both deleterious and beneficial effects on outcome following stroke.(Tuttolomondo, Di Raimondo et al. 2008) There is an unclear period in which these processes turn from deleterious to mediating neuroprotection, tissue repair, and angiogenesis. The function of *TNFα* and *IL1B* signaling has not yet been fully elucidated; although the data support a potentially detrimental role of these cytokines in ischemia. Inhibition of *IL1* converting enzyme decreases infarct volumes in both mice and rats (Relton and Rothwell 1992; Hara, Friedlander et al. 1997) and injection of *TNFα* 24 hours prior to MCAO exacerbates the size of infarction. (Wang, Yue et al. 1994) Following cerebral ischemia there is an initial upregulation of pro-inflammatory cytokines that results in the recruitment of other inflammatory mediators and a subsequent secondary cytokine signaling response.

Animal studies have been helpful in guiding human stroke trials; however inflammatory events in humans are not identical to that of animal models. In addition, the nature of cytokine modulation is extremely complex and is dependent upon severity of injury, duration of ischemia,

and innate mediators of immunity. Therefore the molecular mechanisms that lead to activation and regulation of inflammatory genes in the post-ischemic human brain are even less understood. The development of cDNA microarrays provides a convenient method for identifying therapeutic targets in this neuro-inflammatory cascade since many genes with complex responses are differentially expressed in human cerebral ischemia, and one cannot possibly conduct candidate studies on all of them. (Wong and Crack 2008)

2.1.2 Clinical Diagnosis

Clinical diagnosis of ischemic stroke is often difficult, complicated by its multiple etiologies and variable clinical presentation. In most hospitals, diagnosis is made when the patient presents with symptoms suggestive of acute cerebral ischemia in conjunction with pathologic findings on cerebral imaging that are most likely associated with the presenting symptoms. When possible, history is obtained via inquisition of the following: personal and family history; discussion of history of stroke or symptoms suggestive of stroke; time and activity at the onset of symptoms; temporal progression of symptoms; and whether or not they are accompanied by other factors, such as headache or nausea.(Caplan 2000) Unfortunately more often than not, this information is unobtainable secondary to severity of the stroke and whether or not the patient has family available that can provide the history. At this point the physical examination and brain imaging findings are used to make the definitive diagnosis. Identifying the physical location of the stroke is made by assessment of neurologic status via the neurologic examination, which may include National Institutes of Health Stroke scale (NIHSS) score and the presence of pathologic findings on computed tomography (CT) or magnetic resonance imaging (MRI).

The majority of hospitals in the United States use CT to rule out stroke; however it has been found that CT is less than optimal for identifying acute ischemia.(Chalela, Kidwell et al. 2007) A recent study of emergency room (ER) neurology consults found that the initial diagnosis of the ER physician agrees with the final diagnosis ~60% of the time.(Moeller, Kurniawan et al. 2008) There was a significant pattern of mis-diagnosis for stroke and seizure; other benign medical conditions (e.g. migraine) and psychiatric disorders were originally diagnosed and medically treated as stroke. Although over-diagnosis of stroke early may appear to err on the side of patient safety, it puts a percentage of patients into a category where they are being treated for an acute stroke that they do not have. Given the complications associated with bleeding following rtPA administration, this practice is quite risky. For this reason, a lot of ER physicians are reluctant to treat a patient with rtPA unless they are completely sure of a stroke diagnosis.

Where possible, hospitals are moving toward using MRI for acute diagnosis of stroke; however this is facilitated best by the presence of a dedicated stroke clinical team and only possible in facilities with 24 hour MRI availability. Additionally, even though rtPA is FDA approved, only a small number of stroke patients actually receive the drug. The Brain Attack Coalition has recommended a movement toward the creation of widespread primary stroke centers to increase the utilization of rtPA and creation of standards of care for ischemic stroke patients.(Alberts, Hademenos et al. 2000) In a small community hospital located in Bethesda, MD the establishment of a primary stroke team resulted in a 7-fold increase in the proportion of stroke patients treated with rtPA within 24 months after the onset of the program.(Lattimore, Chalela et al. 2003) This paradigm can be applied to other small community hospitals and has the potential to increase the numbers of stroke patients treated with rtPA by an additional 30,000 patients per year. More and more hospitals are moving towards the establishment of primary

stroke teams; however there are still quite a few hospitals that rely solely on the ER physician's expertise in diagnosing ischemic stroke.

Quick and definitive diagnosis in the acute care setting is essential to separate stroke from non-stroke, distinguish hemorrhage from ischemia, and identify the potential cause of the infarction, but most importantly to determine eligibility for thrombolytic therapy to begin treatment within the three hour window of opportunity. An additional diagnostic measure, such as a serologic blood test or a screen of a panel of markers, would be extremely beneficial in obtaining a definitive diagnosis of acute stroke and increasing the utilization of rtPA, especially in hospitals where primary stroke centers are non-existent.

2.1.2.1 Imaging Biomarkers

Until recently a CT scan was the only radiologic examination routinely performed during the acute clinical assessment of stroke patients. There is growing evidence that MRI is now more sensitive in identifying acute ischemic changes and is the imaging mode of choice when determining eligibility for rtPA or stratifying patients for clinical trials.(Chalela, Kidwell et al. 2007) CT has low sensitivity for ischemic changes in the hyperacute stage; it is difficult to determine the age of the infarct, and cannot identify hypoperfused but viable penumbral tissue. However, CT is more widely available and less expensive than MRI; it can be performed in patients with pacemakers or other metallic foreign bodies, and is less sensitive to patient motion. MRI on the other hand is very sensitive to acute ischemic changes and the age of the infarct, and it can be optimized to identify the penumbral area. However, it is much more expensive than CT, cannot be performed in patients with pacemakers or metal within the body, is quite sensitive to artifact from motion of the patient and requires a trained eye to identify small but significant

lesions. There are pros and cons to both modalities and often the imaging platform of choice will depend on the training of the physicians involved during the acute stroke assessment.

MRI has the ability to image the ischemic penumbra through perfusion/ diffusion mismatch identification, which can be used in clinical trials or even decisions to treat with rtPA to increase the time window for ischemic stroke treatment. Diffusion weighted imaging can detect a reduction in cerebral blood flow within minutes from onset of ischemia and therefore presents a picture of the ischemic core. Perfusion weighted imaging reveals the ischemic core and tissue at risk, so at times the area of compromised perfusion may be larger then that of the ischemic core, indicating penumbral tissue. The diffusion weighted imaging evaluation for understanding stroke evolution (DEFUSE) and echoplanar imaging thrombolytic evaluation trial (EPITHET) randomized clinical studies have identified the use of perfusion-diffusion mismatch for penumbral identification. (Albers, Thijs et al. 2006; Davis, Donnan et al. 2008) The benefits of using perfusion/diffusion mismatch in selecting patients for clinical trials was recently shown in a clinical trial in patients treated with rtPA within 3-6 hours from onset (Albers, Thijs et al. 2006) and an additional trial of Desmoteplase treatment with in 3-9 hours from symptom onset.(Hacke, Albers et al. 2005)

In addition to mismatch identification, contrast enhanced imaging using gadolinium derivatives allows for the assessment of blood brain barrier integrity and cerebral blood vessel patency. Gadolinium enhancement of parenchymal tissue has been used as reference for BBB disruption in multiple sclerosis (Bonzano, Roccatagliata et al. 2008) and only recently identified as an imaging biomarker in acute ischemic stroke. (Latour, Kang et al. 2004)

2.1.2.2 Blood Biomarkers

A rapid blood test to confirm the diagnosis of ischemic stroke would transform stroke care in the US and across the world. Most patients with acute stroke are not assessed by stroke-certified neurologists; especially in hospitals that are not primary stroke centers. Interpretation of CT scan and MRI images by non-neurologists is difficult and often leads to misdiagnosis, inappropriate use or non-use of rtPA, and clinical mismanagement. There has been a substantial attempt to identify blood biomarkers for ischemic stroke; however the task has proven difficult. Many potential blood markers of ischemia and inflammation are also found in other conditions that may mimic stroke, which aggravates the ability to identify a specific biomarker of stroke. A recent meta-analysis of published blood biomarker studies for stroke revealed significant weaknesses associated with methodological designs, such as small sample size, poor references, poor choice of control subjects and lack of validation. All of which lead to a recommendation of a redesign of blood biomarker studies. (Whiteley, Tseng et al. 2008)

A recent study published by Laskowitz et al has identified a "biomarker panel" for stroke that may help in definitive diagnosis and the implementation of rapid treatment.(Laskowitz, Kasner et al. 2009) The concept of a biomarker panel for stroke is identical to the use of serum troponin and CK-MB (creatinine kinase, muscle and brain) values for the evaluation of myocardial infarction and congestive heart failure. Unfortunately the brain poses huge obstacles to the identification of biomarkers specific for brain injury and no single blood biomarker study for stroke has proven clinically useful. The authors explored the feasibility of using a four panel blood biomarker approach to provide adjunctive information for definitive diagnosis of acute ischemic stroke. The four biomarkers, matrix metalloproteinase-9 (*MMP9*), D-dimer, *S100β*, and B-type natriuretic peptide (*BNP*) were evaluated as a point-of-care test and a separate validation

study was performed on 343 patients using a logistic regression model for interpretation. The model demonstrated modest discriminating power that resulted in a sensitivity of 86% for detecting stroke. However the model performed better for discriminating between hemorrhagic stroke and ischemic stroke than stroke mimic and stroke. This has huge clinical implications, since hemorrhage on MRI or CT is most times obvious to detect, therefore this blood test may not provide any additional information for those patients without a clear picture. In addition, the model performed poorly beyond 3 hours from onset; which makes the test only applicable to the small percentage of patients who make it to the ER within 3 hours from onset. Even given these limitations, the study demonstrates the feasibility of using a "biomarker panel" to aid in stroke diagnosis and suggests that a biomarker-based assay when used in conjunction with imaging parameters has tremendous clinical utility.

Most likely, a panel of serum biomarkers will be identified as definitive for ischemic stroke. (Montaner, Perea-Gainza et al. 2008) An ideal biomarker panel should distinguish ischemic stroke from stroke mimic and hemorrhage, be available in small centers without the need for interpretation outside of the facility, and easily accessible. A diagnostic assay, similar to what was used in the BRAIN Study, has been trialed by Biosite Diagnostics. However, the company has withdrawn its premarket approval application with the FDA secondary to ongoing issues regarding the results of their clinical trials and methodological issues in trial design.

2.1.3 Treatment

In spite of the large amount of knowledge acquired over the last 25 years in stroke research, there are still very few therapies available for the treatment of acute ischemic stroke. (Segura, Calleja

et al. 2008) The only FDA approved treatments for ischemic stroke are rtPA and the MERCI retrieval device.

2.1.3.1 Thrombolysis

Thrombolysis in ischemic stroke is aimed at reperfusion to restore cerebral blood flow. Thrombolytic agents, rtPA-alteplase, enhance the formation of fibrinolysin which disrupts fibrin and lysis blood clots. In 1996, the FDA granted approval for the use of intravenous rtPA in the treatment of ischemic stroke within three hours from onset of symptoms based on the findings of the NINDS rtPA study. (1995) In Europe, approval of rtPA was not until 2002, secondary to unsuccessful results of rtPA in the European Cooperative Acute Stroke Studies (ECASS and ECASS II). (1995; Steiner, Bluhmki et al. 1998) Intra-arterial thrombolysis has been shown effective in middle cerebral artery (MCA) occlusions up to 6 hours following stroke onset. (Furlan, Higashida et al. 1999)

There are recommended evidence-based clinical practice guidelines put forth by the American College of Chest Physicians for the administration of rtPA.(Albers, Amarenco et al. 2008) For eligible patients, IV rtPA is administered in a dose of 0.9mg/kg with a maximum dose of 90mg, with 10% of the total dose given as an initial bolus, then the remainder administered over one hour. Patients are treated with rtPA based on the following inclusion and exclusion criteria on the following page:

Table 1. Inclusion and exclusion criteria for administration of rtPA

Inclusion criteria for rtPA administration:

- Age ≥18
- diagnosis of stroke with a clinically meaningful neurologic deficit
- clearly defined time of onset < 3hours before treatment
- baseline CT or MRI scan negative for intracranial hemorrhage

Exclusion criteria for rtPA administration:

- minor or rapidly improving symptoms
- CT or MRI is positive for intracranial hemorrhage
- history of intracranial hemorrhage
- seizure at stroke onset
- stroke or serious head injury in the past 3 months
- major surgery or trauma in the past 2 weeks
- GI or urinary tract hemorrhage within 3 weeks
- systolic BP>185 mmHg
- diastolic BP>110 mmHg
- aggressive treatment required to lower BP
- glucose <50mg/dL or >400 mg/dL
- symptoms of subarchnoid hemorrhage
- arterial puncture at a noncompressible site
- lumber puncture within 1 week
- platelet count <100,000/mm3
- heparin therapy within 48 hour associated with ↑ PTT
- post-myocardial infarction pericarditis
- pregnant women
- international normalized ratio (INR) >1.7

The recent results of the ECASS III study support the use of rtPA beyond 3 hours from onset of injury.(Hacke, Kaste et al. 2008) A total of 821 patients were randomly assigned to receive either alteplase (rtPA) or placebo. Treatment was initiated between 3-4.5 hours from symptom onset. When compared to placebo, alteplase treatment significantly improved outcomes following ischemic stroke; the incidence of symptomatic hemorrhage was higher in the alteplase group. Patients in each treatment group were similar by age, gender and presence of comorbidities. However, the placebo group presented with higher baseline NIHSS scores and was more likely to have a history of stroke, which has significant implications for the interpretation of the

primary end-point of the study (good outcome as determined by the modified rankin score). There are skeptics to using rtPA beyond 3 hours and raise the argument that ECASS III is only one of two studies that have shown efficacy of rtPA beyond 3 hours.(Clark and Madden 2009) The ATLANTIS (Alteplase Thrombolysis for Acute Noninterventional Therapy in Ischemic Stroke) study was conducted in the US to determine the efficacy of rtPA administration between 3-5 hours from symptom onset.(Clark, Wissman et al. 1999) There was no benefit of rtPA therapy post 3 hours from onset, which completely contradicts the findings of the ECASS III study. In addition, post-hoc analyses of the NINDS rtPA trial also suggest no additional benefit of rtPA beyond 3 hours. (Marler, Tilley et al. 2000) Throughout medicine there have been many conflicting results between US and European studies. Even though the ECASS III results are promising, investigators urge clinicians to use caution when interpreting the results, and continue to push for treatment as early as feasibly possible. Even the ECASS III investigators acknowledge, "Having more time does not mean we should be allowed to take more time."(Hacke, Kaste et al. 2008)

2.1.3.2 Other treatments

Endovascular mechanical thromboectomy provides an alternative to rtPA therapy for patients with large vessel occlusion. Endovascular clot retrievers restore cerebral blood flow between 41% and 54% of the time.(Berlis, Lutsep et al. 2004; Smith, Sung et al. 2005; Smith 2006) The MERCI retriever consists of a flexible nickel titanium wire that once passed through a guide catheter conforms to a helical shape. The catheter/wire is advanced through the groin to the brain distally to the site of the thrombus. The clot becomes trapped in the helical shape of the wire and is then withdrawn from the cerebral vessels. The MERCI retrieval device received FDA approval in 2004, yet its clinical utility has yet to be established and is still up for debate. (Becker and

Brott 2005; Smith, Sung et al. 2005) TEven so, this option is particularly promising for patients who do not respond to conventional intravenous rtPA therapy or those who are ineligible for rtPA therapy.

There are multiple other clinically relevant treatments and models of care in use by Stroke Neurologists. Body positioning with head of bed flat to promote cerebral blood flow has been shown to increase CBF by 20% and is implemented for most stroke patients; however it is unclear in which time frame this should be implemented, and for how long. (Wojner-Alexander, Garami et al. 2005) Additionally, anticoagulants and antiplatelets, statin therapy, antihypertensive therapy, and glucose management are all areas of active clinical management of acute stroke patients. Take together; the basic advances in acute care medicine over the last decade have resulted in better outcomes for patients with ischemic stroke.

2.1.3.3 Neuroprotection

There has been an emphasis in the last few years to identify agents that could mitigate the effects of cerebral ischemia and possibly offset the negative effects of reperfusion after rtPA administration. The most extensively explored are a variety of neuroprotective agents targeting calcium antagonists, N-methyl-D-aspartate (NMDA) antagonists, free radical scavengers, agents that stabilize the neuronal membrane and various others. (Chacon, Jensen et al. 2008) Disappointingly, almost all human trials have failed to produce clinically significant results. (Kidwell, Liebeskind et al. 2001) Various reasons have been proposed for these unsuccessful attempts at translational science including inappropriate subject selection, wrong stroke subtype and absence of viable penumbra, outcome measures, and medication dosage and duration. (Gladstone, Black et al. 2002) There are a number of ongoing clinical trials evaluating the effects of hypothermia and therapeutic agents (e.g. Caffeinol, Albumin) in the setting of ischemic

stroke. Recently, the free radical scavenging agent NXY-059 was reported to show a significant effect for acute ischemic stroke patients; which is being accepted as possibly the first neuroprotective trial to show clinical benefit.

The SAINT I study was a randomized trial comparing intravenous administration of NXY-059, a nitrone free radical spin trap agent that serves as a free radical scavenger, with placebo. SAINT II was a replication/confirmation of the first trial. NXY-059 was infused within 6 hours from stroke for a total of 72 hours and the results showed an improved distribution of scores on the modified rankin scale (MRS) and a significantly lower risk of symptomatic intracerebral hemorrhage (ICH) after rrtPA. (Lees, Zivin et al. 2006) This trial has been criticized because the improvement in outcome was only ~5% indicating limited clinical utility. Unfortunately the results of the larger SAINT II study failed on all study end-points, failing to confirm the positive findings of SAINT I.(Shuaib, Lees et al. 2007) Possible reasons given for this include errors in clinical trial design and preclinical drug study problems.(Savitz and Schabitz 2008) Given the disappointment with NXY-059 future neuroprotective studies should emphasize the importance of more rigorous study methods. Savitz and Schabitz (Savitz and Schabitz 2008) offer the following suggestions:

1. Animal studies should adhere to more rigorous quality controls for blinding, randomization, and cerebral blood flow monitoring along with reproducibility of efficacy across different laboratories.
2. Determination of which behavioral tests and at what time points should these tests be performed in rodents and higher order species to determine clinical utility in humans.
3. Use MRI in future studies to separate cortical gray matter stroke from white matter stroke.
4. Study neuroprotection and reperfusion separately.
5. Consider the use of drugs with both neuroprotective and regenerative properties to achieve long lasting effects on functional outcome.

The Stroke Therapy Academic Industry Round table (STAIR) forum developed out of need for a venue in which academia and industry could come together to deal with the issues in stroke drug development.(1999; 2001; Fisher 2003; Fisher, Albers et al. 2005) The guidelines put forth by the forum have been influential in guiding stroke drug research, but given the recent failures of many neuroprotective agents, including the SAINT trials that followed the STAIR recommendations, it appears a different approach may be necessary. It is suggested that a consortium between academia, government and the pharmaceutical industries be created and the parties involved began focusing more on translation medicine to achieve much needed success in the design of stroke therapeutics.(Feuerstein, Zaleska et al. 2008)

2.2 BLOOD BRAIN BARRIER DISRUPTION

The blood brain barrier (BBB) refers to a specialized system of brain endothelial cells (BEC) and accessory cells (astrocytes, microglia, pericytes, etc.) that work in concert to separate the brain from the circulating blood. Homeostasis of the brain environment is maintained by controlling the entry and exit of blood-borne substances into the central nervous system. This is required for proper synaptic transmission, remodeling, angiogenesis and other necessary networks within the brain.

Normal blood brain barrier permeability is limited to molecules smaller than ~400 Daltons (DA). (Pardridge 2007) For perspective, albumin is about 65,000-66,000 DA. Therefore most molecules are not allowed easy access into the cerebral environment and must do so by way of active or facilitated transport mechanisms. Glucose, amino acids, and other metabolites must be ushered into the brain via transport by specific proteins such as insulin, transferrin and other

plasma proteins that aid in endocytosis. (Pardridge 1998) Lipophilic compounds enter the brain more readily than hydrophilic compounds. Such tight regulation is particularly troublesome for drug delivery across the BBB, but necessary to maintain cerebral homeostasis.

The BBB consists of a monolayer of endothelial cells that line the entire cerebral vasculature. These BEC's are connected to one another through tight junctions (TJ's), adherens junctions, and gap junctions. The composition of the TJ's consists of integral membrane proteins, such as occludin, claudin, and junctional adhesion molecules. These proteins inhibit even small molecules from diffusing through the endothelial cells of the vessels into the brain parenchyma. In addition cerebral vascular cells lack fenestrations, in contrast with other vascular cells of the body. TJ's anchor the BEC's to the surrounding cytoskeleton through use of scaffold proteins (ZO-1, -2, -3). Adheren junctions are found together with TJ's and are dependent on signals from the cadherin's and catenin's (e.g. VE-cadherin and β-catenin). The structure of these juctions are reinforced by astrocytes and maintained possibly by signals from BBB cells including endothelial cells of the vasculature, and possibly neurons and microglia. The stability of the microvessels is maintained by the basement membrane (which anchors the BEC's), extracellular matrix proteins, pericytes, microglia, and astrocytes.(Zlokovic 2008) (Persidsky, Ramirez et al. 2006)

The BBB, neurons, and non-neuronal cells (e.g. pericytes, astrocytes, etc) together form what is referred to as the Neurovascular Unit. During ischemia the viability of this unit is compromised, either through direct injury by free radicals or other proteolytic processes or diminished protein synthesis and those molecules once prevented access to the brain, easily cross through loosened tight junction gaps which further contributes to BBB impairment.

2.2.1 Pathophysiology following ischemic stroke

The human brain receives approximately 20% of cardiac output. Small reductions of cerebral blood flow (CBF) by 20% result in decreased protein synthesis of most cells in the brain (Hossmann 1994); however more severe reductions in CBF lead to decreased delivery of oxygen and ultimately a switch to anaerobic metabolism and accumulation of waste by-products.(Drake and Iadecola 2007) Reductions in CBF >80% result in ischemic neuronal cell death ad degeneration of the neurovascular unit.

The integrity of the BBB following ischemic stroke is dependent upon the patient's medical history and subsequent comorbidities along with the mechanism, severity, and duration of ischemia. (Rosenberg 1999; Brott and Bogousslavsky 2000) The cerebral ischemic response results in white blood cell invasion and the release of proteases, particularly matrix metalloproteinases that degrade the basement membrane of BBB vessels and loosen the tight junction proteins. This results in cytotoxic and vasogenic edema. Transient ischemia can be worse than permanent occlusion for BBB cells, attributable to proteolytic properties of molecules released by reperfusion related oxidative stress. (Rosenberg 1999)

BBB opening following stroke is biphasic, particularly following reperfusion. (Kuroiwa, Ting et al. 1985) The first opening is minutes to hours following the onset of ischemia followed by a second opening between 22 and 46 hours post-reperfusion. (Huang, Xue et al. 1999) A proposed complication of BBB opening is cerebral swelling; however there is room for debate regarding the direct role of BBB permeability of the presence of cerebral edema. Some studies suggest that cerebral swelling following ischemia/reperfusion can occur in the presence or absence of BBB disruption and attribute the cerebral edema to disruptions in ion homeostasis, increased tissue osmolality, and increased hydrostatic pressure. (Gartshore, Patterson et al. 1997)

Blood brain barrier (BBB) disruption following ischemic brain injury propagates a series of detrimental events, escalating secondary injury and the likelihood of poor outcome (Latour, Kang et al. 2004; Warach and Latour 2004; Dinapoli, Huber et al. 2007). The identification of factors contributing to impaired BBB integrity following ischemia and reperfusion is crucial to developing effective stroke therapies. There is a vast amount of empirical evidence, both human and animal, implying that activation of matrix metalloproteinase's (MMP's), specifically MMP9, contributes to proteolysis of the BBB basal lamina (Pfefferkorn and Rosenberg 2003; Rosenberg and Yang 2007). Proteolytic breakdown of the BBB vasculature increases the permeability of the barrier resulting in vasogenic edema, leukocyte infiltration, and hemorrhagic transformation (HT).

MMP's are zinc and calcium dependent endopeptidases responsible for regulation of the extracellular matrix (ECM). ECM homeostasis is maintained by a balance between pro- and anti-proteolytic factors including *MMP9* and its natural inhibitor tissue inhibitor of matrix metalloproteinase -1 (*TIMP1*). The *MMP9*/*TIMP1* ratio provides an in vivo assessment of the proteolytic potential of MMP9 to degrade the ECM. The balance between *MMP9* and *TIMP1* likely plays a role in reperfusion injury. (Rosenberg, Estrada et al. 1998)

Ischemia and reperfusion injury results in oxidative stress that mediates BBB disruption through metalloproteinase activation. (Gasche, Fujimura et al. 1999; Gasche, Copin et al. 2001) The tight junction protein, occludin, is a specific target in oxidative stress induced microvascular injury. (Maier, Hsieh et al. 2006) Occludin is integral to the maintenance of the BBB and once it is damaged, permeability of the barrier increases, allowing substances free movement between blood and brain parenchyma. A recent publication from Kelly et al identified a significant association between F2-isoprostane (*F2IP*) levels, which is a valid biomarker of oxidative stress,

and plasma MMP9 in rtPA-treated stroke patients (Kelly, Morrow et al. 2008) which implies a relationship between oxidative stress and proteolytic activity. *MMP9* expression is the result of activated leukocytes (particularly neutrophils), (Gidday, Gasche et al. 2005) and results in *IL1beta* activation (Russo, Siviglia et al. 2007) and initiation of the inflammatory cascade (Kolev, Skopal et al. 2003), further contributing to BBB impairment. Matrix metalloproteinases are closely related to endogenous rtPA concentrations (Sumii and Lo 2002; Kahles, Foerch et al. 2005; Wang, Lee et al. 2006; Lee, Guo et al. 2007) in that endogenous rtPA enhances *MMP9* expression and plays a role in *MMP9*/heparin induced hemorrhagic transformation (HT). (Zhao, Ikeda et al. 2004) Early inhibition of *MMP9* or *MMP9* gene knockout mice models consistently show decreased infarct volumes and attenuation of BBB disruption and inflammation. (Amantea, Russo et al. 2007; Svedin, Hagberg et al. 2007) However, late inhibition of *MMP9* can be detrimental, suggesting a role for *MMP9* in neurovascular remodeling and recovery following ischemic brain injury. (Zhao, Wang et al. 2006) Taken together, the data suggest that early *MMP9* inhibition in stroke may be beneficial, especially when used in combination with thrombolytic therapy to attenuate inflammation and BBB disruption.

2.2.1.1 Age and the BBB

Age-related changes in the BBB can be attributed to alterations in ion and nutrient transport, hemodynamic changes in the microcirculation, and changes in neurotransmitter activity.(Mooradian 1994) The number of brain capillary endothelial cells decreases with age in conjunction with capillary wall thinning.(Mooradian 1988) There is also a decreased amount of occludin (one of the essential accessory proteins of tight junctions) in cerebral tissue of aged rats. (Mooradian, Haas et al. 2003) It is well known that aging is associated with morphological changes in the BBB that increases susceptibility to brain injury during ischemia. However the

mechanisms by which aging mediates the response of the BBB to ischemia are not well understood. In addition, most animal models of stroke fail to incorporate aged animals for comparison to young. The disparity found between laboratory animal models and clinical studies of stroke may be associated with age related changes in the BBB. Dinopali et al demonstrated that aged rats (17-18 months) have more severe infarcts and greater disruption of the BBB following embolic MCAO compared to younger rats (3-4 months).(DiNapoli, Huber et al. 2008) The group identified that BBB disruption occurs prior to neuronal damage and age has an influence on this response.

2.2.2 Hyperintense acute reperfusion injury marker

The integrity of the BBB can be assessed by specific MRI sequences following the administration of the contrast agent gadolinium-diethylene triamine penta-acetic acid (Gd-DTPA). Gd-DTPA does not cross an intact BBB and the visualization of contrast enhancement within the sulcal spaces of cerebral tissue is therefore indicative of a compromised BBB. Extravasation of GD-DTPA into the cerebrospinal fluid (CSF) results in hyperintensity within the CSF space on fluid-attenuated inversion recovery (FLAIR) MRI. (Mathews, Caldemeyer et al. 1999) (Henning, Latour et al. 2008)

Warach and Latour first aimed to describe the phenomena of post-contrast FLAIR enhancement of the CSF space in acute stroke patients in 2004 and have since termed this observation *H*yperintense *A*cute *R*eperfusion injury *M*arker (HARM). (Warach, Latour et al. 2003; Latour, Kang et al. 2004; Warach and Latour 2004) An analysis of 213 ischemic stroke patients, over a 22-month period, imaged using 1.5 Tesla MRI within 24 hours of symptom onset revealed that 33% of stroke patients develop BBB disruption as HARM on MRI. (Latour, Kang

et al. 2004) Reperfusion was the strongest predictor of HARM, suggesting that HARM may be an indicative sign of reperfusion related injury to the BBB. Additionally, HARM was more common in patients with HT. This relationship was stronger when the patients were treated with rtPA. The presence of HARM was also associated with poor clinical outcome defined by Modified Rankin Score >2. This association was maintained after baseline risk factors, such as NIHSS and HT, were adjusted for in a logistic regression model. A second study provided the first evidence of the toxic effect of thrombolytic therapy in humans, reporting that the rate of HARM was greatest in patients treated with intra-arterial rtPA, following by the intravenous rtPA group, and was lowest in the MERCI retrieval and no thrombolytic therapy groups. (Kidwell, Latour et al. 2008) Findings from these studies suggest that early BBB disruption following ischemic stroke is related to significant adverse effects, and treatment with rtPA may exacerbate these complications. (Kidwell, Latour et al. 2008)

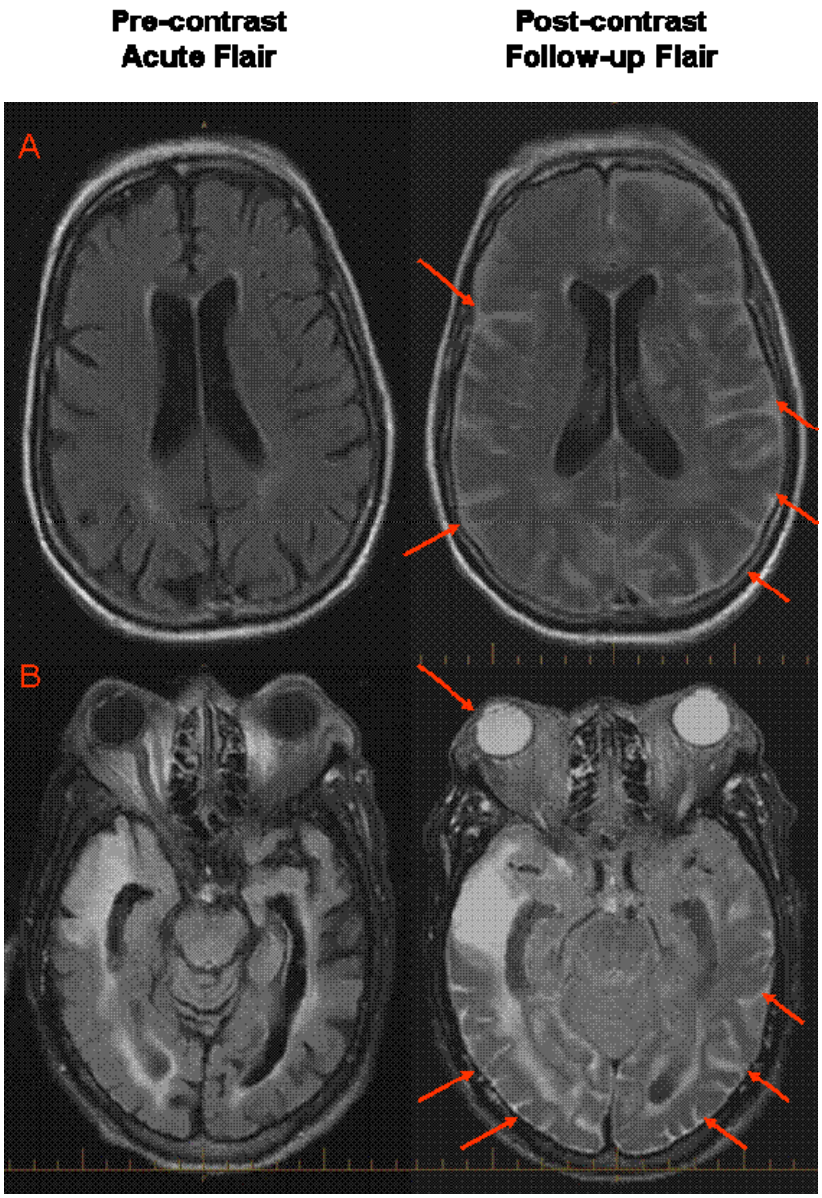


Figure 3. HARM Examples

HT following ischemic stroke has tremendous implications for treatment and outcome, however the molecular underpinnings of the process are not completely understood, nor are there undisputable predictors of HT. Stroke severity, age, and thrombolytic administration have been associated with the development of HT. More recently elevated plasma *MMP9* has emerged as a predictor of HT (Castellanos, Leira et al. 2003; Castellanos, Sobrino et al. 2007), especially in

the presence of rtPA administration (Montaner, Fernandez-Cadenas et al. 2003). If an association between plasma *MMP9* and HARM can be found, it may be possible to select patients who will benefit from thrombolytic therapy, without the risk of HT. The use of an *MMP9* inhibitor to augment rtPA therapy and attenuate HT may serve as an additional option for the treatment of acute ischemic stroke. Validation of preclinical animal models and human studies concerning the association between *MMP9* and BBB disruption provides justification for the use of *MMP9* inhibition in humans.

BBB disruption is a primary event initiating HT, and given associations between plasma *MMP9* and the occurrence of HT have already been made, it is hypothesized that plasma *MMP9* will be associated with HARM on MRI. At this time MRI cannot allow for the identification of the mechanism of injury behind BBB disruption; however peripheral biomarker analysis can. Combining an imaging marker (HARM) with peripheral biomarker protein analysis (*MMP9* and *TIMP1*) allows for the identification of feasibly observable clinical indicators of BBB disruption.

Gd-DTPA has a relatively short half life of about 1 ½ hours in the plasma. Therefore patients who present with HARM on follow up imaging must have BBB disruption around the time of the acute contrast injection. The occurrence of BBB disruption and the time window for thrombolytic therapy make HARM a relevant clinical indicator of the therapeutic response to tPA. By examining the factors that predispose patients to HARM, it may be possible to allocate treatment to those who will have the greatest benefit.

2.3 WHOLE GENOME EXPRESSION

The utilization of microarray gene expression analysis in the study of ischemic stroke has the potential to reveal the mechanisms of disease and pathways associated with ischemic stroke progression and recovery and contribute to the detection of therapeutic targets and novel drug discoveries. However, as in the purpose of this study, the technique can also be used to aid in the identification of diagnostic biomarkers irrespective of etiology.

2.3.1 Ischemic Stroke and Genetics

Twin and family studies have shown that there is a significant familial component involved in the occurrence of stroke. (Brass, Isaacsohn et al. 1992; Liao, Myers et al. 1997; Bak, Gaist et al. 2002) An even larger number of well known studies have identified environmental factors such as hypertension, smoking, and diabetes as potential culprits. There is no doubt that ischemic stroke has both environmental and genetic components involved in its pathogenesis and course of recovery. Therefore the complexity involved in determining associated genetic factors is tremendous. However, there have been some advances in identifying purely genetic elements responsible for stroke onset and recovery. Joutel et al (1996) characterized human Notch3 gene mutations affecting the Notch signaling pathway as an associated factor in the development of Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy (CADASIL). (Joutel, Corpechot et al. 1996) CADASIL is characterized by relapsing strokes with neuropsychiatric symptoms clearly distinct from arteriopathy and amyloid angiopathy that affects relatively young adults of equal numbers in both sexes. (Tournier-Lasserve, Joutel et al.

1993) In addition to the findings associated with CADASIL, others have attempted to establish a relationship between the angiotensin converting enzyme (ACE) insertion/deletion (I/D) polymorphism and the occurrence of stroke with mixed results. Markus et al (1995) identified the deletion polymorphism in the ACE gene as an independent risk factor for lacunar stroke. (Markus, Barley et al. 1995) However, Ueda et al (1995) only found evidence of an association between ACE genotype and presence of ischemic stroke in a subgroup of hypertensive patients. (Ueda, Weir et al. 1995) An even larger prospective study found no association between the ACE genotype and subsequent stroke risk, further complicating the interpretation of the results. (Zee, Ridker et al. 1999) Others have attempted to correlate the presence of APOE polymorphisms and genotype with ischemic stroke with both success (Luo, Chen et al. 2003) and failure (Sturgeon, Folsom et al. 2005). Clearly these inconsistencies demonstrate that the answers to genetic stroke risk and recovery may not be associated with one specific gene mutation, but rather a collection of mutations that subsequently interact with environmental factors to produce ischemic stroke and varying degrees of BBB disruption. It is also these same mutations that may play a significant role in the recovery from an ischemic event. The ability to assess multiple sets of genes at one time, such as what is proposed in this project using whole genome wide expression, associated with BBB disruption may provide more information on the pathophysiologic processes of ischemic stroke rather than studying candidate genes alone.

The first whole genome association study of ischemic stroke was performed by Matarin and colleagues in 2007. (Matarin, Brown et al. 2007) Over 400,000 unique SNPs from the Illumina Infinium Human-1 and HumanHap300 were genotyped in a cohort of 278 patients with ischemic stroke and 275 neurologically normal control subjects. The analysis revealed hundreds of statistically significant markers with the challenge now to distinguish the true associations

from the false positives. Some of these SNPs are within or near interesting candidate loci. Three significant SNPs were found within the *KCNK17* gene; a member of the acid-sensitive subfamily of tandem pore K+ channels contributing to cellular resting membrane potential. In the heart, background K^+ currents are thought to modulate the cardiac action potential.

2.3.2 Ischemic stroke and whole genome expression

The success of the human genome project and the ability of gene expression microarrays to assess the 25,000 or so genes in the human genome have rapidly advanced the science of genomics. Whereas genetics is the study of single genes and their effects, genomics is the study of the functions and interactions of all the genes in the genome. The study of genomics will most likely make its greatest contribution to health by revealing mechanisms of common diseases. (Guttmacher and Collins 2002)

The future of genomic profiling in ischemic stroke is quite promising. (Sharp, Xu et al. 2006) Recent studies have demonstrated that different patterns of gene expression are associated with different diseases, and these patterns can be used for diagnostic verification and treatment decision making. Progress in genomic approaches to neurological diseases has been slow secondary to the inability of obtaining brain tissue. However, findings from recent studies proving that genomic profiling of blood may serve as a surrogate marker of brain disease offers promise for the application of genomics to the study of ischemic stroke.

Gene expression profiling simultaneously assesses the approximately 25,000 genes of the human genome. It has proven to be a powerful and effective approach to identify genes, pathways and interactions correlated with a phenotype (e.g. leukemia disease classification(Andersson, Ritz et al. 2007; Basso, Case et al. 2007)); the technology has also been

used to identify genes and gene interactions for the prediction of a phenotype (e.g. positive versus negative response to chemotherapeutics (Cardoso, Van't-Verr et al. 2008)). Given these advancements it is possible that gene expression profiling can be used to diagnosis stroke from stroke mimic or predict the occurrence of good versus bad outcome. The tissue with the most specificity for identifying the genomic response to ischemic stroke or neurological disease is brain; however access to human brain tissue is unfeasible in most clinical situations, being accessible only post-mortem. The use of post-mortem tissue complicates the interpretation of findings and the relevancy of the candidate genes/proteins identified in this manner to the acute response to ischemic brain injury.

DNA microarrays are most commonly used to examine gene expression profiles between two different cell populations. Recently the technique of constructing the arrays has been slightly modified to detect single nucleotide polymorphisms (SNPs) or copy number variations across different samples for use in genome wide association studies (GWA) to identify SNPs with variability. In addition, the microarray can be custom designed to target specific molecular pathways (e.g. targeted analysis of cancer related genes or genes in an inflammatory pathway). Microarray is a high-throughput technology necessary in the post-genome era. Since the completion of the Human Genome project in 2003, attention has been turned to assessing DNA variation, the function of the 20-25,000 genes present in the human genome and identifying mediators of gene expression (Guttmacher and Collins 2002). The field of functional genomics is rapidly expanding and the microarray is a valuable tool for use in understanding the complex interactions between the genes of our genome.

Gene expression microarrays today generally fall into one of two categories: either two-color cDNA or oligonucleotide. cDNA arrays contain long sequences of cDNA (~50 bases)

generated from gene libraries and amplified using polymerase chain reaction (PCR), which are then printed into spotted matrices onto glass slides. Each spot corresponds to a specific gene or transcript (probe). RNA is extracted from the cells of interest and amplified using PCR in which two types of fluorescent base pairs (Cy3 and Cy5) are inserted into the generated cDNA. These fluorescently labeled cDNA's from both cell lines are then allowed to hybridize to the glass chip with the cDNA transcripts generated from the gene libraries. The amount of hybridization to the cDNA transcripts on the glass chip is proportional to the amount of mRNA expression in the cell and can be quantified as a fold change in expression between the two fluorescent tags.

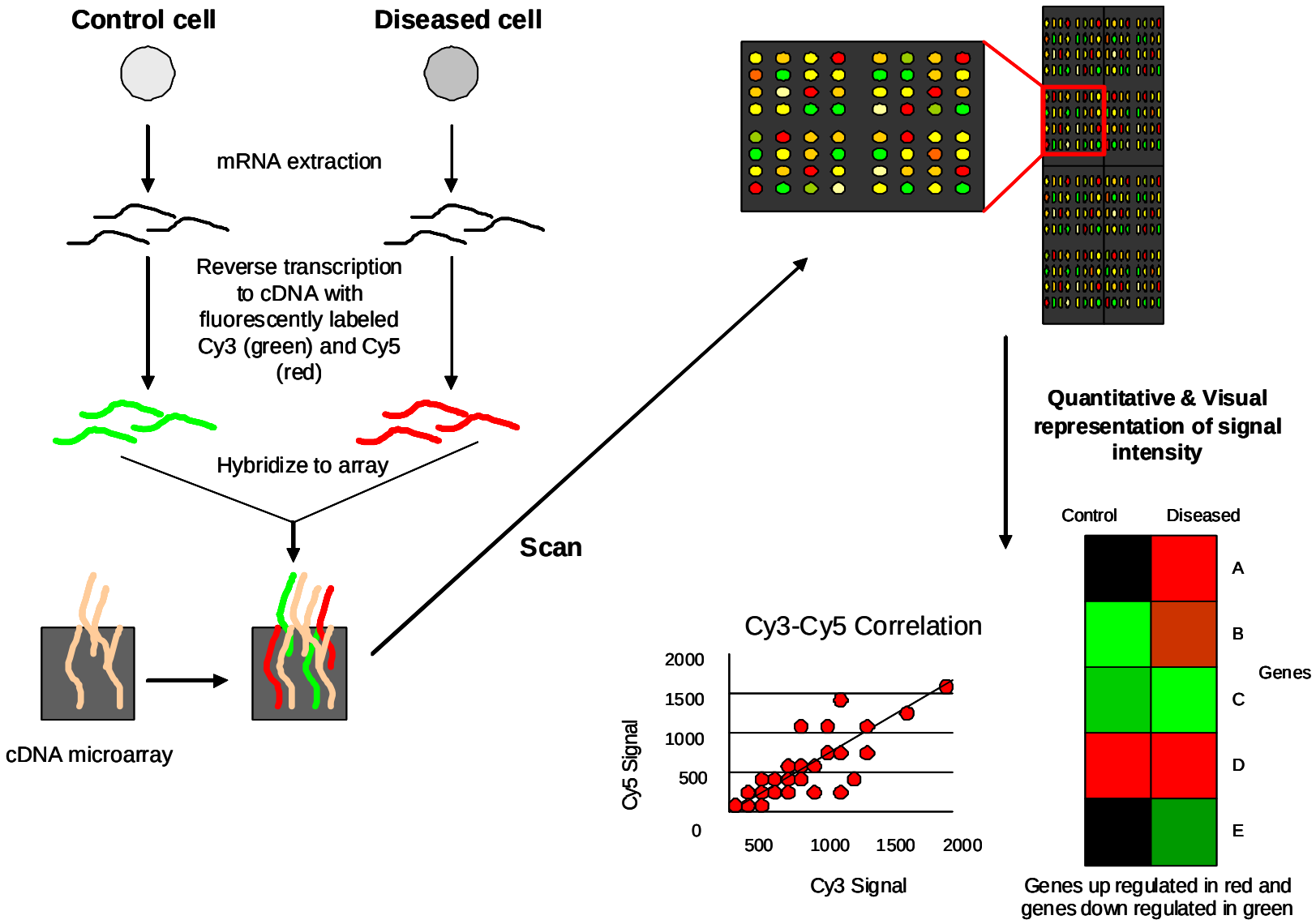


Figure 4. Two color cDNA microarray

Oligonucleotide arrays consist of shorter nucleotide sequences and have been pioneered by the commercial companies Affymetrix© and Illumina© (Lipshutz, Fodor et al. 1999). Since the lengths of the oligonucleotides are generally 25-50 bases, the density of these chips is much greater than that of cDNA arrays, allowing the user to assess greater numbers of gene products at the same time. Oligonucleotide arrays can be manufactured as traditional cDNA arrays where the probes are spotted or synthesized on a 2-dimensional substrate or by using BeadArray technology. Beadchips are constructed by introducing oligonucleotide bearing 3-micron beads into microwells etched into the surface of a slide-sized silicon substrate. Using the Illumina© platform, the beads self assemble onto the beadchips resulting in an average of 30-fold redundancy of every full-length oligonucleotide. After random bead assembly, 29-mer address sequences present on each bead are used to map the array, identifying the location of each bead. Oligonucleotide sequences are selected based on their uniqueness to the target genes and may require the use of several matched sequences for high specificity to a single target. Similar to cDNA arrays, RNA is extracted from target cells and allowed to hybridize to the oligonucleotide array. However, with oligo arrays, only a single fluorescent channel is used, thus only a single sample can be measured on one array.

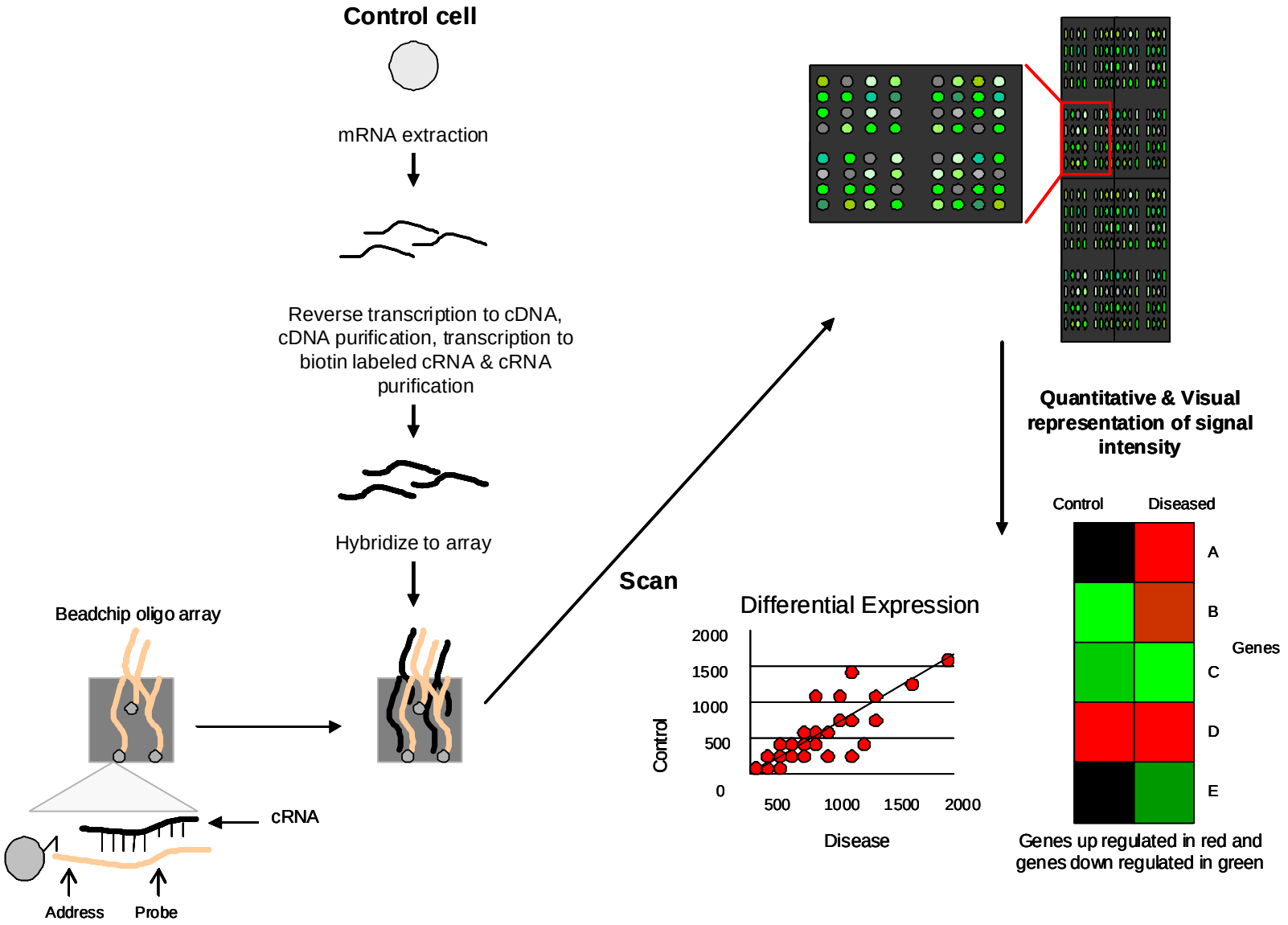


Figure 5. Beadchip oligonucleotide cDNA microarray

There are a number of other microarray platforms available, however the use of commercially developed products are encouraged to ensure robust and reproducible results. Before this method can be applied in routine clinical practice and research consensus on the validity of data generated across platforms must be reached. Efforts to examine the inter-platform variability have been undertaken by the Microarray Quality Control (MAQC) project as part of the FDA's Critical Path Initiative. http://www.fda.gov/nctr/science/centers/toxicoinformatics/maqc/ The data reveal promising results regarding the consistency of findings across different platforms and laboratories (Guo, Lobenhofer et al. 2006; Patterson, Lobenhofer et al. 2006; Shi, Reid et al. 2006). Even so, some still claim there is a significant divergence of conclusions (2006). Those who support the findings of the MAQC project suggest that reliable estimates of gene expression can be obtained equally across platforms (Woo, Affourtit et al. 2004; Barnes, Freudenberg et al. 2005; Maouche, Poirier et al. 2008). Inconsistencies across platforms can be attributed to different background correction methods and normalization techniques, cross-hybridization problems, technical noise and ultimately investigator interpretation. All of which can lead to over or under estimates of expression levels (Shi, Reid et al. 2006). The important point to remember is that dependable results will be produced with either commercial platform as long as the user identifies clear project goals and designs the study appropriately, follows experimental procedures correctly and minimizes procedural variation, and employs appropriate statistical modeling to analyze the differential expression.

2.3.2.1 Animal proof of principle studies

A series of four animal studies were conducted by Sharp and colleagues to examine the relationships between brain and blood genomic profiles in brain injury and whether or not blood

genomic responses could serve as fingerprints of medical or neurological diseases. (Tang, Lu et al. 2001; Tang, Lu et al. 2002; Lu, Tang et al. 2003; Tang, Nee et al. 2003)

The first study examined whether the gene expression profile of systemic white blood cells (WBC) could serve as a fingerprint of different types of neurological insults. (Tang, Lu et al. 2001) Adult male rats were grouped and subjected to one of the following: ischemic stroke; hemorrhagic stroke; kainite induced seizure; insulin-glucose deprivation; hypoxia; untouched controls; and sham. Each condition demonstrated a unique gene expression pattern in the WBCs and it was suggested that the differences found in the WBC profiles could relate to the unique immune responses to the different induced injuries. This was the first study to show the potential for genomic profiling of the blood response as a marker of end-organ disease.

A similar study design was employed with the aim of examining the role of neurologic insults on the gene expression profile of brain tissue. (Tang, Lu et al. 2002) In comparison to the 6 other groups studied (ischemic stroke; hemorrhagic stroke; kainite induced seizure; insulin-glucose deprivation; hypoxia; untouched controls; and sham.), brain ischemia altered the transcription of the most genes in general and induced the greatest number of immune mediated molecules along with a family of hypoxia-responsive genes. This could possibly be due to the fact that ischemia damages all cellular components of the cerebrum including neurons, glia, axons, white matter, and the cerebral vasculature. A surprising finding here was a failure to detect common genes that were down regulated in all conditions, suggesting that transcriptional down regulation may not be a major part of the brain injury genomic response.

The first whole blood expression profiling study of ischemic stroke in animals was performed in 2003. (Tang, Nee et al. 2003) Rats were grouped as before and RNA was isolated from mononuclear and polymorphonuclear cells, including RNA from red blood cells and

platelets. This study verified that gene expression profiling of whole blood demonstrates unique gene signatures for each group studied, suggesting that expression profiling of peripheral whole blood could be used to assess the occurrence of neuronal injury associated with various neurological insults, including ischemic stroke. An important finding of this study was that many regulated genes identified via expression profiling were not shared between isolated mononuclear cells and the whole blood experiments.

A subsequent study of the genomic expression profile of brain tissue identified a series of molecular pathways associated with cerebral ischemia in the rat. (Lu, Tang et al. 2003) The multiple numbers of genes induced after ischemia suggests that future pharmacological approaches should be designed to target multiple pathways simultaneously, instead of targeting single genes or single gene pathways.

These studies were crucial to identifying the utility of genomic expression profiling of peripheral blood in ischemic stroke. The next steps were to replicate the findings in humans, offering a promise to transform stroke research.

2.3.2.2 Human proof of principle studies

Several mechanisms have been proposed to explain the genomic response of peripheral blood cells to cerebral injury. The most supported is that of the interaction between peripheral cells and their ability to migrate and infiltrate ischemic brain tissue.(Kochanek and Hallenbeck 1992) In the clonal selection theory, each lymphocyte is genetically committed to express a unique antibody or receptor that when encountered by external antigens, responds by clonal expansion and differentiation.(Jerne 1985; Neuberger 2008) Therefore various clones of lymphocytes will be selected and expanded in response to external stimuli, and the cumulative changes in the transcriptional pattern of blood could reflect those lymphocytes that have been selected to

respond to cerebral ischemia.(Sharp, Xu et al. 2007) Additionally, various signaling molecules and neurotransmitters are released into the serum in response to neuronal injury. Peripheral white cells contain neurotransmitter receptors and transporters which may play a role in signal transduction pathways and the immune response of the CNS. Therefore surveying the genomic fingerprint of peripheral whole blood can serve as a surrogate for the genomic response of ischemia within the cerebral environment.

The first blood gene expression profiling study of human ischemic stroke was published by Dr. Allison Baird and colleagues at the National institute of Neurologic disorders and stroke (NINDS) in 2005. (Moore, Li et al. 2005) The objective of the study was to evaluate whether it was possible to use whole blood expression profiling in human acute ischemic stroke with the following aims to determine: which genes were expressed in peripheral blood mononuclear cells (PBMC); whether there was a specific genomic profile for stroke; whether the genes found had clinical significance for stroke; and whether a larger study was justifiable. A total of 20 ischemic stroke patients were compared to a referent group of 20 mainly elderly volunteers. An additional group of patients with active multiple sclerosis (MS) (n=12) and control subjects (n=10) was used to represent a positive disease control to address whether the results could be due to a nonspecific stress response. For the ischemic stroke patients, blood was drawn as soon after hospitalization as possible: <24 hours (n=7), 24-48 hours (n=10), and >48 hours (n=3). Whole blood was obtained in EDTA blood tubes and RNA was isolated from PBMCs and hybridized to Affymetrix Human genome U133A microarrays.

Of the 5,060 genes found to be different between stroke patients and control subjects, after Bonferroni correction and prediction analysis for microarray (PAM) classification, 22 genes predominantly involved in activation and differentiation of WBCs were found to be statistically

significant. Refer to Appendix C for the gene list. Validation of the gene list resulted in a sensitivity and specificity of 89% and 95% respectively, indicating that expression profiles strongly differentiated stroke patient from control subject. Even after adjusting for stroke risk factors, there was still a substantial difference in gene expression between the groups. There was also a lack of concordance of the gene expression between stroke patients and MS patients, demonstrating that the expression profile found may indeed be specific for stroke.

Soon after the publication of Dr. Baird's study, Dr. Frank Sharp and colleagues followed up their animal gene expression profiling studies with a human ischemic stroke population.(Tang, Xu et al. 2006) The aim of the study was to examine the genomic changes present in whole blood before 3 hours, at 5 hours, and at 24 hours after ischemic stroke. A total of 15 stroke patients enrolled in a clinical trial (combination approach to lysis utilizing eptifibatide and recombinant tissue-type plasminogen activator (rtPA) (CLEAR)), and 8 healthy control subjects were recruited to address the aims of the study. For stroke patients, whole blood was obtained in Paxgene RNA tubes and for control subjects whole blood was obtained in EDTA blood tubes. RNA was isolated and hybridized to Affymetrix Human U133 Plus2 arrays.

The results identified 18, 22 and 24 genes significantly regulated at 3, 5, and 24 hours respectively compared to healthy control subjects. Nearly all of the genes regulated at 3 hours were also regulated at 5 and 24 hours. PAM classification identified 18 genes specific for ischemic stroke (Appendix D) and revealed that neutrophils and monocytes are the major blood cell types involved in the human blood genomic response to acute ischemic stroke. These findings suggest that the rapid changes of whole blood gene expression may be useful for making early diagnosis of ischemic stroke in humans. Post hoc analyses of this data set revealed

a unique pattern of gene expression for patients with cardioembolic versus large-vessel atherosclerotic disease. (Xu, Tang et al. 2008)

There are significant limitations and differences worth discussing between Dr. Baird's and Dr. Sharp's human studies. Only two genes found to accurately predict stroke were identified by both groups (v-ets Erythroblastosis virus E26 oncogene homolog 2 (avian); and N-acetylneuraminate pyruvate lyase (dihydrodipicolinate synthase) This is likely due in part to the fact that different cell populations were studied. The other major difference is that all of the patients in Dr. Sharp's study were treated with rtPA alone or rtPA plus eptifibatide as part of the CLEAR trial. It cannot be determined whether the changes in gene expression found in Dr. Sharp's study were the result of the ischemic stroke or the treatment of these medications. The groups used different microarray chips, with Dr. Baird's microarray chip containing significantly less probes. Additionally, the time of onset of symptoms was significantly different between the patient groups in each study.

A recent publication by Grond-Ginsbach et al (2008)(Grond-Ginsbach, Hummel et al. 2008) suggests that gene expression profiling of whole blood, compared to peripheral blood mononuclear cell (PBMC) isolation is the most promising for characterizing the blood genomic response to ischemic stroke (Grond-Ginsbach, Hummel et al. 2008). The group sampled acute ischemic stroke patients (n=15) within 22 to 26 hours from symptom onset and compared the expression profiles of PBMC's to an acute TBI cohort (n=15; 14 hours to 4 days from injury), healthy subjects without vascular risk factors (n=15), and stroke survivors (n=15). RNA samples for the groups were pooled into final samples of 4 pools for ischemic stroke, 3 pools for TBI, 3 pools for control subjects, and 3 pools of stroke survivors. Therefore a total of 13 array experiments were conducted. Only a single probe and single gene group yielded a significant

gene expression difference for stroke. Phosphodiesterase 4D (PDE4D) was found to be 50% down-regulated for stroke, whereas genes involved in the inflammatory response were found to be up-regulated. They were not able to replicate the findings of the first two studies performed by Dr. Baird and Dr. Sharp. Possible reasons for this include the use of PBMC's for RNA isolation, blood sampling after 24 hours from onset of stroke symptoms, pooling of RNA samples into 4 stroke groups, small sample size, and the use of different statistical analysis techniques.

2.4 SIGNIFICANCE AND INNOVATION

Despite promising preclinical therapeutics, rtPA is the only FDA approved treatment for acute ischemic stroke. There is an urgent need to reassess the best approach to study the human response to brain injury moving forward (Maas, Marmarou et al. 2007). Most of what we have discovered of the physiologic response to ischemic brain injury has been identified in preclinical animal and cell modeling and treatments based on these findings have been largely unsuccessful, therefore a redirection of the science is necessary. A novel approach to the study of ischemic stroke is the use of gene expression profiling to identify biomarkers associated with ischemic stroke diagnosis. A secondary advantage of gene expression profiling is its ability to unveil the molecular pathways involved in brain recovery and health and elucidate complex genomic interactions that may play a role in outcome. Since brain tissue is neither easily accessible nor practical to obtain in live subjects and post mortem analysis can introduce bias, we need to rely on other mechanisms as the means by which we analyze gene expression in most patients affected by stroke and neurological disease. Peripheral blood is easily accessible in all clinical

situations and therefore provides greater clinical utility. There is the hope that this approach could aid in earlier diagnosis of neurological disease, identify therapeutic targets for brain injury, and help in treatment decision making and predictions regarding prognosis (Baird 2006; Sharp, Xu et al. 2006).

The advantages of this proposed thesis study are the following: peripheral blood samples of stroke patients will be gathered within 24 hours from onset of symptoms; the follow up blood draw will provide an unbiased expression profile; diagnosis of ischemic stroke will be made by MRI; patients will be matched to healthy control subjects in an attempt to control for environmental variation; and molecular pathways will be assessed for their relevance in ischemic stroke.

3.0 PILOT STUDIES

3.1 PILOT STUDY I, MANUSCRIPT I: MMP9 AND HARM

Barr T, Latour LL, Lee KY, Schaewe T, Luby M, Chang G, El-Zammar Z, Alam S, Kidwell C, Warach S.

Early blood brain barrier disruption is associated with plasma matrix metalloproteinase-9 concentration in acute stroke patients.

Targeted Journal: Stroke.

*Podium Presentation International Stroke Conference 2008

Abstract

Objective: Matrix metalloproteinases (MMP's) may play a role in blood brain barrier (BBB) disruption following ischemic stroke. BBB disruption is characterized by gadolinium enhancement of cerebrospinal fluid (CSF) on fluid attenuated inversion recovery (FLAIR) magnetic resonance imaging (MRI) and is termed *H*yperintense *A*cute *R*eperfusion injury *M*arker (HARM). We hypothesized that acute *MMP9* is associated with a marker of BBB disruption on MRI.

Methods: Patients evaluated for acute stroke underwent MRI on presentation and 24 hours later. The presence of HARM on post-contrast FLAIR images was assessed by blinded readers. *MMP9* and tissue inhibitor of matrix metalloproteinase -1 (*TIMP1*) was measured by ELISA. Logistic regression models tested for predictors of HARM on 24 hour follow-up scans separately for *MMP9* and *MMP9*/*TIMP1* ratio.

Results: Forty-one patients were enrolled. Diagnoses were acute ischemic cerebrovascular syndrome 34 (82.9%), intracerebral hemorrhage 6 (14.7%) and stroke mimic 1 (2.4%). HARM was present on 24 hour follow-up in 17 (41.5%) patients. In model 1, HARM was associated with acute plasma *MMP9* concentration: odds ratio (OR) = 1.01 (95% confidence interval (CI) =1.001-1.019), p=0.033. In model 2 HARM was associated with *MMP9*/*TIMP1* ratio: OR=4.94 (95% CI=1.27-19.14), p=0.021.

Interpretation: Acute *MMP9* was a significant predictor of HARM at 24 hour follow-up, supporting the hypotheses that HARM reflects early BBB disruption and MMP9 is associated with BBB disruption. If the association between *MMP9* and HARM is confirmed in future studies, HARM may be a useful imaging marker to evaluate *MMP9* inhibition in ischemic stroke and other populations with BBB disruption.

*Barr, T. performed all ELISA's necessary for the completion of this study.

3.2 PILOT STUDY II: *KCNK17* AND ISCHEMIC STROKE

Barr, Taura; Nalls, Mike; Arepalli, Sampath; Singleton, Andrew; Meschia, James; Hardy, John; Matarin, Mar

Polymorphisms in *KCNK17* are not associated with ischemic stroke

*Poster Presentation Eastern Nursing Research Society Conference 2009

Abstract

Objectives: The recent genome wide association (GWA) study of ischemic stroke (IS) identified hundreds of statistically significant single nucleotide polymorphisms (SNPs) associated with IS. The objective of this study was to attempt to replicate the significant association found between these SNPs in the *KCNK17* gene and ischemic stroke (IS) in a separate cohort of Caucasian stroke patients and control subjects of North American descent.

Methods: A total of 548 stroke patients and 376 control subjects were included in this replication study. All samples were selected from the Ischaemic Stroke Genetics Study (ISGS), a prospective five-centre case-control study in the USA; unrelated IS affected probands from the SWISS study; a sibpair linkage study; and IS cases from the National Institute of Neurological Disorders and Stroke neurogenetics repository. All individuals underwent a detailed medical history and diagnosis of ischemic stroke by trained neurologists. For the 3 SNPs (rs2395721, rs10807204, rs10947803) single-tube reagent-based genotyping assays (TaqMan) were used. Results were validated by direct DNA genotyping in a subset of samples (n=96). From our GWA study in IS, 100 samples were genotyped with the same probes used in this study to test the

concordance between assays. All five exons of *KNCK17* were genotyped in 288 stroke patients and 288 control subjects to examine the variability of the gene.

Results: No significant association was found between genotype of either SNP and IS in 924 individuals after adjusting for stroke risk factors (age, gender, hypertension, diabetes, atria fibrillation, and myocardial infarction). Sequencing of 576 stroke patients and control subejcts across exons 1-5 did not reveal obvious candidates for IS with the exception of one stop codon in exon 5, which was found in only one stroke patient.

Interpretation: The genetic variants examined in the *KCNK17* gene did not reveal a reproducible association with ischemic stroke. The association found in our recent GWA study of ischemic stroke may have been a false positive.

3.3 PILOT STUDY III, MANUSCRIPT II: AGE AND HARM

Barr, T.; Latour, L.; Kidwell, C.; Lee, K.; Merino, J.; Warach, S.

Age is the major risk factor of early blood brain barrier disruption in acute ischemic stroke

Targeted Journal: Stroke.

*Poster Presentation International Stroke Conference 2009

Abstract

Objective: Advanced age is a known predictor of ischemic stroke (IS) and is associated with the presence of *H*yperintense *A*cute *R*eperfusion injury *M*arker (HARM) in IS patients. It is proposed

that the integrity of the blood brain barrier (BBB) is compromised with age. The objective of this study was to further examine the relationship between age and HARM in IS patients and determine whether age remains a significant predictor of HARM after controlling for comorbidities and known risk factors associated with advanced age.

Methods: This is a pooled analysis of 235 patients from studies conducted between June 2000 and June 2007. Clinical demographic data and risk factor history was obtained by the patient or family members during the acute clinical workup. The time of onset was determined as the time the patients were last known to be normal or free of acute symptoms, if exact time of onset was unknown. Patient evaluations and management were standardized. Patients were included if they received a diagnosis of ischemic stroke, had a baseline MRI within 24 hours from time of stroke onset and received a follow up MRI with contrast-enhanced fluid attenuated inversion recovery (FLAIR) imaging. HARM was determined as hyperintensity present in sulci across greater than 10 MRI slices and dichotomized as present or absent. A forward conditional logistic regression with an entry criterion of 0.15 was performed in an attempt to develop a predictive model of HARM based on the following covariates: age, National Institutes of Health Stroke Scale Score (NIHSS), administration of tissue plasminogen activator (tPA), and history of hypertension, diabetes, hyperlipidemia, or ischemic stroke.

Results: Age (OR=1.03; p=0.002), NIHSS (OR=1.08; p<0.000), hypertension (OR=2.18; p=0.033), previous ischemic stroke (OR=2.73; p=0.004), and tPA administration (OR=2.24; p=0.009) were associated with presence of HARM in univariate analysis. In a binary logistic regression model, age >75 years (OR=3.46; p=0.000), NIHSS category (OR=1.46; p=0.018), previous ischemic stroke (OR=2.27; p=0.036), history of diabetes (OR=2.04; p=0.073), and thrombolysis (OR=1.86; p=0.083) were predictors of HARM. The odds of developing HARM in

patients with age greater than 75 years was 3.4 times higher than for younger patients ($p<0.00$). In addition, patients with age greater than 75 years of age who received tPA had a higher risk of developing HARM (OR=3.2; p=0.012) when compared to those younger in age who received tPA (OR=1.7; p=0.26).

Conclusions: After adjusting for risk factors known to be associated with advanced age and presence of HARM, age remained the strongest predictor of BBB disruption. We have confirmed other factors that independently contribute to the risk of BBB disruption. BBB changes associated with age may be the most clinically relevant for stroke and should be accounted for in future studies of BBB biology. It will be important to determine what factors associated with age contribute to HARM in this population.

3.4 REVIEW, MANUSCRIPT III: GENE EXPRESSION PROFILING IN BRAIN INJURY

Barr, Taura; Alexander, Sheila; Conley, Yvette.

Gene expression profiling for discovery of novel targets in human traumatic brain injury

Submitted to Journal of Neurotrauma April 13, 2009

Abstract

Several clinical trials have failed to demonstrate a significant effect on outcome following human traumatic brain injury (TBI) despite promising results obtained in pre-clinical animal studies. This may be due in part to a misinterpretation of the findings obtained in pre-

clinical animal models of TBI; a misunderstanding of the complexity of the human response to TBI; limited knowledge about the biological pathways that interact to contribute to good and bad outcomes after brain injury; and the implications of genomic variability and environment on individual recovery. Recent publications suggest data obtained from gene expression profiling studies of complex neurological diseases, such as Stroke, multiple sclerosis, Alzheimer's and Parkinson's may contribute to a more informed understanding of what contributes to outcome following TBI. This may help to bridge the gap between successful preclinical studies and negative clinical trials in humans to reveal novel targets for therapy. Gene expression profiling has the capability to identify biomarkers associated with response to TBI, elucidate complex genetic interactions that may play a role in outcome following TBI and reveal biological pathways related to brain health. The following review highlights the current state of the literature on gene expression profiling, discuss its ability to aid in unraveling the variable human response to TBI and the potential for it to offer treatment strategies in an area where we currently have limited therapeutic options primarily based on supportive care.

4.0 METHODS

4.1 DESIGN

A whole genome expression analysis with a case control design is proposed to assess whole blood gene expression in acute ischemic stroke patients (cases) compared to neurologically healthy control subjects. The primary aim of the study is to determine which genes are under- and over-expressed in acute ischemic stroke patients compared to neurologically healthy control subjects. The secondary aim of the study is to determine the changes in gene expression that occur in the first 24-48 hours following acute ischemic stroke. The tertiary aim of the study is to determine whether acute ischemic stroke patients with BBB disruption have a specific blood genomic profile compared to AIS patients without BBB disruption.

Gene expression profiles for acute ischemic stroke patients will be assessed at acute and twenty four hour time points to evaluate changes in gene expression over time using a within patient control approach. For the last aim of the study, stroke patients with BBB disruption will be matched to stroke patients without BBB disruption. Whole genome expression microarrays will provide a specific gene expression profile in whole blood for acute ischemic stroke patients, neurologically healthy control subjects, and patients with BBB disruption.

4.2 SAMPLE

A total of 39 acute ischemic stroke patients and 25 neurologically healthy matched control subjects were recruited to address the aims of the study. The NIH Stroke Team is consulted on about 500 cases per year at Suburban Hospital Bethesda, MD. Of those 500 cases, about 60% (n=300) receive a diagnosis of probable or definite acute ischemic cerebrovascular syndrome (AICS) (Kidwell and Warach 2003). All patients consulted were eligible for the study. Those enrolled included patients with diagnosis of definite acute cerebrovascular syndrome (ischemic stroke) and non-stroke patients without neurologic deficits that could be used as control subjects. Recruitment took place weekdays Monday-Friday during regular working hours. To achieve the original goal of 22 acute ischemic stroke patients, over sampling of acute ischemic stroke patients occurred for a goal of 40.

To increase the success of healthy volunteer enrollment in future studies, a Healthy Volunteer Protocol has been written and submitted to the Institutional review board (IRB) to allow for the enrollment of patient family members or significant others. Once this is approved, family members or significant others of patients enrolled in the NIH Natural History study will be approached for enrollment into subsequent gene expression profiling studies. This approach has the advantage of attempting to control for some environmental factors associated with differences in gene expression profiles. Non-hospital admission volunteers will be recruited and evaluated at Suburban Hospital. Subjects will be considered for enrollment if they contact the research team and would like to volunteer to participate in the study or are a family member/significant other of a patient enrolled in the Natural History Disease Pathogenesis of Stroke study. Following enrollment, volunteers will be assigned a date and time in which to

return to the magnetic resonance imaging (MRI) suite. Healthy subjects will not be followed over time.

For the present study, a healthy volunteer cohort of 25 subjects has been recruited under a separate IRB approved protocol of Dr. Andrew Singleton's. The population consists of friends or family members of patients affected with amyotrophic lateral sclerosis (ALS). Medical history, family history and medication history was obtained on all control subejcts. None of these volunteers underwent MRI testing nor were they followed over time.

Peripheral blood samples were collected from patients who met the following inclusion criteria: age ≥18 years with diagnosis of definite acute ischemic cerebrovascular syndrome (acute ischemic stroke) or those who were non-stroke neurologically healthy control subjects who presented to Suburban Hospital, Bethesda Maryland. Exclusion criteria included pre-existing neurological disease, patients presenting past 24 hours following onset of symptoms and those patients with contraindications to MRI scanning. All patients received an MRI of the brain on admission and follow-up on day two. Non-hospital admission healthy control subjects were recruited on a volunteer basis. Upon enrollment the subjects had an MRI performed and the subject's blood was drawn. For those patients with acute ischemic stroke blood was redrawn 24 hours following onset of symptoms to determine changes in gene expression profiles over time. Acute ischemic stroke patients were frequency matched to healthy control subjects on a 1:2 basis by gender, age within +/- 10 years and race. There were only three African American (AA) patients recruited at Suburban hospital and no AA control subjects were recruited; therefore the AA patients were left out of this study to avoid potential problems with population stratification. A subsequent sample collected at Washington Hospital Center, Washington DC will be analyzed

to determine the validity of the gene expression profile identified in this study for an AA population.

4.3 RATIONAL FOR SAMPLE SIZE

Sample size calculations for microarray analysis are based on the number of genes available on the chip, probability of Type 1 error, probability of Type 2 error, random error, and effect size. Sample size calculations have been conducted using PASS: Power analysis and sample size system and JMP software. A 2.0 fold change is generally expected in genes of significance; however a smaller fold change of 1.5 may be used to identify genes with similar but slight differences in expression between the two groups. For Aim 1, a total of 22 patients and 22 healthy control subjects achieves 90.68% power for each gene to detect a true difference in expression with at least a 1.5 fold change and an estimated standard deviation of 1.5 with a false discovery rate of 0.05 using a two-sided one-sample t-test. Of the 50 genes with anticipated true mean difference in expression >1.5, 45 are expected to be detected. For Aim two, a total of 22 patients with two time points will achieve the same: 90.68% power for each gene to detect a true difference in expression with at least a 1.5 fold change and an estimated standard deviation of 1.5 with a false discovery rate of 0.05 using a two-sided one-sample t-test. For Aim three, eight patients with BBB disruption and eight patients without BBB disruption achieves 90% power for each gene to detect a true difference in expression with a 2.0 fold change and estimated standard deviation of 1.1 with a false discovery rate of 0.05 using a two-sided one-sample t-test. See Appendix E for sample size calculations.

4.4 SETTING

Patients were recruited from the Suburban Hospital Stroke Team Center. All patients were initially evaluated in the hospital Emergency Department or an inpatient unit and followed for 24 hours. Suburban hospital is a community owned, not-for-profit 228 bed hospital located in Bethesda Maryland. In 1999 the NIH stroke program at Suburban hospital was established and became the first acute stroke program in Montgomery County Maryland. The acute stroke team is available 24 hours a day seven days a week and patients evaluated have direct access to the NIH MRI center with a 1.5T MRI.

4.5 RECRUITMENT

Subjects were chosen for this study from patients enrolled in a larger prospective NIH Natural History of Stroke study conducted by the NIH/NINDS Stroke Program at Suburban Hospital. Patients were consented either by T. Barr or an associate investigator of the larger NIH study. Each patient received an oral and written explanation of the purposes, procedures, and potential risks of the study. Copies of the signed informed consent were obtained and placed in the patient's medical chart, research chart, and one copy given to the patient. Due to the need to include all patients who presented to the emergency department with suspected acute stroke there were patients who were cognitively impaired as a result of their medical condition. Surrogate consent was obtained for these individuals. Exclusion criteria included: patient's less than 18 years of age; patients presenting >24 hours following onset of symptoms; and patients with contraindication to MRI scanning including patients with the following devices or conditions: a.

Central nervous system aneurysm clips b. Implanted neural stimulator c. Implanted cardiac pacemaker or defibrillator d. Cochlear implant e. Ocular foreign body (e.g. metal shavings) f. Insulin pump g. Metal shrapnel or bullet h. Any implanted device that is incompatible with MRI; patients with a condition precluding entry into the scanner (e.g. morbid obesity, claustrophobia, etc.).

4.6 STANDARD MEDICAL CARE

All patients recieved standardized medical care based on management decisions established by Class 1 evidence and recommendations of professional societies. All members of the clinical stroke team follow the "Standard Non-Investigational Inpatient *Stroke Care Protocol* for Acute Ischemic Cerebrovascular Syndromes of the NINDS Stroke Diagnostics and Therapeutics Section". Acute management includes stabilization of the patient to maximize cerebral perfusion (HOB flat, IV normal saline); no treatment of blood pressure except as required for thrombolytic therapy or SBP>220 or DBP>120; stroke labs; stroke MRI stat (or CT if MRI contraindicated); and accurate determination of last known to be free of symptoms to determine eligibility for rtPA therapy. National Institutes of Health Stroke scale (NIHSS) is performed during the initial clinical workup and at subsequent neurologic examinations. The NIHSS is a 15-item assessment tool that provides a quantitative measure of neurologic deficit. Patients were admitted to the Stroke Unit or the Intensive care unit (ICU) and received care to maximize cerebral perfusion pressure; started on deep vein thrombosis (DVT) therapy; strict glucose monitoring and control; and received a complete work up for stroke etiologies and risk factors. Secondary stroke prevention was started as early as possible.

4.7 DATA COLLECTION

Demographic and medical data was collected as part of the clinical work up and recorded on the clinical data sheets by a member of the stroke team. Case report forms (CRFs) were developed for this study (Appendix F) and were filled out by T. Barr using the clinical data sheets, discussion with the patient and a clinical member of the stroke team who was present during the patient's acute work up. Laboratory data and in-hospital medication administration data was gathered from Suburban hospitals Meditech system for the patients hospital stay and placed with the CRFs and clinical data sheets in the research chart. Patient research charts are stored in a locked office. All data was entered and verified into an SPSS database, which has been designed specifically for this project.

Acute peripheral blood samples were obtained via standard phlebotomy procedures by T. Barr following consent. Follow up blood draws were performed by T. Barr as close to 24 hours following onset of symptoms. All blood draws were performed via venipuncture for a total of 5 ml of blood at each time point. Specimens were immediately taken to the Suburban hospital laboratory and placed in a -80°C freezer until RNA isolation. All data and specimen collection, processing, and laboratory analysis was conducted by T. Barr under the supervision of Dr. Steven Warach and Dr. Andrew Singleton.

4.8 DATA MANAGEMENT

Subjects were assigned a unique identification code upon admission to the study that was used to link all clinical and medical data to the appropriate laboratory specimens. Data was entered into

an SPSS database and verified at a separate time by the T. Barr. All hard copy data sheets are kept in a locked office and the SPSS database is kept on a password protected computer.

5.0 VARIABLES, MEASURES, AND LEVEL OF MEASUREMENT

5.1 GENE EXPRESSION

5.1.1 Paxgene Blood RNA system

The best tissue for gene expression studies of stroke would be human brain tissue, however this is clearly not feasible given the fact that most ischemic strokes are relatively mild and therefore do not provide an opportunity to collect brain tissue samples (either through decompressive craniotomy or autopsy after death). The alternative is gene expression profiling of peripheral blood. Peripheral blood analysis is a powerful tool for biomarker discovery in the clinical setting. Although peripheral blood samples are collected routinely in practice, it is relatively difficult to maintain the integrity and biological signature of RNA because mRNA since it is constantly being degraded in the cytoplasm and therefore the half life is short lived. A widely used method for RNA isolation requires the isolation of peripheral blood mononuclear cells (PBMC). This additional step adds to both the time and instrumentation requirements for RNA isolation, and may potentially cause a change in the biological signature of the RNA (Debey, Schoenbeck et al. 2004).

A new technology that has recently gained entrance into the clinical arena, especially clinical trials research is the Paxgene Blood RNA isolation system. Paxgene blood RNA tubes

contain a reagent that protects RNA from degradation, preserving the RNA expression profile during and after phlebotomy. Using Paxgene RNA tubes eliminates the isolation of PBMC by integrating and consolidating nucleic acid stabilization and RNA purification thereby reducing the unpredictability associated with RNA processing of PBMC isolation and therefore enhances the accuracy of RNA expression analysis. Paxgene RNA tubes are a reliable source of RNA isolation and have been shown to be a valid approach to RNA extraction from human whole blood (Rainen, Oelmueller et al. 2002). Proof of principle studies have been performed in our laboratory verifying the Paxgene RNA isolation method (Barr, Unpublished data). For its ease of use in the clinical setting, and its ability to maintain the specific biological signatures at the time of the blood draw, Paxgene blood RNA tubes were used to collect 2.5ml of peripheral blood from subjects using standard phlebotomy procedures then immediately inverted 8-10 times to ensure adequate red blood cell lysis.

5.1.2 RNA extraction

Paxgene blood RNA tubes were immediately placed in a -80°C freezer at Suburban Hospital until RNA isolation and analysis. All frozen whole blood specimens were allowed to thaw at room temperature for 24 hours on a rotating bed prior to RNA isolation procedures to ensure complete red blood cell lysis, per manufacturer recommendations. Once thawed, purification began with centrifugation to pellet the nucleic acids. The pellet was washed and resuspended in buffer with proteinase K to optimize protein digestion. The lysate was then centrifuged through a Paxgene shredder spin column for homogeneity and to remove cell debris. Then through a process of washes and centrifugation, RNA was eluted in the buffer. Total RNA purified using

the Paxgene system is highly pure, with *A*260/*A*280 values between 1.8 and 2.2 and ≤1.0% (w/w) genomic DNA. RNA yields are ≥3 μg from 2.5ml of whole blood. Appendix G

RNA isolated from whole blood typically consists of a high proportion of mRNA encoding globin polypeptides. Some have been concerned that this high proportion of globin mRNA results in reduced sensitivity of hybridization on some microarray platforms. A recent study demonstrated that globin reduction does not increase the number of differentially expressed transcripts when hybridizing to HumanRef-8 v2 beadchips and therefore has little impact on probe detection when using the Illumina platform. (2007) Therefore, globin reduction was not conducted on any sample in this study.

5.1.3 RNA amplification

The Illumina TotalPrep RNA amplification kit was employed to generate biotinylated, amplified RNA for hybridization to the arrays. The procedure consisted of reverse transcription with an oligo (dT) primer and a reverse transcriptase designed to produce higher yields of first strand cDNA. The cDNA underwent a second strand synthesis and clean up to become a template for in vitro transcription. The in vitro transcription resulted in biotin labeled antisense cRNA copies of each mRNA in a sample which will be hybridized to the arrays. Appendix H

5.1.4 Human Ref-8 v2 expression bead chips

Illumina humanRef-8 v2 expression bead chips were used for gene expression analysis and have the capability to analyze 18,631 unique curated genes at once. The beadchips contain up to date content derived from the National Center for Biotechnology Information reference sequence

(NCBI RefSeq) database. The multi-sample format allows for up to eight samples to be arrayed in parallel, increasing throughput and decreasing experimental variability. Reproducibility has been demonstrated by high concordance and precision between hybridization replicates. The expression beadchips are constructed by introducing oligonucleotide bearing 3-micron beads into microwells etched into the surface of a slide-sized silicon substrate. The beads self assemble onto the beadchips resulting in an average of 30-fold redundancy of every full-length oligonucleotide. After random bead assembly, 29-mer address sequences present on each bead are used to map the array, identifying the location of each bead. cRNA will be fragmented prior to hybridization to the gene chip. Sample labeling, hybridization, and scanning were conducted using standard Illumina protocols. Appendix I

5.1.5 Quantitative real time PCR validation

Taqman gene expression probes exploit the 5' nuclease activity of Taq DNA polymerase to detect the accumulation of target PCR product by using a cleaved fluorescent probe. Most are available as "off-the-shelf" assays and all have been designed to have high amplification efficiency and specificity for the target gene, eliminating non-specific amplification. A housekeeping gene (Beta-actin) was chosen from the microarray data for normalization of target genes because the expression between stroke patients and control subjects was relatively the same for Beta-actin. cDNA was generated from total RNA per standard laboratory technique and RT-PCR was performed on 5 of the significant genes determined by array analysis normalized by Beta-actin on all 39 stroke patients and 24 control subjects in triplicate for each gene. These 5 genes were chosen because four were also found to be significant in the first gene

expression profiling study for stroke and one was the only down-regulated gene in stroke. See Appendix J for cDNA synthesis and RT-PCR protocol.

A key attribute to PCR is that there is a doubling of PCR product with every PCR cycle. Identification of the PCR cycle when the exponential growth phase is first detectable (cycle threshold=C_T) is an accurate measurement of quantitative gene expression. (2006)When using Taqman gene expression assays the comparative C_T ($\triangle\triangle C_{T)}$ method for determining relative fold change correlates well with expected fold change values.(2006) The $\triangle\triangle C_T$ method assumes the amplification efficiency is the same across all probes. C_T values greater then 35 approach the sensitivity limits of the detection system and were therefore eliminated from analysis. The average C_T value for each sample was identified across the triplicate wells. Then an overall C_T average for stroke subjects and control subjects was determined. The difference between the Beta-actin and the target gene average was determined for stroke subjects and control subjects separately and then the $\triangle\triangle C_T$ values between the target and Beta-actin were converted to relative expression (RQ) values using the following equation: $2^{-\triangle\triangle CT}$. Validation was determined positive if the relative fold change in expression was similar to what was identified with the microarray results; t-test analysis revealed significance and there was a positive correlation between PCR and microarray results.

5.2 HYPERINTENSE ACUTE REPERFUSION INJURY MARKER

MRI was performed using a 1.5-Tesla clinical MR system on patients upon admission or onset of symptoms and follow up at 24 hours. MR imaging of healthy control subjects was not performed in this study. The scanning protocol for patients was standardized for sequence parameters and

order of acquisition to include: diffusion weighted imaging (DWI), T2*-weighted gradient recalled echo (GRE), FLAIR, and perfusion-weighted imaging (PWI). Perfusion weighted imaging was obtained using the bolus passage of contrast method by injecting Gd-DTPA at a dose of 0.1 mmol/kg via power injector. Image analyses were performed independently and blind to clinical information. MRI was assessed for location of HARM (e.g., sulcal, bilateral, ventricles, vitreous) and level (none, mild, moderate, or severe). HARM was identified positive when the intensity in the cerebrospinal fluid (CSF) in the sulci or ventricles appeared hyperintense in comparison with the previous examination. Level of HARM was identified as a categorical variable: 1. No HARM: Black CSF or 1-2 point like hyperintense regions; 2. Mild: Numerous point-like hyperintense regions; 3. Moderate HARM: Linear hyperintense regions confined to less than 10 MRI slices; 4. Severe HARM: Linear and continuous hyperintense regions present in at least 10 MRI slices. For the purpose of analysis, previous work conducted by investigators in the Section on Stroke Diagnostics and Therapeutics, NINDS/NIH have found that Severe HARM is the only clinically relevant presentation of HARM, therefore HARM was further categorized as a binary variable: Severe HARM yes/no. Differential gene expression was assessed across all groups then by HARM Severe yes/no. See Appendix B for NINDS Stroke Team-HARM Rating Scale developed by investigators in the Section on Stroke Diagnostics and Therapeutics, NINDS/NIH.(Latour, Kang et al. 2004)

5.3 TIME

Time of onset was identified as the time the patient was last known to be normal or free of symptoms. At times this was hard to determine, especially if the patient lived alone or had

neglect and was unaware of their symptoms. In this case, the time of onset was determined as when the patient was last seen normal. If a patient fell asleep normal and woke up in the morning with symptoms, the time of onset was noted as the time the patient fell asleep. All attempts to determine an accurate time of onset were made by the stroke neurologist, especially when the patient was eligible for rtPA therapy.

The acute blood draw was performed as early after time of onset, but not greater than 24 hours from time of onset. The follow up blood draw was drawn as close to 24 hours from time of onset, or last known normal as possible. In times where the acute blood drawn was performed near 24 hours from time of onset, the follow up blood draw was timed as 24 hours from acute blood draw.

5.4 STROKE PATIENTS AND CONTROL SUBJECTS

Cases were classified by AICS diagnostic criteria (Kidwell and Warach 2003) during the stroke neurologists assessment at the acute work up. This classification scheme for ischemic stroke defines four categories ranging from “Definite AICS” to “Not AICS”, based on evidenced-based neuroimaging diagnostic certainty. Transient ischemic attack (TIA) generally refers to an episode in which symptoms resolve completely and has been considered a different clinical entity than ischemic stroke. However, MRI and MR spectroscopy have identified that the majority of TIA patients have imaging evidence of permanent ischemia. For this reason, a redefinition of TIA based on tissue pathophysiology rather than an arbitrary time cutoff was warranted. This type of diagnostic certainty provides universally applicable and standardized definitions important for clinical therapeutic decision making and rigorous research studies and clinical trials. All patients

with imaging positive ischemia are categorized as Definite AICS, regardless if the symptoms resolve. For the purposes of this study, only patients with a diagnosis of "definite" AICS were included. Patients with a diagnosis of "probable" or "possible" AICS were excluded from this study. Refer to appendix A for the classification categories of AICS.

For stroke patients, ischemic stroke was classified using the TOAST (Trial of ORG 10172 in Acute Stroke Treatment) subtype criterion which classifies stroke into 5 core pathologic groups: large-artery atherosclerosis (LAA), including large-artery thrombosis and artery-to-artery embolism; cardioembolism (CE); small artery occlusion (SAO); stroke of other determined cause (OC); and stroke of undetermined cause (UND).

Three month outcome was determined using the Modified Rankin Scale score (MRS). The MRS measures degree of disability following stroke on a 0-6 scale, from no symptoms to death: 0- No symptoms at all; 1- No significant disability despite symptoms; able to carry out all usual duties and activities; 2-Slight disability; unable to carry out all previous activities, but able to look after own affairs without assistance; 3- Moderate disability; requiring some help, but able to walk without assistance; 4- Moderately severe disability; unable to walk without assistance and unable to attend to own bodily needs without assistance; 5-Severe disability; bedridden, incontinent and requiring constant nursing care and attention; 6- Dead.

Volunteers recruited under Dr. Singleton's protocol completed a neurologic Clinical Data Elements (CDE) form which included information about medical and family history and an additional form for medication history. Refer to Appendix K for a copy of the CDE's used to obtain medical histories.

6.0 DATA ANALYSIS

6.1 DESCRIPTIVE STATISTICS

Data analysis for gene expression was conducted using Illumina BeadStudio Gene Expression (GX) Module (version 1, Illumina ®, San Diego CA) and GeneSpring GX v10 ® (Agilent technologies). Ingenuity pathways analysis was used to evaluate potential biomarkers associated with ischemic stroke and to elucidate the biological pathway associated with ischemic stroke and BBB disruption. Baseline descriptive statistics for the sample were computed using SPSS (version 15, SPSS, Inc., Chicago, IL). Descriptive and frequency analysis was conducted for all demographic and clinical data. Baseline demographic and clinical characteristics were compared between acute ischemic stroke patients and healthy control subjects using chi-square analysis for the following categorical variables: gender, race, presence of comorbidities (HTN, DM, etc.), and medication history. Differences between patients with and without HARM were also compared using chi-square analysis for severity and rtPA administration. In addition Student's t-test (or Mann-Whitney) was used to analyze the difference between ischemic stroke and control subjects by age. To determine the change in medication usage from baseline to follow up in stroke patients McNemar's χ^2 test for matched pairs was used. For patients with and without HARM, Student's t-test (or Mann-Whitney) was used to analyze the difference on the following continuous variables: age, admission National Institutes of Health Stroke Scare score (NIHSS),

Modified Rankin scale score (MRS), onset time to blood draw and onset time to imaging. The level of significance for these descriptive comparisons was established at a p value of 0.05 for two-sided hypothesis testing.

6.2 DATA SCREENING PROCEDURES

Univariate descriptive statistics were used to examine continuous baseline variables to ensure the values were within the appropriate ranges, and means and standard deviations are plausible. Categorical variables were examined to determine if they were within the appropriate categorical ranges and the coded values were appropriately programmed. Outliers were detected by graphical methods (histograms, box plots, and normality probability plots) and statistical procedures (Z-score). The Shapiro-Wilks test and graphical methods (histogram) was employed to assess for normality of the variables. Missing data was analyzed for the degree to which the data were missing and the patterns associated with the missing data.

6.3 GENE EXPRESSION QUALITY ASSURANCE

6.3.1 Quality Checks

The ratio of absorbance at 260 nm and 280 nm was used to assess purity of RNA using a Nanodrop. A ratio of ~2.0 is generally accepted as "pure" for RNA. Further more, RNA quality was examined by assessing RNA degradation. Twenty five percent of the RNA samples were run

on agarose gels prepared with tris/acetate/EDTA (TAE). The presence of 18S and 28S ribosomal RNA without smearing using gel electrophoresis was evidence that the RNA is intact. Each microarray chip contained a set of positive and negative control probes. The signal of both probes was assessed and the array was of good quality when the negative controls reported low signals and the positive controls showed uniform intensities across the microarray chip.

6.3.2 Unsupervised and Supervised Clustering

First, the raw microarray data was clustered based on how the samples grouped, without knowledge of diagnosis (stroke patients vs. control subjects and stroke patients with BBB disruption vs. stroke patients without BBB disruption) to determine the phylogenetic distances between the samples. When the phylogenetic distances were larger than 0.1 the sample was considered an outlier and removed prior to analysis. Once outliers were removed, the raw microarray data were clustered again based on the true sample groups (supervised) (stroke patients vs. control subjects and stroke patients with BBB disruption vs. stroke patients without BBB disruption) to determine the phylogenetic distances between the samples. An un-normalized correlation matrix was calculated between subjects using the Pearson product-moment correlation coefficient to determine if arrays had an average inter-subject correlation <2 or 3 SDs below the mean; if so they would have been excluded, however no arrays were excluded from this analysis. 2D heat maps were generated to represent the relative level of expression of the genes across the samples. There are several data visualization functions available in the BeadStudio gene expression module and the GeneSpring GX v10 software including: scatter plots, bar plots, line plots, box plots, heat maps, cluster analysis dendograms,

principal components analysis, control summary reports, histograms and images. Additional visualization methods were chosen based on sample grouping.

6.3.3 Normalization of Array Data

After scanning the array, primary image data were analyzed with the aims of background elimination, filtration, and normalization to enable group comparisons. Normalization algorithms adjust sample signals in an attempt to minimize variation from non-biological factors. For all algorithms, normalization is computed with respect to a mathematically calculated sample that represents average probe intensities across all samples in the experiment. Two different normalization approaches were employed within BeadStudio to ensure that the data was of good quality: Quantile and Rank invariant. Quantile normalization is used to make the distribution, median and mean of probe intensities the same for every sample. The normalization distribution is chosen by averaging each quantile across the samples and is not that much affected by outliers and works best with non-linear data. Rank invariant normalization uses a set of probes that is rank invariant between a given sample and a virtual sample. The algorithm operates under the assumption that probes with similar ranking between samples have similar expression levels. When the data is of high quality, different normalization techniques will likely not lead to large differences in results. The raw intensity values of all genes on a scatter plot will be inspected in linear and logarithmic transformation to determine the best method based on the absence of curvatures in the data.

Robust multi-array analysis (RMA) normalization was conducted in GeneSpring on the un-normalized, un-background corrected raw signal intensities from BeadStudio occurring in the following order: 1. Background correction (using perfect match probe information and correct

values on each array separately); 2. Quantile normalization (probe level normalization performed across all arrays which causes all distributions to be the same); 3. Summarization (expression measure summary performed in log base scale 2 which uses the median to fit a linear model). Probesets are then filtered in GeneSpring via signal values based on the percentile of expression to determine whether genes are of low or high expression. This procedure tends to over-normalize at low signal intensities making it difficult to detect differential expression for genes with low expression.

6.3.4 Differential gene expression

After normalization, expression levels for each gene were calculated based on the absolute value of the intensity of the spot per gene on the array and saved into the Illumina BeadStudio GX Module software. All differential expression algorithms compare a group of samples to a reference group (cases vs. controls). The Illumina custom algorithm was used in BeadStudio and *t*-test analysis was employed in GeneSpring to determine the similarity of the results using two different programs. The Illumina custom model assumes that target signal intensity is normally distributed among replicates corresponding to a biological condition. To attempt to compensate for technical differences between microarray chips, 100 housekeeping genes included on all chips were re-scaled so the average values were equal across the chips. Array outlier's were also identified at this point by both visual inspection and normalization values.

6.4 DATA ANALYSIS PROCEDURES

6.4.1 Analysis of Aims 1, 2 and 3

Specific Aim 1: Determine which genes are under- and over-expressed in acute ischemic stroke patients compared to neurologically healthy age- matched control subjects.

RQ1. Is there a specific blood genomic profile associated with AIS that can be used to identify candidates for diagnostic biomarkers of acute ischemic stroke?

Specific Aim 2: Determine the changes in gene expression that occur in the first 24-48 hours following acute ischemic stroke.

RG2. How will blood gene expression profiles change between the acute phase of ischemic stroke (0-24 hours) and 24-48 hours following onset of symptoms?

Specific Aim 3: Determine whether acute ischemic stroke patients with BBB disruption have a specific blood genomic profile compared to AIS patients without BBB disruption.

RQ3. Is there a specific blood genomic profile associated with the development of BBB disruption as HARM on MRI after AIS?

Stroke patients were matched to healthy control subjects by age +/- 10 years and race. Stroke patients with BBB disruption were frequency matched to stroke patients without BBB disruption by age +/- 10 years and race. Since fold change comparisons do not address reproducibility and cannot be used to determine statistical significance, the gene expression sets were compared in a univariate manner between stroke patients and control subjects through the

use of Illumina's custom algorithm (Appendix L) in BeadStudio and with *t*-test comparisons in GeneSpring (Appendix M). The uncorrected probability values were assigned a cutoff threshold value of significance of <0.05. Inflation of type one error from multiple hypothesis testing was corrected by Benjamini-Hochberg false discovery rate (FDR) technique in BeadStudio and with Bonferroni Family wise error (FWER) in GeneSpring and a false detection rate of <.05 after correction was considered statistically significant. Benjamini-Hochberg technique uses only the genes that pass through the first filter of 0.05 for the correction; therefore it is lest stringent and thus results in larger gene lists, but more false positives. On the other hand, the Bonferroni technique uses all of the genes available on the array for the correction and is thus more stringent and dramatically reduces the number of statistically significant genes but does not tolerate false positives. Therefore the gene set identified in BeadStudio was compared to the gene set identified in GeneSpring to determine the most likely significant genes. Hierarchical cluster analysis was then performed on the gene subsets found to be significantly different between patients and control subjects by using pairwise correlations calculated for each gene along with subsequent calculations of a distance matrix forming the basis of the clustering. The distance matrix was calculated by the dissimilarity measure (Djk) between two observations, one from group one and one from group two. (Djk=|| xj-xk|| for j and k observations) Ingenuity Systems Pathway analysis module was used to identify relevant biological pathways in the whole blood following acute ischemic stroke. Appendix N.

6.4.1.1 Identification of RNA profile associated with HARM

A logistic regression with stepwise model selection was performed in SAS to identify an RNA profile associated with HARM based on the genes significant between stroke patients and control subjects. Four models were tested. Two models of HARM (a. none, mild, moderate or severe; b.

severe HARM yes/no) with either one of the following covariate groups: 1.intensity data of the 16 genes specific for stroke, age, gender, rtPA treatment, hypertension, diabetes, hyperlipidemia, and smoking; and 2. intensity data of the 16 genes specific for stroke, age, gender, and rtPA treatment.

6.4.2 Ingenuity systems pathway analysis

Samples were phenotypically anchored into one of two groups, either stroke patient or control subject, or patients with or without BBB disruption. This approach allows for a systems level biological interpretation of gene expression profiles. Since it takes into consideration the biological system as a whole, a systems biology approach has significant potential in prioritization of biomarker candidates. This system wide data analysis is derived by a knowledge base that contains materials from well annotated databases and scientific literature gathered by the Gene Ontology consortium.(Ganter, Zidek et al. 2008) The ingenuity systems pathway filter determines what biological processes and molecular functions are most significantly associated with the phenotype of interest.

Genes with a 1.5 fold difference in expression between stroke patients and control subjects were identified. The data set that contained gene identifiers and their corresponding expression signal intensities was uploaded into the ingenuity systems program. Each probe set was mapped to its corresponding data base gene object to designate focus genes, which were genes that fall into the <0.05 significance threshold and they directly interact with other genes in the global molecular network. The ingenuity systems software queried the knowledge base and generated a set of networks with a network size of 35 genes/gene products. A score, which was derived from a p-value, was generated for each network according to the fit of the set of

significant genes. Scores of 2 or higher were considered to have at least a 99% confidence of not being generated by chance alone. Biological functions were then calculated and assigned to each network. Networks were displayed graphically as nodes (genes/gene products) and edges (the biological relationships between the nodes). The intensity of the node indicates the degree of up regulation (red) or down regulation (green).(Schoch, Dugas et al. 2004)

The significance value associated with the identified biological pathways is a measure of how likely it is that genes from the dataset file participate in that function. The significance is expressed as a p-value, which is calculated using the right-tailed Fisher's Exact Test. In this method, the p-value is calculated by comparing the number of user-specified genes of interest that participate in a given function or pathway, relative to the total number of occurrences of these genes in all functional/pathway annotations stored in the Pathways Knowledge Base.

7.0 RESULTS

Data collection was conducted from October 2007 to August 2008. During that time a total of 92 subjects (67 stroke patients and 25 healthy control subjects) were recruited by T. Barr. For this study to occur under the larger NINDS/NIH Natural History of Stroke study T. Barr was responsible for IRB amendments to the original protocol and consent, establishing and maintaining collaboration with Suburban hospital's laboratory department for space in the laboratory and freezer, maintaining supplies necessary for the project, performing all phlebotomy blood draws, processing of all specimens, and maintaining the research database. In addition, T. Barr was also responsible for recruiting and submitting samples to the Specialized Program of Translational Research in Acute Stroke (SPOTRIAS) and Coriell repositories. During the period of recruitment, plasma samples from 30 patients and DNA from 20 patients was submitted to the repositories. All patients recruited for this thesis, also had blood drawn and processed by T. Barr for plasma and serum at both acute and follow up time points.

7.1 SAMPLE CHARACTERISTICS

Of the 67 stroke patients enrolled: 39 stroke patients were Definite AICS patients with acute blood drawn within 24 hours. Of the 25 control subjects recruited, only one sample was an outlier based on unsupervised dendogram analysis and supervised PCA and appropriately

removed, for a total of 24 for analysis. Therefore for the analysis of Aim 1—we compared 39 stroke patients to 24 healthy control subjects. Of the 39 definite AICS patients, 33 have a follow up blood draw. Therefore for the analysis of Aim 2---we compared the acute and follow up gene expression profiles in 33 definite AICS patients. Finally, of the 39 definite AICS patients with acute blood draw, 30 have a follow up MRI for the analysis of HARM. A total of 4 patients, 8 have mild HARM, 9 have moderate HARM and 9 have severe HARM. When HARM is categorized as a binary variable (Severe HARM yes/no) for the analysis of Aim 3---we can compare 21 patients without BBB disruption with 9 patients with BBB disruption.

7.1.1 Sample characteristics for Aim 1

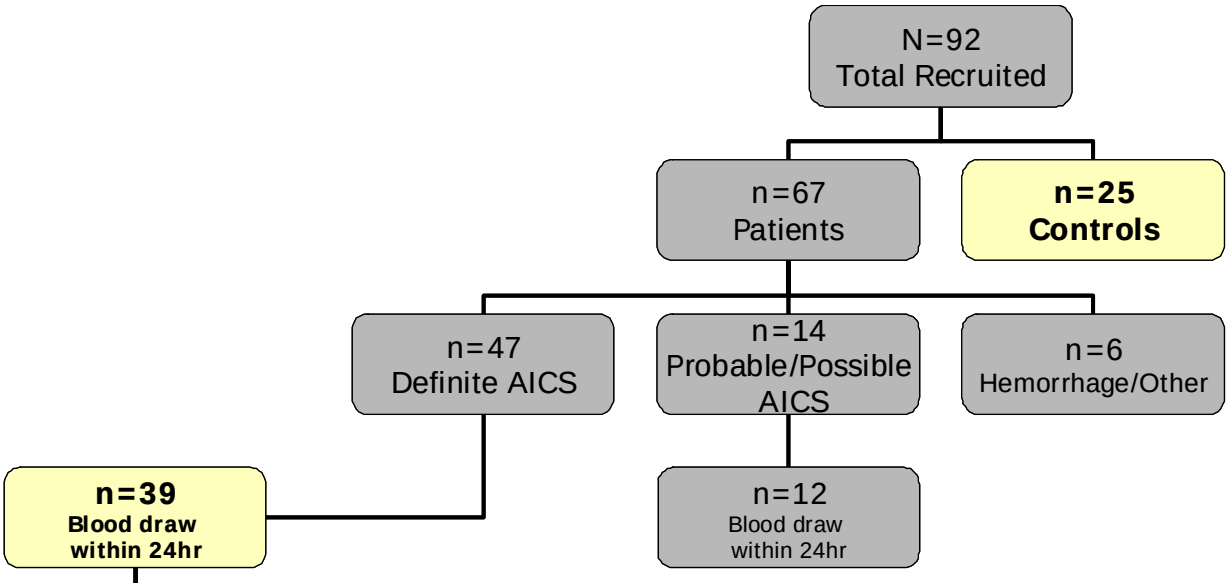


Figure 6. Aim 1 Sample

A total of 39 stroke patients and 24 control subjects were analyzed to address Aim 1. Of the 39 stroke patients 100% were Caucasian and 43.6% were male. Cardioembolic stroke accounted for 43.6% of stroke etiologies. Over 59% of the patients were discharged to home. The presence of comorbidities was prevalent with the stroke population, with 64% having a history of hypertension, 28% with a history of diabetes, 15% with a history of atrial fibrillation, 15% with a history of myocardial infarction, 46% with a history of dyslipidemia, 15.4% with a history of prior stroke, and 38% with a smoking history (previous or current). Over half of the patients were on anticoagulants or antiplatelets and 74% were being treated for their hypertension. Nine (23.1%) of the patients received rtPA, of which only one patient had their blood drawn before rtPA administration. The mean age of the patients is 73.05±14. The mean time from symptom onset to acute blood draw is 10:06 hours ±6:31. The patients have a pre stroke MRS median score of zero with an SD of 1.2. The severity of injury for the patient group was mild with a median baseline NIHSS of 3 with a range from 0-23 and a discharge NIHSS median of 0 with a range from 0-10. Results below are presented as mean ± standard deviation.

Table 2. Patient Clinical Characteristics

	Stroke
rtPA administration	9 (231%)
rtPA given before baseline blood draw	8 (88.9%)
Onset time to Acute blood draw	10:06±6:31
Onset time to Follow up blood draw	29:24±7:10
Median Pre-Stroke MRS	0 (range 0-4)
Median Baseline NIHSS	3 (range 0-23)
30 day MRS	1 (range 0-6)
30 day NIHSS	0 (range 0-8)

Table 3. TOAST Classification of Patients

	TOAST
Large Artery Embolus/thrombus	5 (12.8%)
Cardioembolic	17 (43.6%)
Small vessel (lacune)	3 (7.7%)
Other etiology	3 (7.7%)
Undetermined Two or more causes	2 (5.1%)
Undetermined Negative evaluation	8 (20.5%)
Undetermined Incomplete evaluation	1 (2.6%)

Table 4.Discharge Disposition

	Discharge Disposition
Home	24 (61.5%)
Rehabilitation	11 (28.3%)
Nursing Home	2 (5.1%)
Expired	2 (5.1%)

Of the 24 healthy control subjects 100% were Caucasian and 41.7% were male. The presence of comorbidities was not as prevalent in the control group. Approximately 30% of the control subjects had a history of hypertension of which only 4% were receiving treatment; 8% had a history of diabetes, 62.5% had a smoking history (previous or current), 8.3% had a history of stroke and none of the control subjects claimed a history of atrial fibrillation, myocardial infarction, or dyslipidemia. The mean age of the control subjects was slightly less than the patient group with a mean of 59.9±9.73.

Differences in baseline clinical demographic data between stroke patients and control subjects was determined by *t*-test for age and by Pearson $\chi 2$ tests using 2x2 contingency table analysis for gender, risk factor history, and medication history. Risk estimates as odds ratios were obtained with the F test for categorical variables. There was no difference by race or gender between the groups. However, stroke patients were significantly older than control subjects (t=-4.03; p=0.000) and stroke patients were more likely to have the presence of comorbidities for which they were receiving medication.

Table 5. Stroke patients and control subjects characteristics

	Total Sample N=63	**Stroke** n=39 (61.9%)	**Control** n=24 (38.1%)	**Statistic/df**	***p* value**
Gender (% female)	36 (57.1%)	22 (61.1%)	14 (38.9%)	χ^2 0.02/1	0.883
Mean age, years	68.1±14.02	73.1±14.0	59.9±9.73	*t* -4.1/61	**0.000**
Hypertension	32 (50.8%)	25 (78.1%)	7 (21.9%)	χ^2 6.6/1	**0.010**
Diabetes	13 (20.6%)	11 (84.6%)	2 (15.4%)	χ^2 3.3/1	0.068
Dyslipidemia	18 (28.6%)	18 (100%)	0	χ^2 14.9/1	**0.000**
Atrial Fibrillation	6 (9.5%)	6 (100%)	0	χ^2 3.9/1	**0.048**
Myocardial Infarction	6 (9.5%)	6 (100%)	0	χ^2 3.9/1	**0.048**
Previous Ischemic Stroke	8 (12.7%)	6 (75%)	2 (25%)	χ^2 0.7/1	0.414
Previous or Current Smoker	30 (47.6%)	15 (50%)	15 (50%)	χ^2 7.8/2	**0.020**
Hypertension Medication	37 (47.6%)	29 (78.4%)	8 (21.6%)	χ^2 10.3/1	**0.001**
Diabetes Medication	8 (12.7%)	7 (87.5%)	1 (12.5%)	χ^2 2.66/1	0.103
Cholesterol Medication	22 (34.9%)	17 (77.3%)	5 (22.7%)	χ^2 3.39/1	0.066
Anticoagulant or Antiplatelet	21 (33.3%)	20 (95.2%)	1 (4.8%)	χ^2 14.8/1	**0.000**
Family history of Stroke	19 (30.2%)	15 (78.9%)	4 (21.1%)	χ^2 3.6/2	0.169

7.1.2 Sample Characteristics for Aim 2

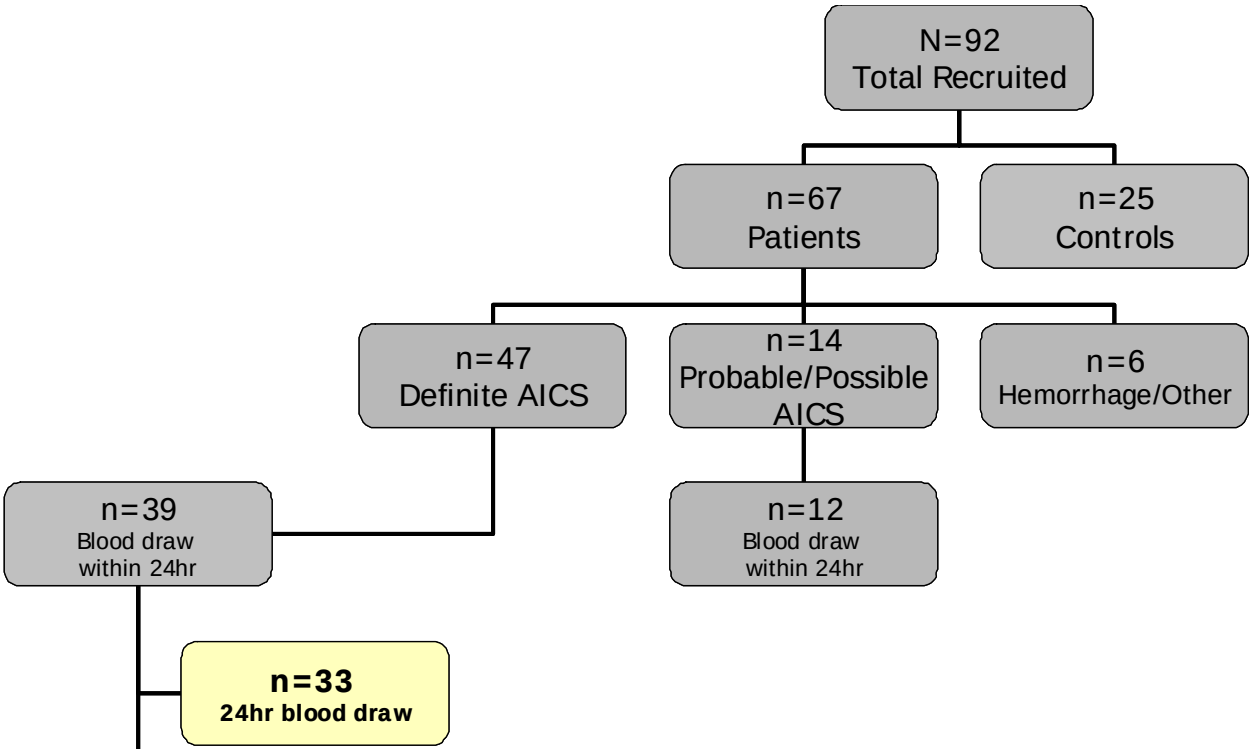


Figure 7. Aim 2 Sample

Of the 34 Definite AICS patients with acute and follow up blood draws, the mean age of the sample is 71.94±14.6. Mean time from symptom onset to acute blood draw is 9:29±6:19 (range 2:35-23:02); to follow up blood draw is 29:24±7:10 (range 18:45-43:30). Mean time between acute and follow up blood draw is 19:55±3:26 (range 13:30-27:32). Median pre stroke MRS is 0; median baseline NIHSS is 3 with a range of 0-24.

Table 6. Aim 2 Sample Characteristics

	Total N=34
Gender (% female)	20 (58.8%)
Age, years	71.94±14.6 (range 43-96)
Baseline MRS	0 (range 0-4)
Baseline NIHSS	2 (range 0-23)
Received rtPA	6 (17.6%)
Onset to Acute blood	9:29±6:19 (range 2:35-23:02)
Onset to Follow-up Blood	29:24±7:10 (range 18:45-43:30)
Acute to Follow-up Blood	19:55±3:26 (range 13:30-27:32)

Table 7.Aim Two In-hospital medication change

	Baseline	**24 hour Follow-up**	**p-value**
Hypertension Medication	24 (70.6%)	7 (20.6%)	0.000
Cholesterol Medication	13 (38.2%)	27 (79.4%)	0.003
Diabetes Medication	7 (20.6%)	13 (38.2%)	0.031
Antiplatelet/ Anticoagulant	17 (50%)	27 (79.4%)	0.021

*p-values generated from the McNemar χ^2 test for matched-pairs

7.1.3 Sample Characteristics for Aim 3

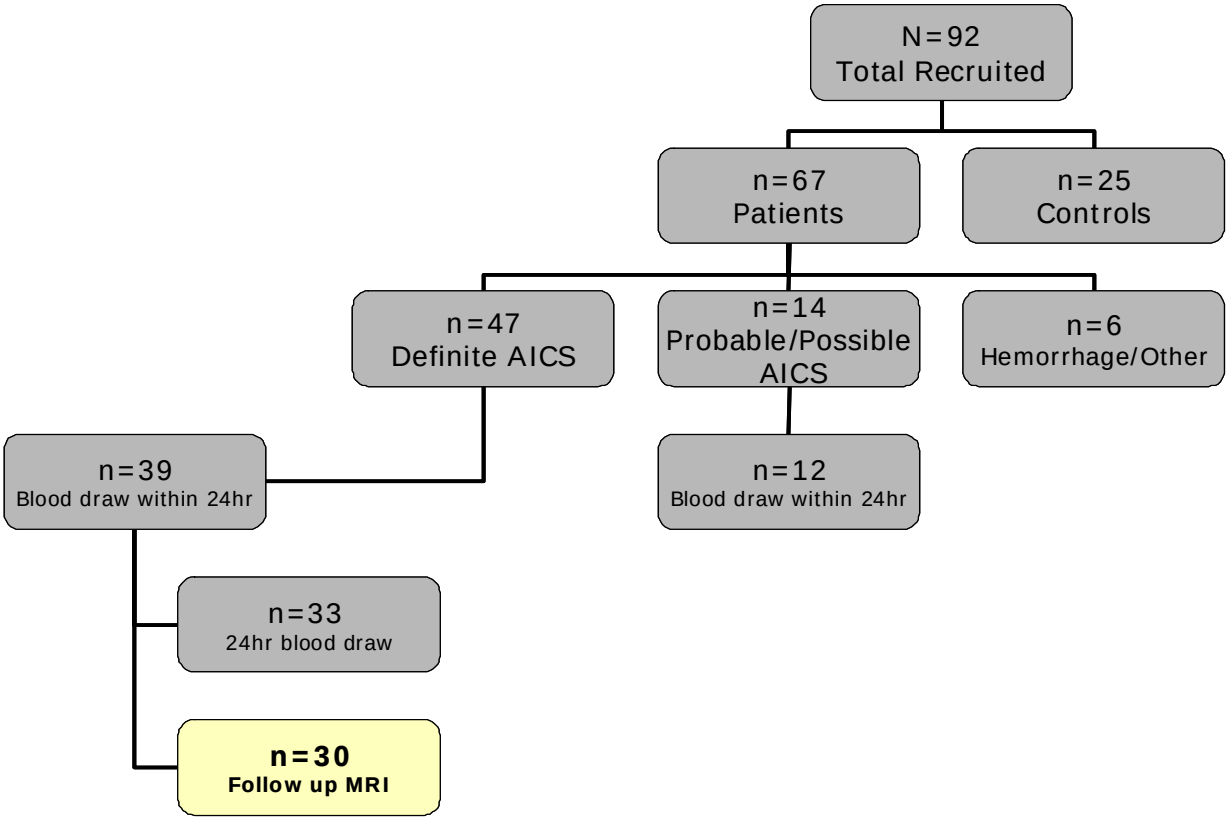


Figure 8. Aim 3 Sample

Of the 39 Definite AICS patients with acute blood draw, 30 have a follow up MRI Therefore a total of 30 patients can be appropriately matched; the mean age of the sample is 72.8±14.1. Mean time from symptom onset to acute blood draw is 10:26±6:24. Median pre stroke MRS is 0; median baseline NIHSS is 3 with a range of 0-19. The mean time from onset to the Acute MRI (where the patient would have received the first dose of GD-DTPA contrast) is 4.41 h ± 5.03 with a range from 0-19 hours. There are 4 (13.3%) in the no HARM group; 8 (26.7%) in the mild HARM group; 9 (30%) in the moderate HARM group and 9 (30%) in the severe HARM group.

Table 8. Aim 3 Sample Characteristics

	Total N=30	**None** 4 (13.3%)	**Mild** 8 (26.7%)	**Moderate** 9 (30%)	**Severe** 9 (30%)	**Statistic/df**	**p-value**
Gender (%female)	16 (53.3%)	3 (18.8%)	4 (25%)	4 (25%)	5 (31.2%)	χ^2 1.1/3	0.779
Age, years	72.1±14.5	53.5±11.5	74.1±9.6	72.9±1.5	77.7±0.7	F 3.4/3	**0.034**
Baseline MRS	0 (0-4)	0	0	0 (0-4)	0 (0-2)	F 1.6/3	0.214
Baseline NIHSS	3 (0-18)	3 (0-4)	1 (0-7)	3 (0-16)	5 (0-18)	F 1.7/3	0.186
Received rtPA	9 (30%)	0	1 (11.1%)	3 (33.3%)	5 (55.6%)	χ^2 5.73/3	0.126
Hypertension	19 (63.3%)	2 (10.5%)	4 (21.1%)	6 (31.6%)	7 (36.8%)	χ^2 1.77/3	0.621
Diabetes	2 (6.7%)	0	1 (50%)	0	1 (50%)	χ^2 1.6/3	0.659
Dyslipidemia	16 (53.3%)	0	5 (31.3%)	4 (25%)	7 (43.7%)	χ^2 7.29/3	0.063
Atrial Fibrillation	2 (6.7%)	0	1 (50%)	0	1 (50%)	χ^2 1.65/3	0.648
Myocardial Infarction	4 (13.3%)	0	2 (50%)	1 (25%)	1 (25%)	χ^2 1.64/3	0.652
Previous Ischemic Stroke	4 (13.3%)	0	0	3 (75%)	1 (25%)	χ^2 5/3	0.172

Table 9. Aim Three Medications

	Total N=30	**None** 4 (13.3%)	**Mild** 8 (26.7%)	**Moderate** 9 (30%)	**Severe** 9 (30%)	**Statistic/df**	**p-value**
Hypertension Medication	20 (66.7%)	1 (5%)	4 (20%)	8 (40%)	7 (35%)	χ^2 6.6/3	0.085
Cholesterol Medication	14 (46.7%)	0	5 (35.7%)	4 (28.6%)	5 (35.7%)	χ^2 4.6/3	0.203
Diabetes Medication	2 (6.7%)	0	2 (100%)	0	0	χ^2 5.9/3	0.117
Antiplatet/ Anticoagulant	15 (50%)	0	5 (33.3%)	5 (33.3%)	5 (33.3%)	χ^2 4.7/3	0.193

Table 10. Aim Three: Time Variables

	Total N=30	**None** 4 (13.3%)	**Mild** 8 (26.7%)	**Moderate** 9 (30%)	**Severe** 9 (30%)	**Statistic/df**	**p-value**
Onset to Baseline MRI	4.2±5	4.25±4	5.25±6.3	4.33±5.4	3.11±4.5	F 0.23/3	0.870
Onset to Follow-up MRI	27.13±5.4	26.50±7.8	29.50±5	25.67±4.9	26.78±5.8	F 074/3	0.536
Baseline MRI to Follow-up MRI	22.5±4.6	21.75±3.3	23.88±2.2	21±7.9	23.22±1.6	F 0.63/3	0.602
Onset to Baseline Blood draw	10:11±625	9:58±6:18	12:18±8:08	8:15±4:39	10:20±6:49	F 0.54/3	0.660
Baseline Blood Draw to Follow-up MRI	2253±4.6	16:50±8:43	17:25±8:28	17:67±7:07	16:56±7:73	F 0.04/3	0.989

7.2 RNA EXTRACTION AND QUALITY

RNA extraction was performed according to the Paxgene blood RNA handbook. All samples were allowed to thaw at room temperature for at least 24 hours prior to beginning the extraction. Due to low initial yield, a portion of the samples (n=34) were placed in the vacuum dryer for 15 minutes at a temperature of 45°C. Following vacuum dry the samples went from a final volume of 80µl to ~40µl. Total mean sample RNA concentration is 82.6ng/µl with a range between 49.1-282.2ng/µl in a total volume between 40-80µl. Mean 260/280 ratio is 2.14±0.1 with a range from 1.96-2.8. Total RNA concentration following amplification for the patient group was 938.7ng/ul ±296.5 and the mean 260/280 ratio was 2.15±0.03. Total RNA concentration following amplification for the control group was 854.2ng/ul±265.7 and the mean 260/280 ratio was 2.14±0.06. A portion of the samples (n=45) were also ran on agarose gels to assess quality and presence of degradation. All samples showed clear 28s and 18s bands without smearing, indicative of good quality, intact RNA. There were no significant differences between patients and control subjects for RNA concentration ($t=-1.14$; $p=0.258$) or quality by 260/280 ratio ($t=-0.524$; $p=0.602$).

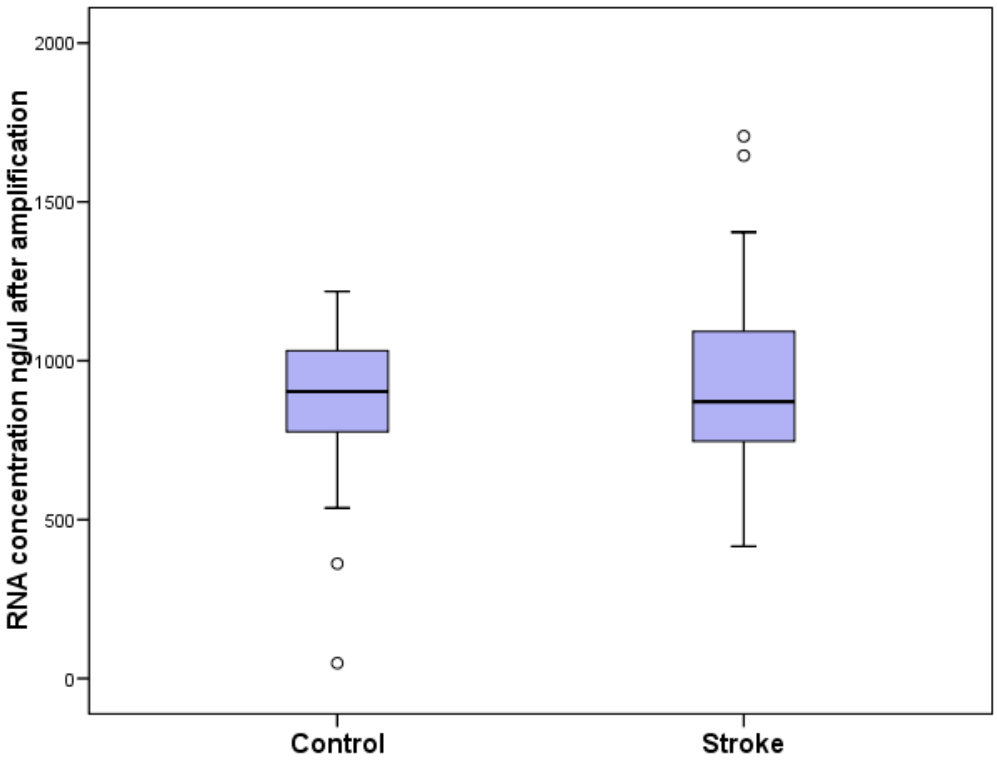


Figure 9. RNA concentration ng/ul following amplification

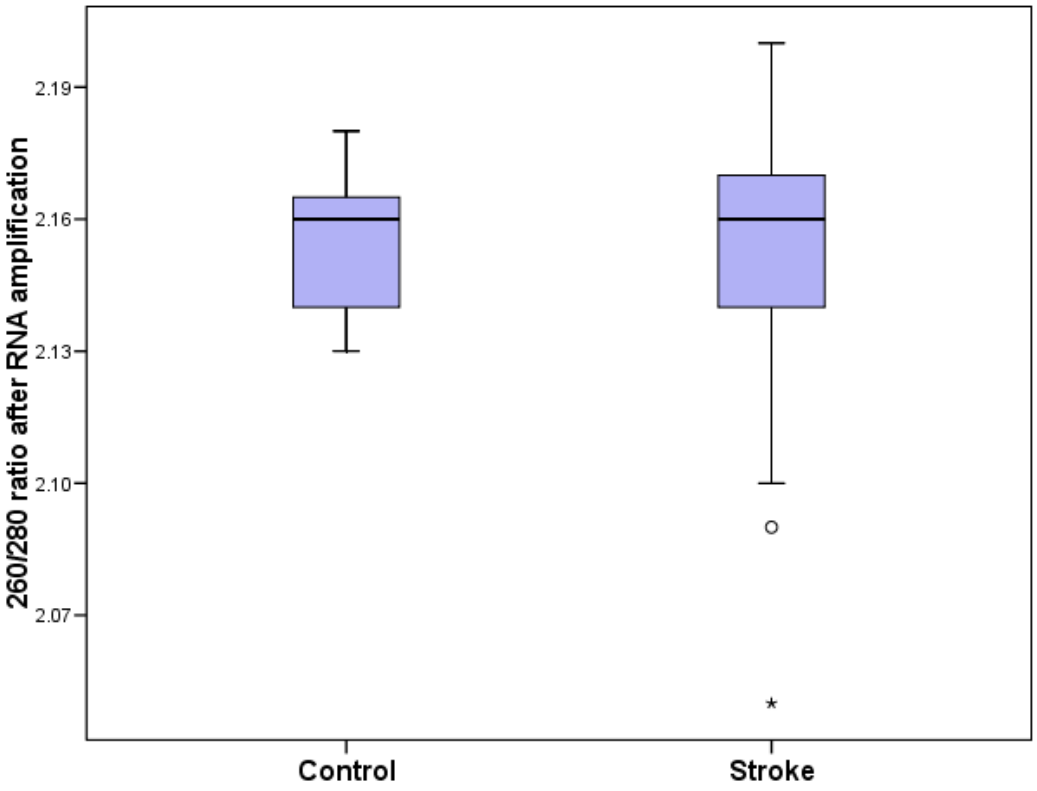
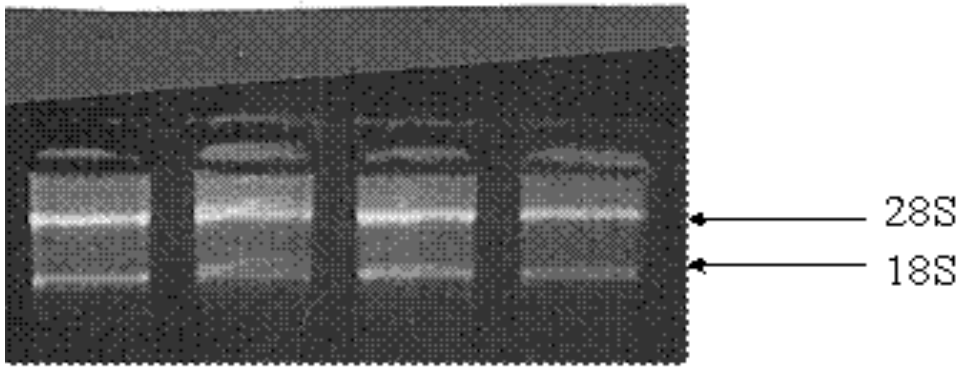
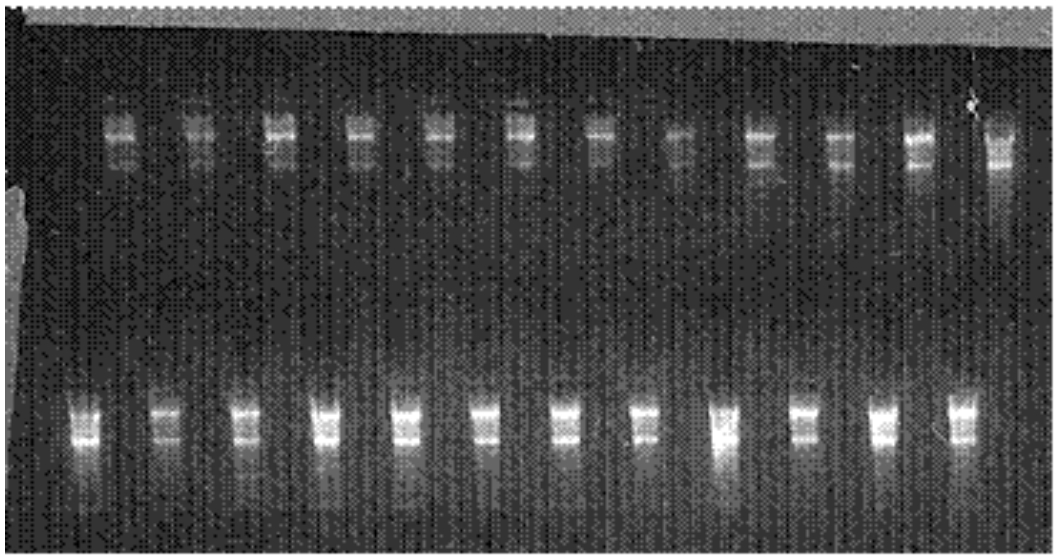


Figure 10. RNA quality 260/280 ratios following amplification



*Four patient samples



*24 additional patient samples

Figure 11. RNA Gel

7.3 ILLUMINA QUALITY CONTROL ASSESSMENTS

7.3.1 Quality control for arrays

BeadArrays were visually inspected in BeadStudio for marks or cracks etc, which could interfere with hybridization or scanning. All arrays were found to be clear of imperfections.

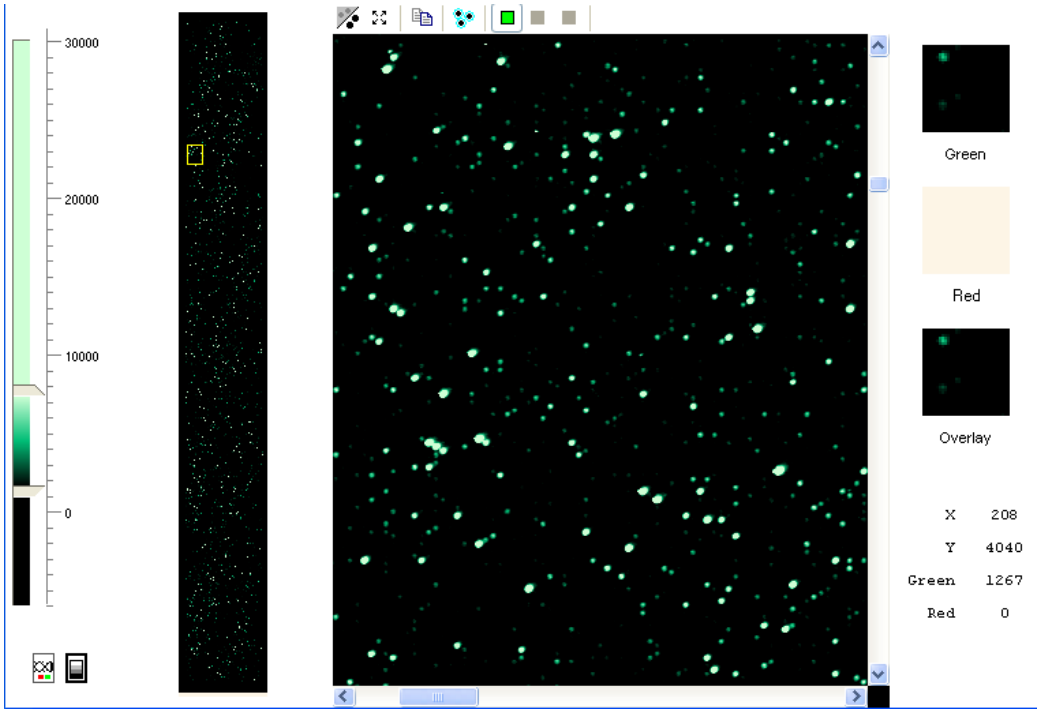


Figure 12. BeadArray Image

The BeadStudio Gene Expression module displays a graphic summary for quality control assessment of arrays based on built-in controls. All control plots look comparable to what is available for review in the BeadStudio handbook, except the High Stringency plot. A representative from Illumina stated that the high stringency probes are no longer on the v3 beadchips, so there should not be signal there; the rest of the plots are consistent with high

quality data. Hybridization controls were appropriate for low, medium and high. Negative control, background, and noise signals were low (<200) across all arrays and housekeeping and biotin signals were high (>20,000). The average signal for internal controls across the arrays was similar.

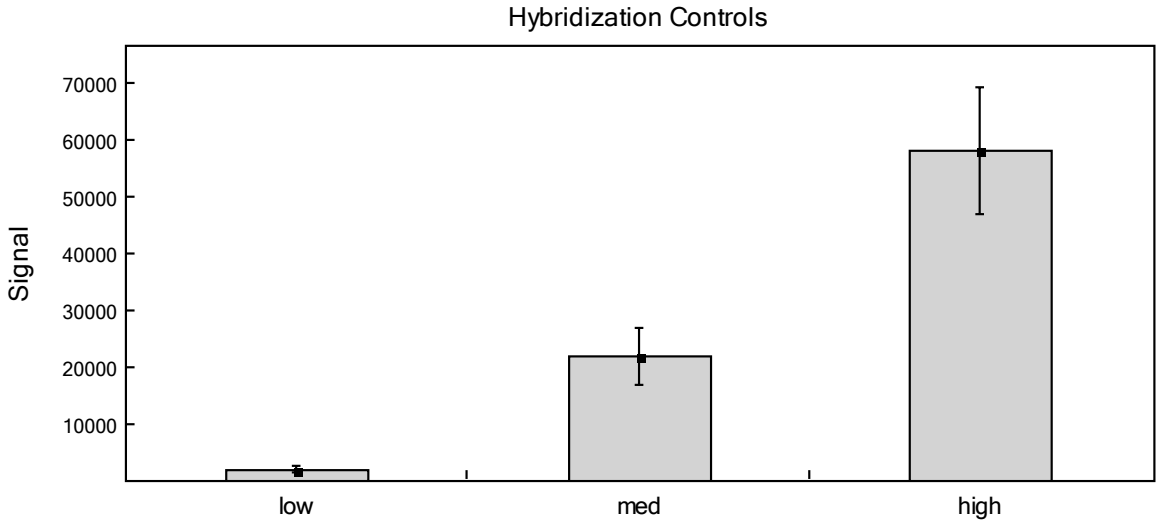


Figure 13. Hybridization Controls

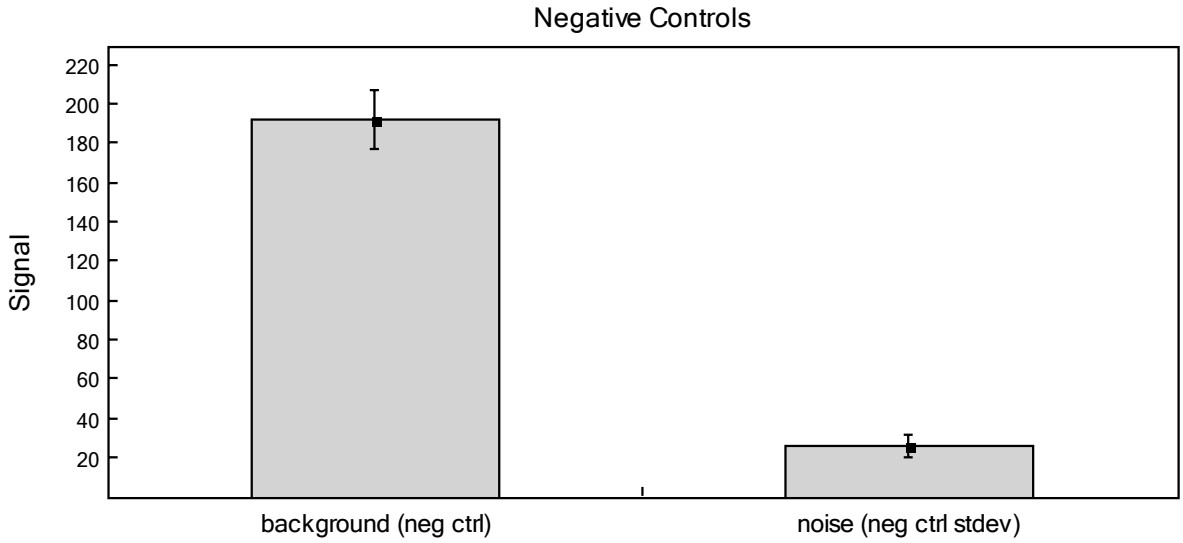


Figure 14. Negative Controls

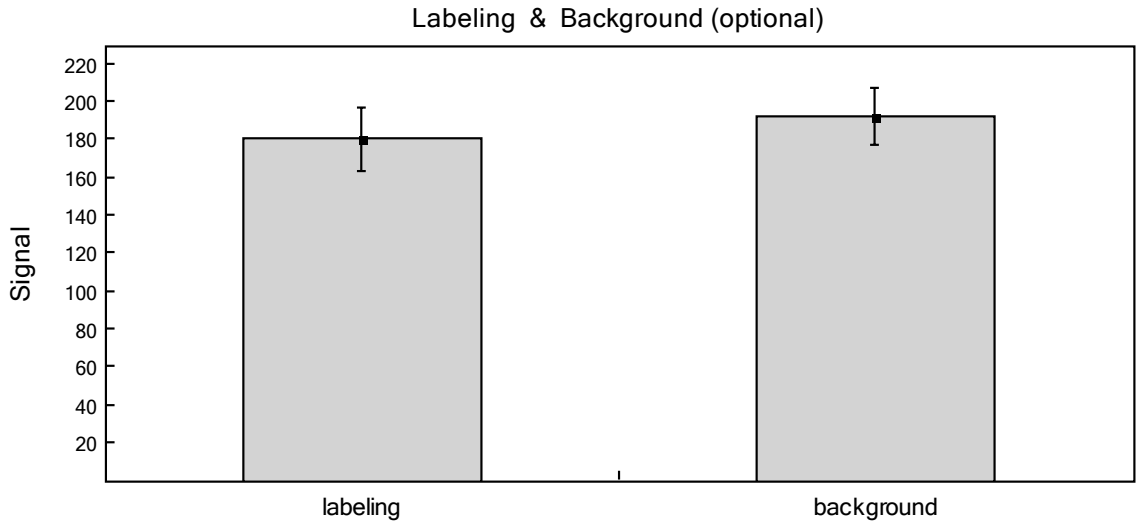


Figure 15. Labeling and Background

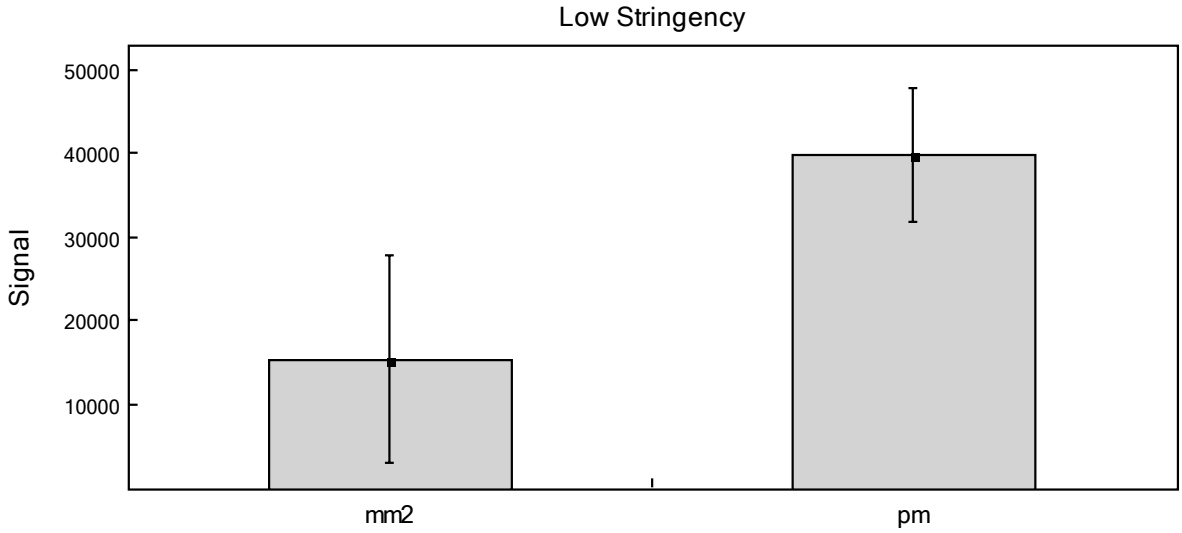


Figure 16. Low Stringency

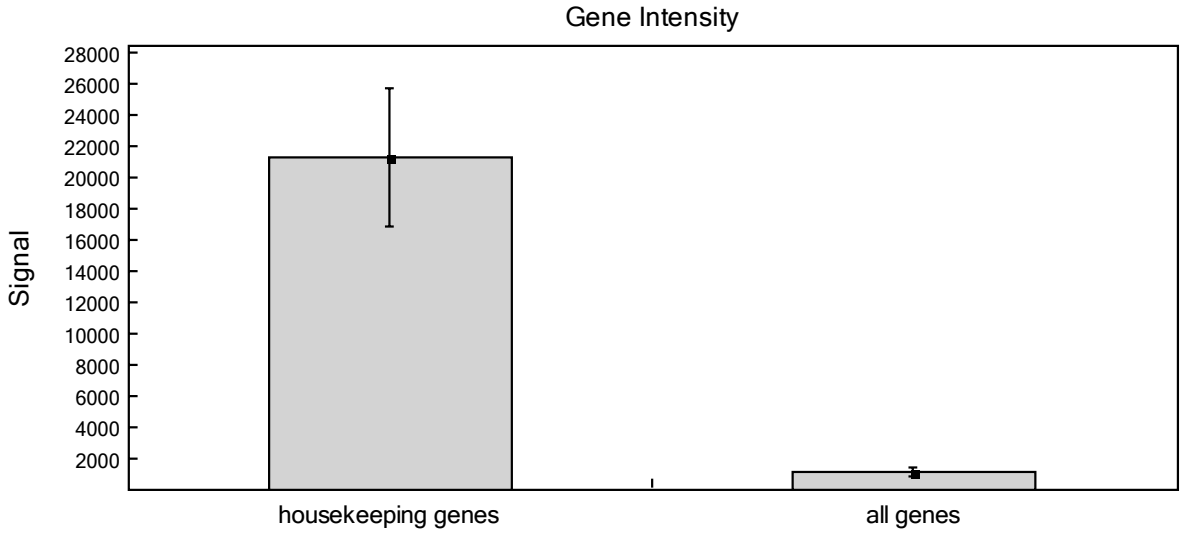


Figure 17. Housekeeping Gene Intensity

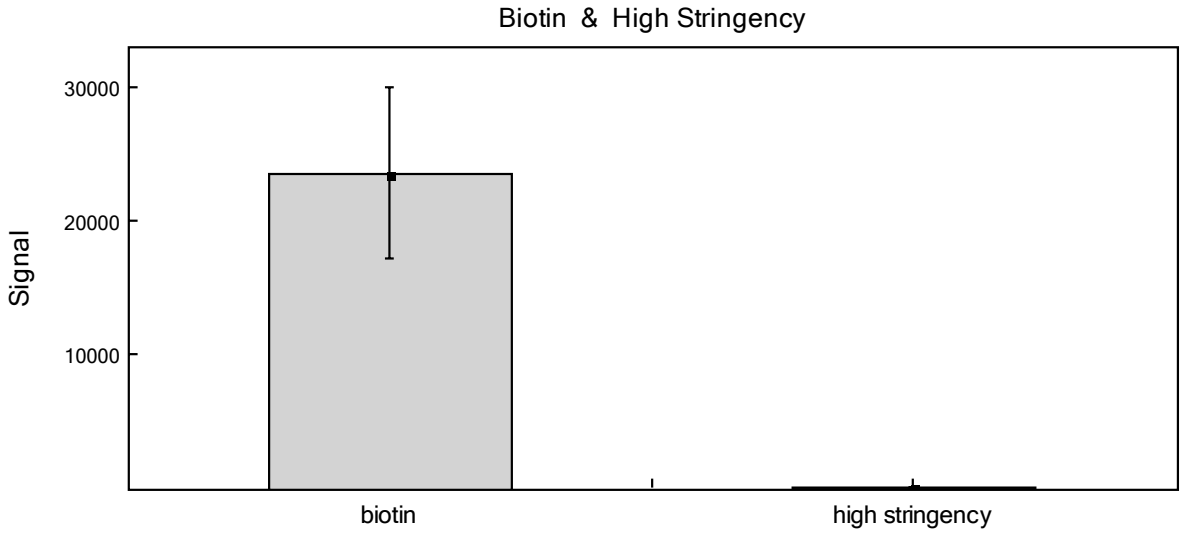


Figure 18. Biotin

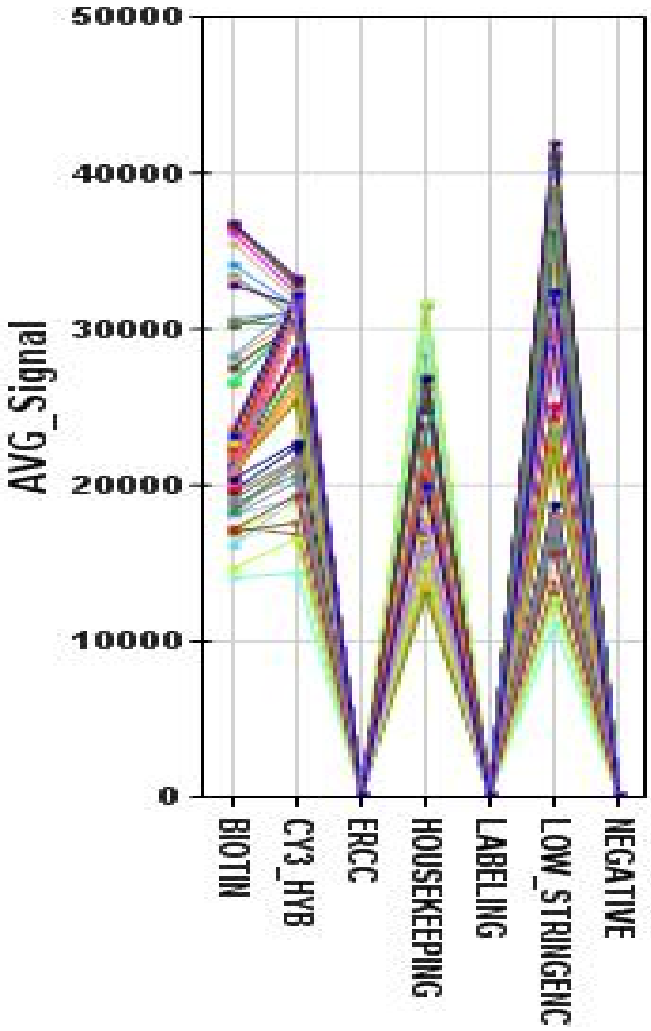


Figure 19. Average signal for internal controls across arrays

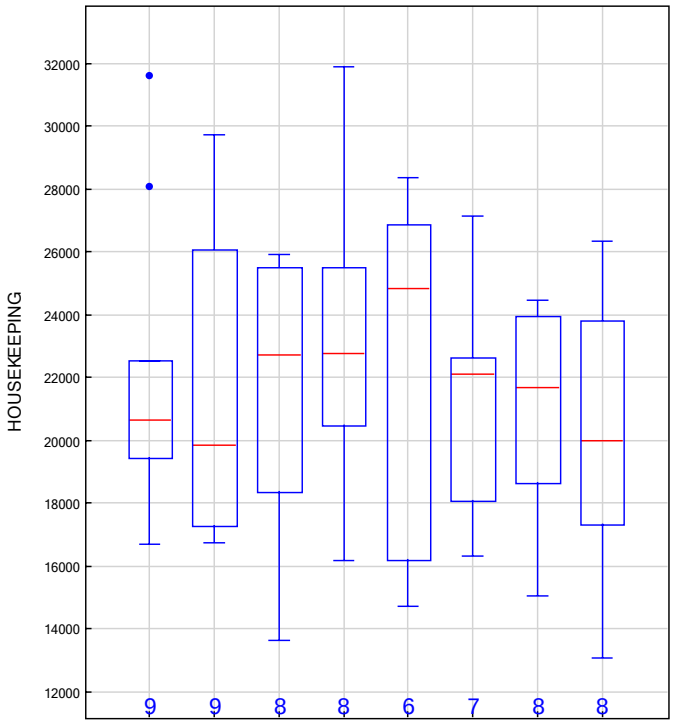


Figure 20. Housekeeping gene signal per array

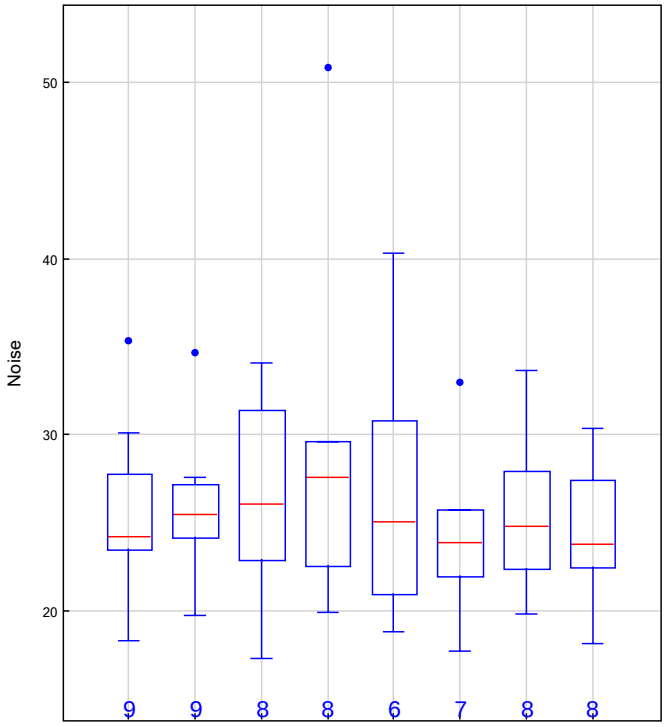


Figure 21. Noise signal per array

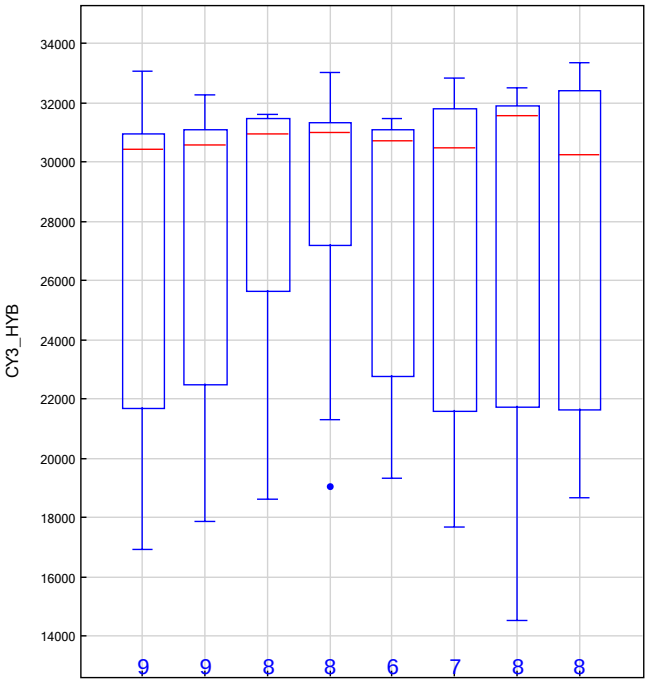


Figure 22. CY3 Hybridization signal per array

7.3.2 Quality control for samples

7.3.2.1 Unsupervised Clustering

Unsupervised clustering of samples using all methods in BeadStudio (correlation; absolute correlation; manhattan; Euclidean) revealed two outliers in the data (one stroke patient and one control subject) with distances on the dendogram greater than 0.1. Round table discussions amongst investigators resulted in the elimination of only the control subject from analysis. It was decided that it is impossible to determine if the patient sample was an outlier because of technical or biological variability and to keep credibility of the dataset, the patient outlier was kept in the analysis.

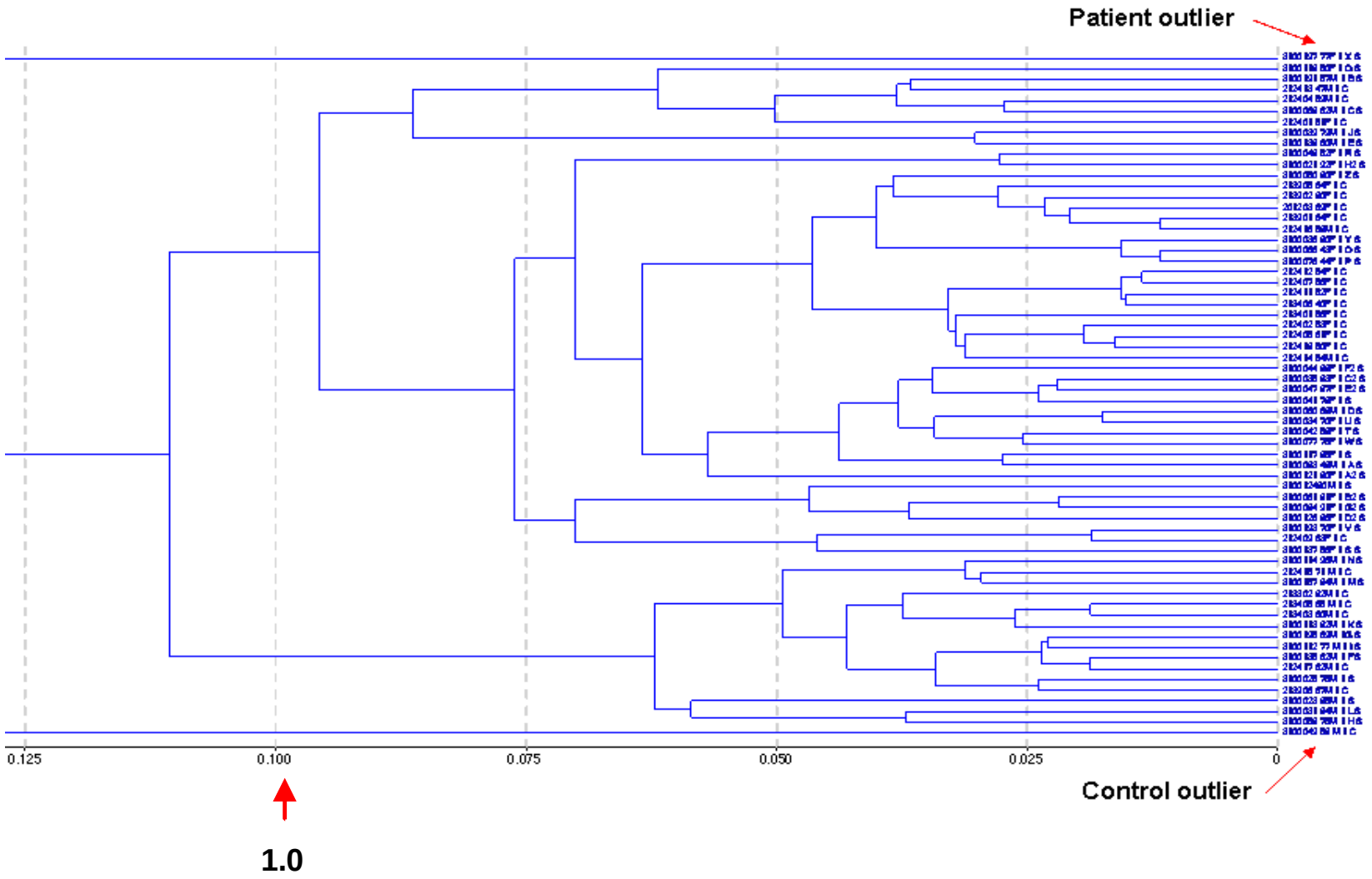


Figure 23. Unsupervised sample cluster

7.3.3 Probe level analysis

Probes were filtered in GeneSpring based on signal intensity values. The raw data was filtered by percentile with a lower cut-off of 20 and an upper cut-off of 100. The stringency of the filter was set at 1 out of 63 minimum number of samples in which the probe must pass the filter. 60 probes did not pass the first filter. The probes were then filtered based on present and marginal flags with at least 1 of 63 with acceptable values. An additional 42 probes did not pass this second filter. This resulted in a final probe set of 24,424 to be used in analysis.

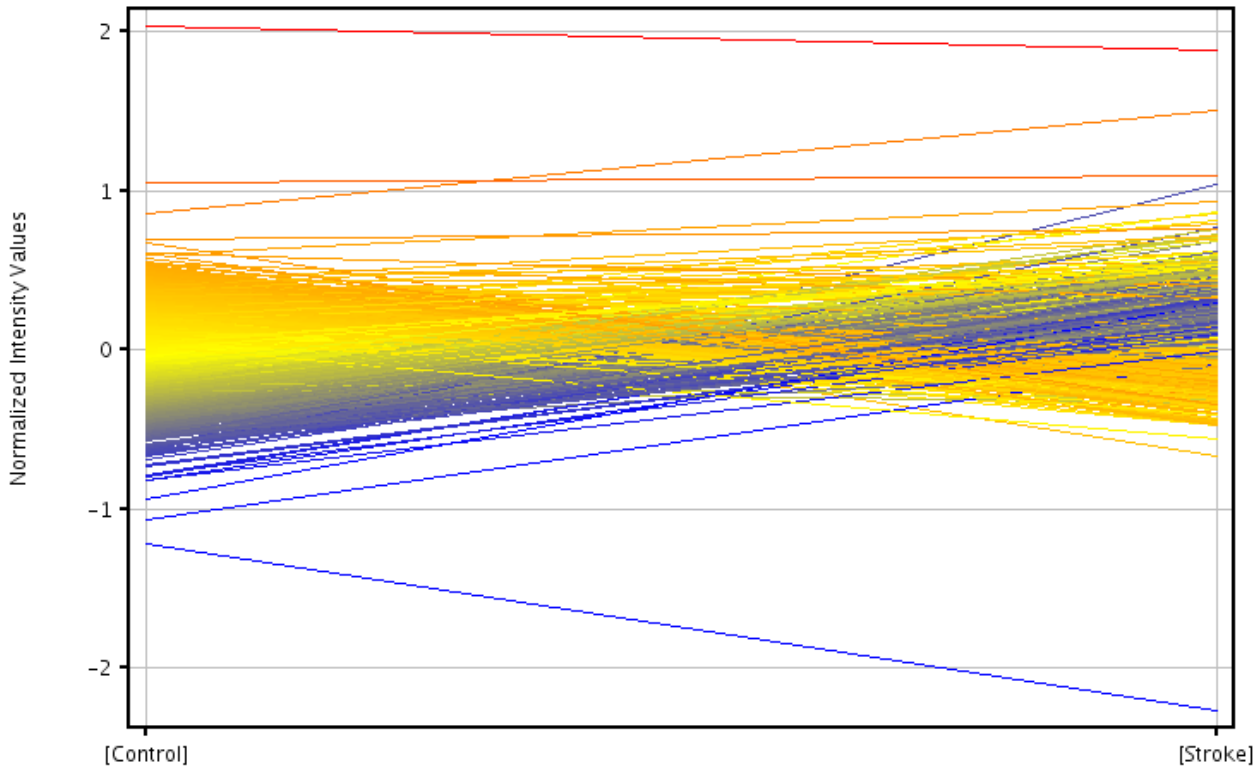


Figure 24. Probes filtered based on expression and flags

7.3.4 Normalization

Normalization was conducted under two methods (quantile and rank invariant) to determine the most appropriate technique for this dataset. Rank invariant normalization resulted in a larger gene set specific for stroke (100 genes with 2 fold difference, $p<0.05$), but contained all of the transcripts identified with quantile normalization (16 genes with 2 fold difference, $p<0.05$). Quantile normalization reduced variability of expression between stroke patients and control subjects, but was chosen as the most appropriate method because the patient outlier was left in the analysis and quantile normalization is resistant to outliers. In addition quantile normalization is based on transforming each of the array specific distributions of intensities so that they all have the same values at specific quantiles. In order to take care of array to array bias and variation this is the method more strongly advocated.

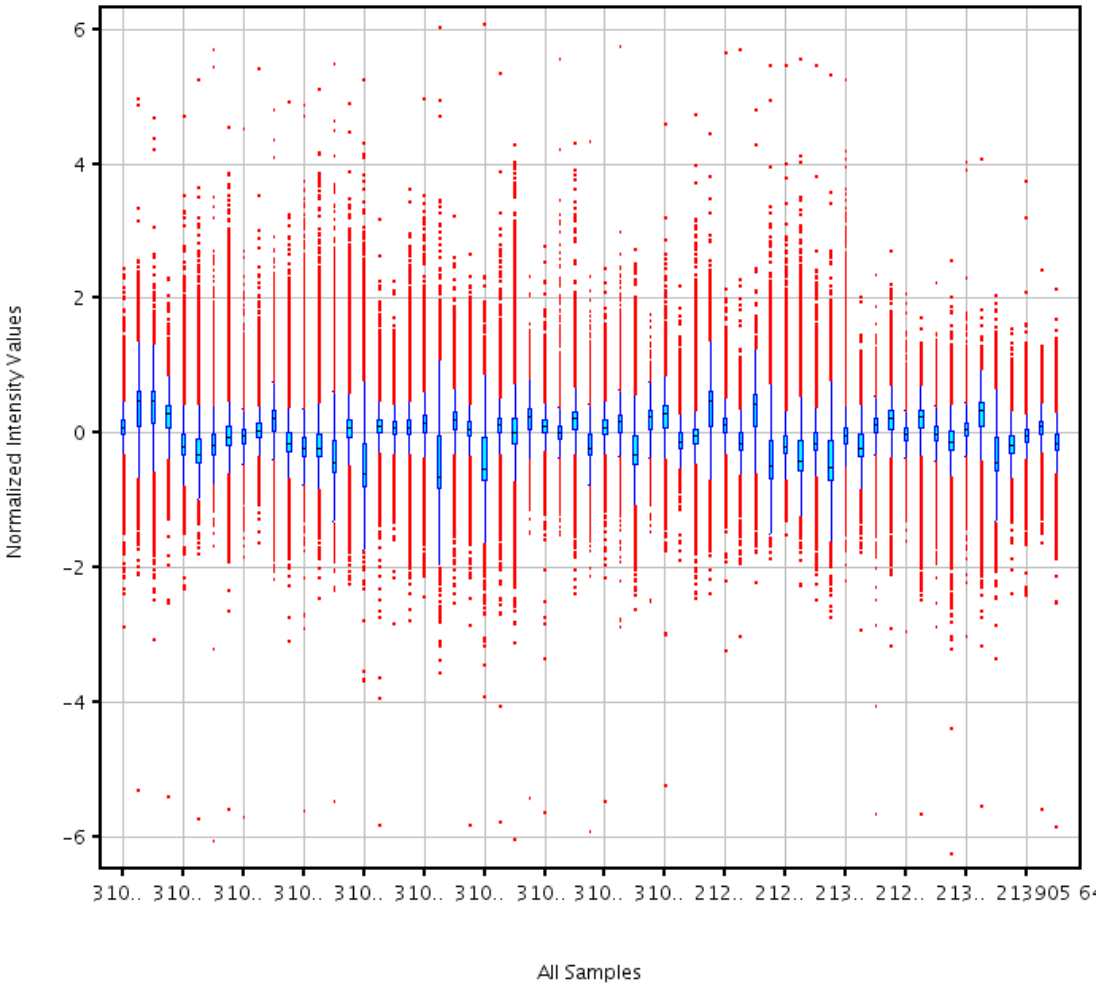


Figure 25. Normalized intensity by sample

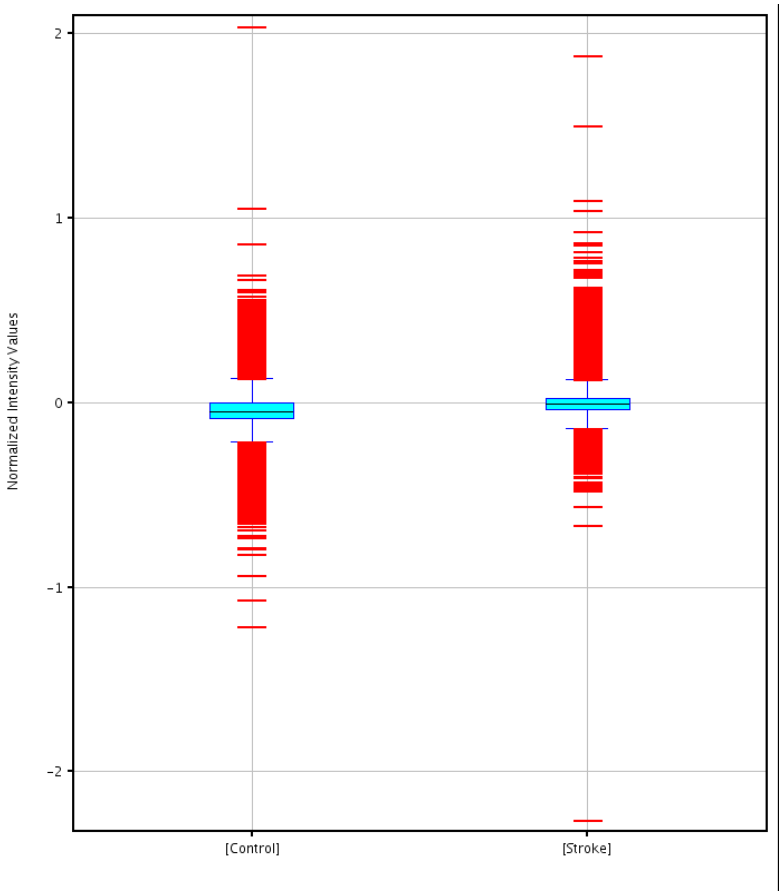


Figure 26. Normalized intensity by group

A logarithmic transformation (log base 2 scale, baseline transformation to the median) was applied to the normalized signal intensities to summarize the data. Log^2 transformation provided a greater number of normally distributed data points than linear scale.

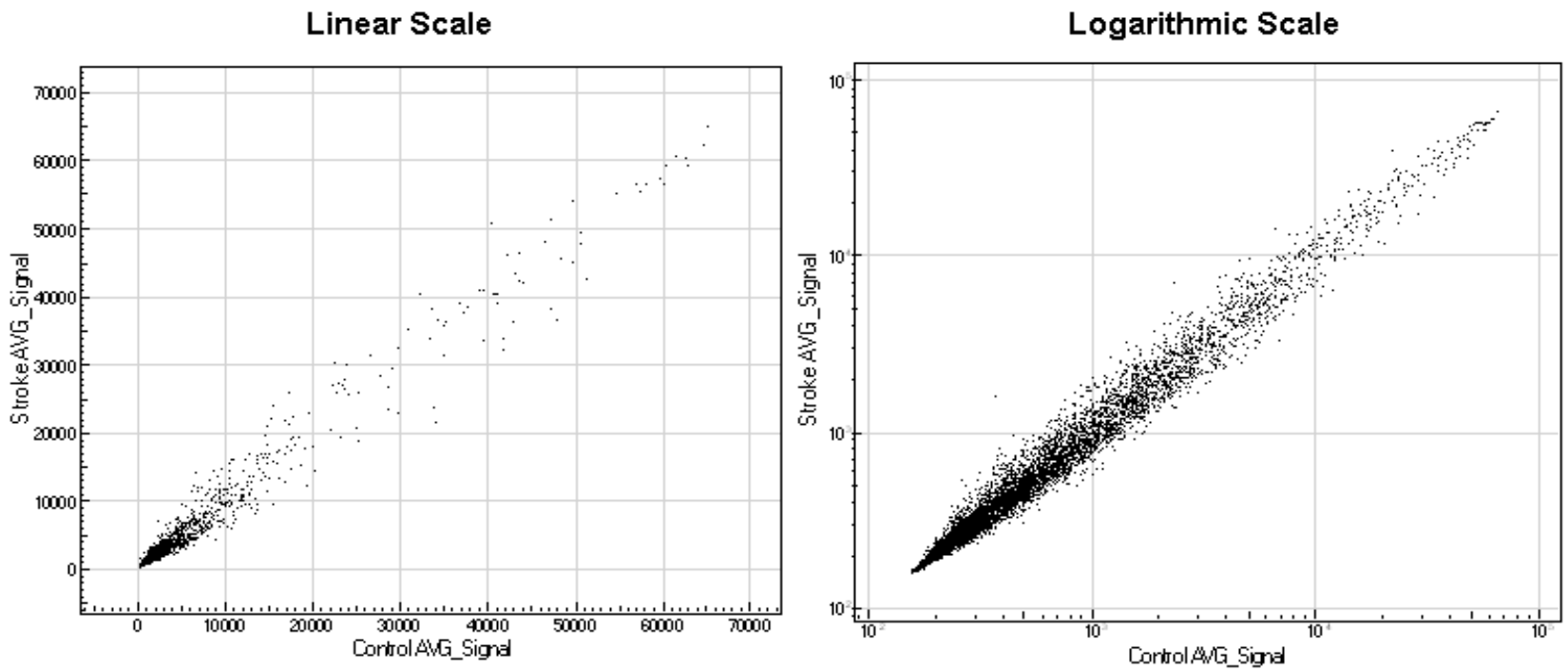


Figure 27. Logarithmic transformation

7.3.5 Supervised clustering

In GeneSpring, supervised clustering with principal components analysis (PCA) was also performed to identify sample outliers after the removal of the control subject outlier. PCA assumes that samples within a group should be more similar to one another than with those from different conditions (case vs. control). It is a variable reduction method that performs eigenvalue-eignevector decomposition on the covariance matrix of gene expression for all samples. One would expect that cases and controls group separately. Similar to the unsupervised clustering performed in Bead Studio, the PCA analysis demonstrated a homogenous group of stroke patients and control subjects with the same stroke patient outlier.

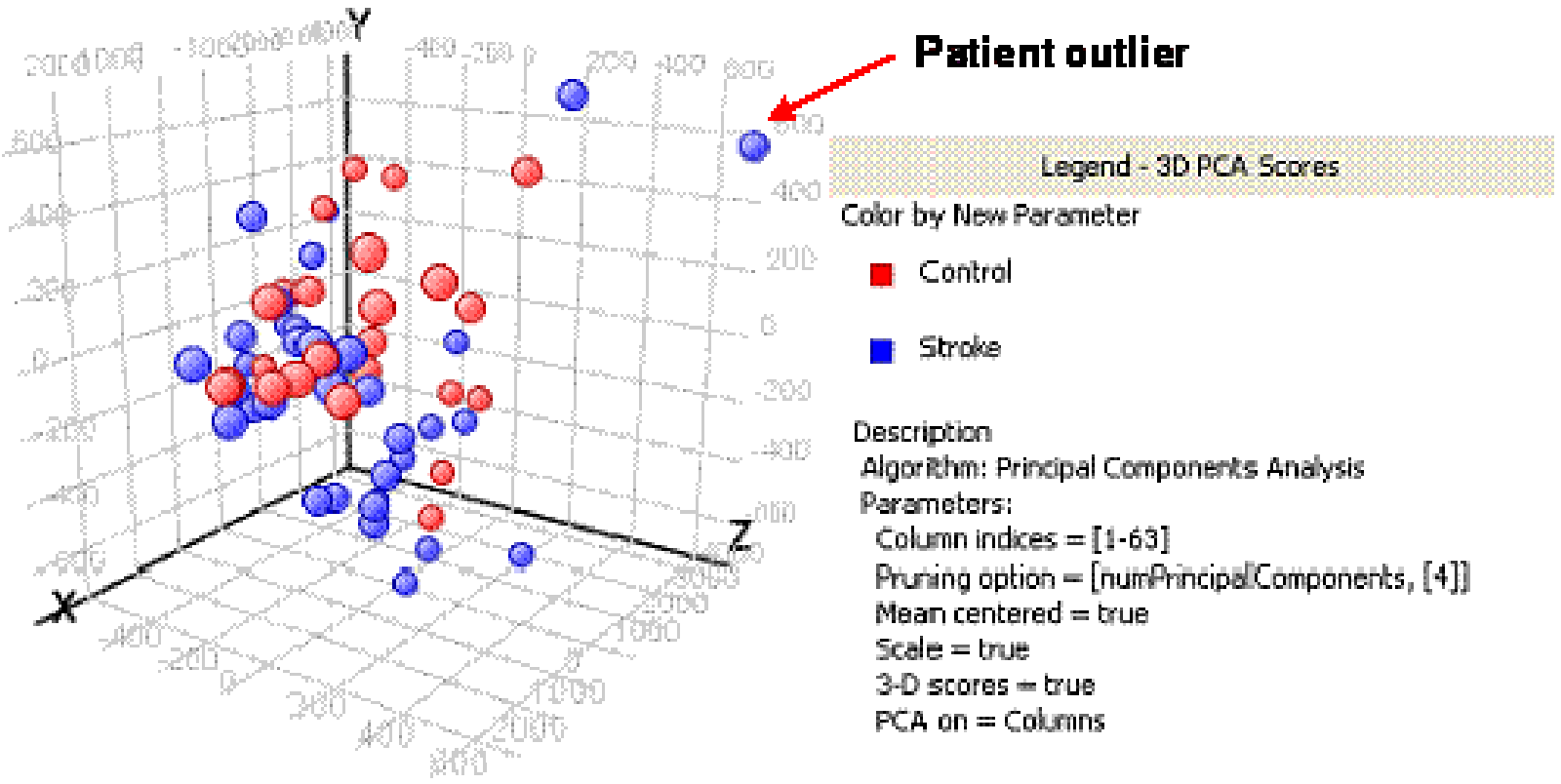


Figure 28. PCA on samples

7.4 PRIMARY ANALYSIS RESULTS

7.4.1 Analysis of Specific Aim One

Specific Aim 1: Determine which genes are under- and over-expressed in acute ischemic stroke patients compared to neurologically healthy age- matched control subjects.

RQ1. Is there a specific blood genomic profile associated with AIS that can be used to identify candidates for diagnostic biomarkers of acute ischemic stroke?

7.4.1.1 Peripheral blood gene profile for Stroke

All analyses were conducted first in Illumina Bead Studio and then in GeneSpring to verify the findings. In Bead Studio, there were 344 genes with a 1.5 fold difference in expression with a diff score > 13 (corrected $p<0.05$) between stroke patients and control subjects, Appendix 0. There were 19 genes with a 2 fold difference in expression with a diff score >13 (corrected $p<0.05$), Appendix P. Results were comparable in GeneSpring with 355 genes with 1.5 fold difference in expression (bonferroni corrected $p<0.05$) (Appendix Q) and 16 genes with 2 fold difference in expression between stroke patients and control subjects (Appendix R), bonferroni corrected $p<0.05$. After comparison there were 9 genes identified by both packages with 2 fold difference in expression, corrected $p<0.05$ significantly different between stroke patients and control subjects.

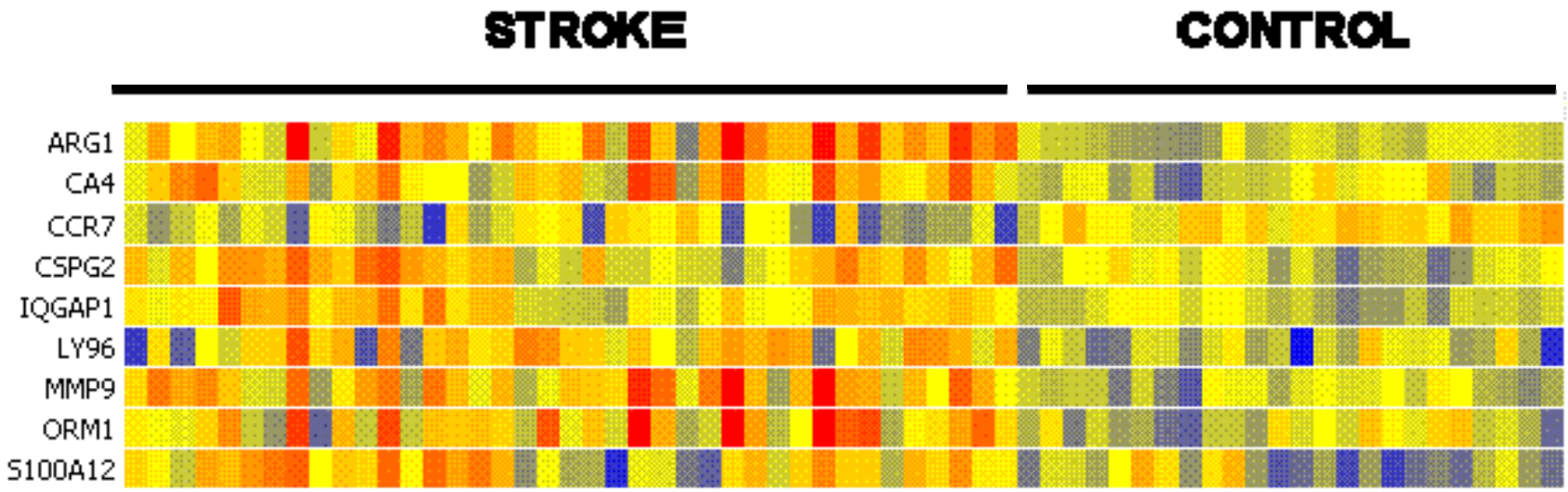


Figure 29. HeatMap for 9 gene list

Table 11. Gene List for Ischemic Stroke

Gene	*p*-value	Fold Change	Regulation	Description
ARG1	2.84E-07	3.175	up	Arginase-1
CA4	2.0E-04	2.122	up	Carbonic anhydrase-4
CCR7	4.37E-05	2.094	down	Chemokine CC Motif Receptor 7
CSPG2	3.45E-05	2.087	up	Chondroitin sufate proteoglycan 2
IQGAP1	7.97E-07	2.031	up	IQ motif-containing GTPase-activating protein 1
LY96	0.001	2.159	up	Lymphocyte antigen 96; MD2 protein
MMP9	1.11E-05	2.644	up	Matrix metalloproteinase 9; gelatinase B
ORM1	0.006	2.246	up	Orosomucoid 1; alpha 1 acid glycoprotein
S100A12	3.87E-04	2.354	up	s100 calcium binding protein A12; calgranulin C

Class prediction models are generally used to identify prognostics for classification of disease. This model identifies expression signatures of groups under study and uses these expression signatures to predict whether an unknown is case or control. A class prediction decision tree algorithm was built from these nine genes using the following criteria: pessimistic error pruning method; information gain goodness function; local 1.0 leaf impurity; leave one out validation; 3 folds; 10 repeats; and the attribute fraction of nodes set at 1.0. The accuracy of these genes to predict stroke was 94.9% with an overall prediction of 95.2%.

Table 12. Prediction model

	Control (Predicted)	Stroke (Predicted)	Accuracy
Control (True)	23	1	95.8%
Stroke (True)	2	37	94.9%
Overall Accuracy			**95.2%**

Five of the 9 genes identified in this study were also found to be significant in the first whole blood gene expression profiling study of stroke (*ARG1; CA4; LY96; MMP9; S100A12*).(Tang, Xu et al. 2006).

Table 13. Comparison of Gene List to Previous Study

Gene	*p*-value	Fold Change	*p*-value	Fold Change
	In this study		Tang et al 2006	
ARG1	2.84E-07	3.2	5.03E-04	3.8
CA4	2.0E-04	2.1	3.54E-05	2.2
LY96	0.001	2.2	3.67E-03	2.1
MMP9	1.11E-05	2.6	3.54E-05	3.2
S100A12	3.87E-04	2.4	2.59E-04	2.2

Since there were significant differences between stroke patients and control subjects by age, an analysis was conducted to determine which genes were significantly regulated by age. To ensure there was a relatively equal distribution of control subjects in each group, age was divided into two groups at 60 years. This resulted in four groups: Control <60 years (n=13); Stroke <60 years (n=9); Control >60 years (n=11); Stroke >60 years (n=30). T-test analysis revealed there were 31 genes significantly different by age for stroke patients (fold change >2 and corrected $p<0.05$) (Appendix S) and 77 genes significantly different by age for control subjects (fold change >2 and corrected $p<0.05$) (Appendix T). An analysis of variance (ANOVA) was performed to determine the changes in gene expression by age between all four groups, which revealed two genes with fold change >2 and corrected $p<0.05$: *IFI27* and *s100A12*.

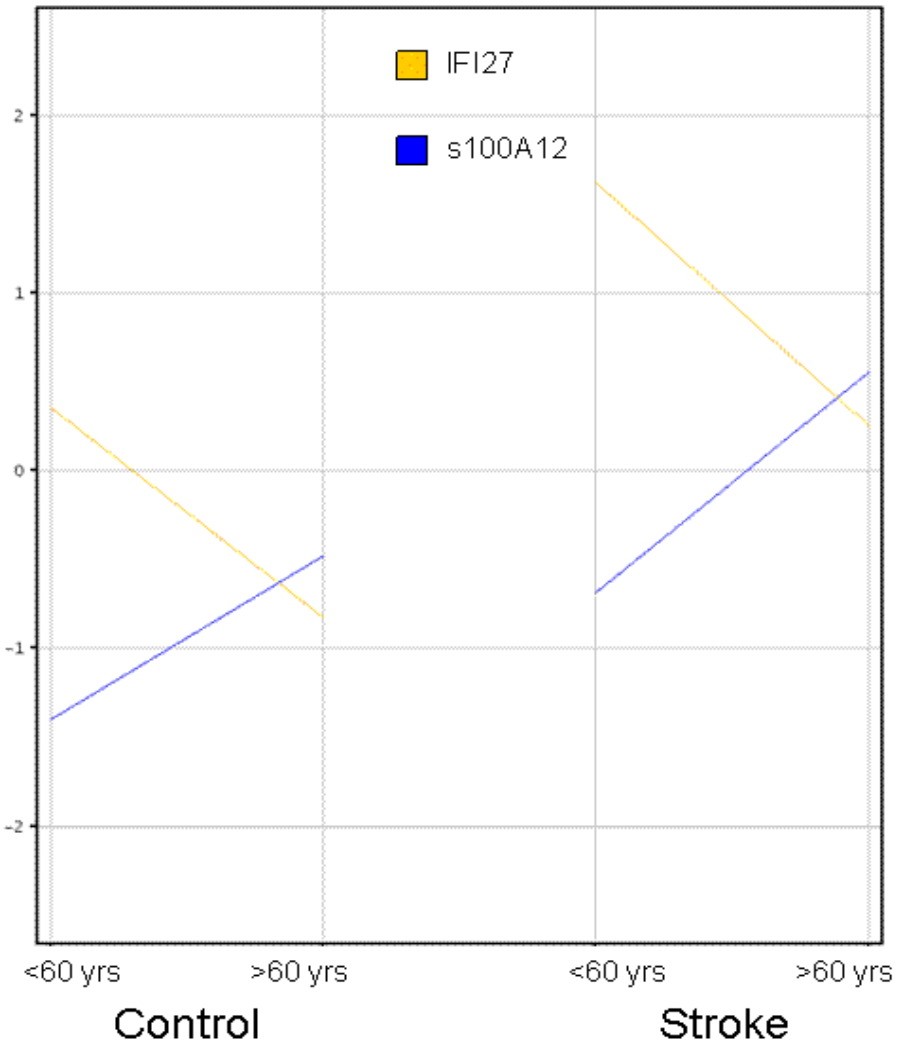


Figure 30. Genes associated with age

Baseline *s100A12* signal intensity was significantly correlated with age (r=0.61; p=0.00). *S100A12* signal intensity was significantly higher in those with age > 60 years (t=-3.9; p=0.00).

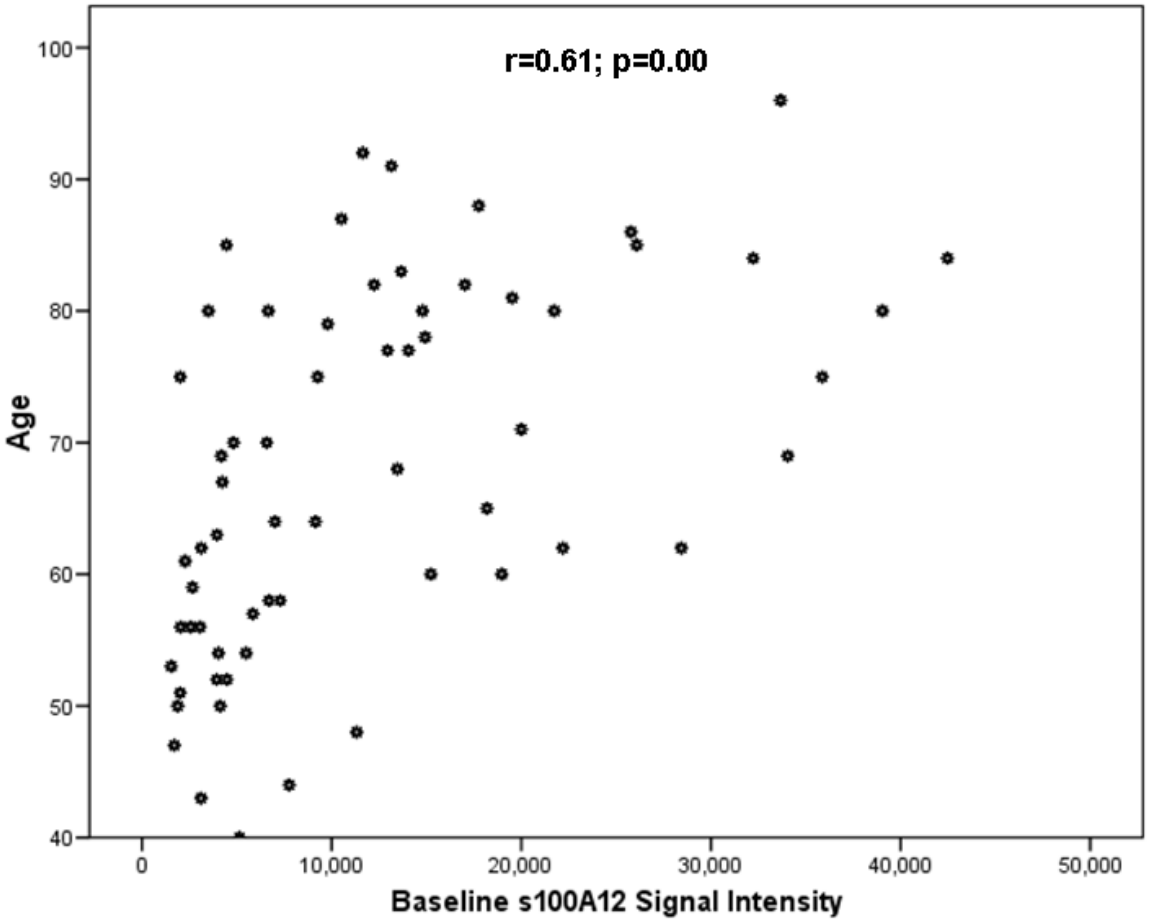


Figure 31. Correlation between *s100A12* and Age

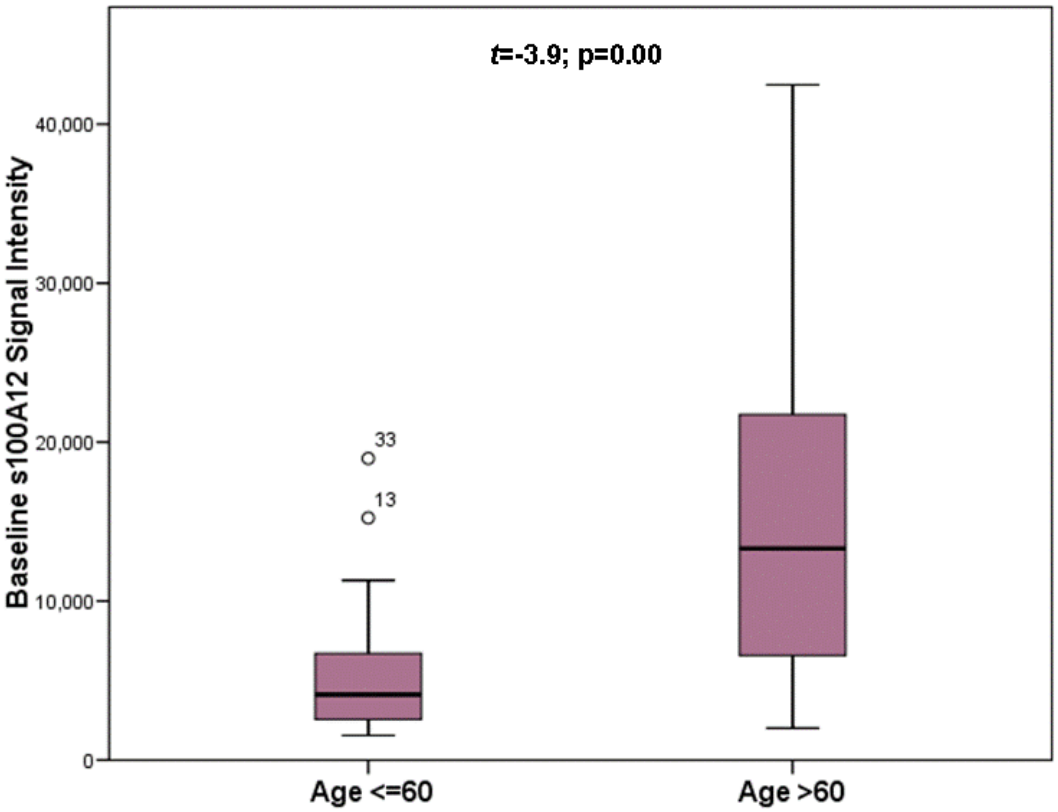


Figure 32. *S100A12* by Age Group

In addition, to the correlation with age, baseline *s100A12* signal intensity was significantly higher in males versus females for both control subjects and stroke patients (*t*=3.45; p=0.002 and t=3.52; p=0.001 respectively). Males and females were not significantly different by presence of hypertension (χ^2=0.43; p=0.51) or dyslipidemia (χ^2=3.4; p=0.06).

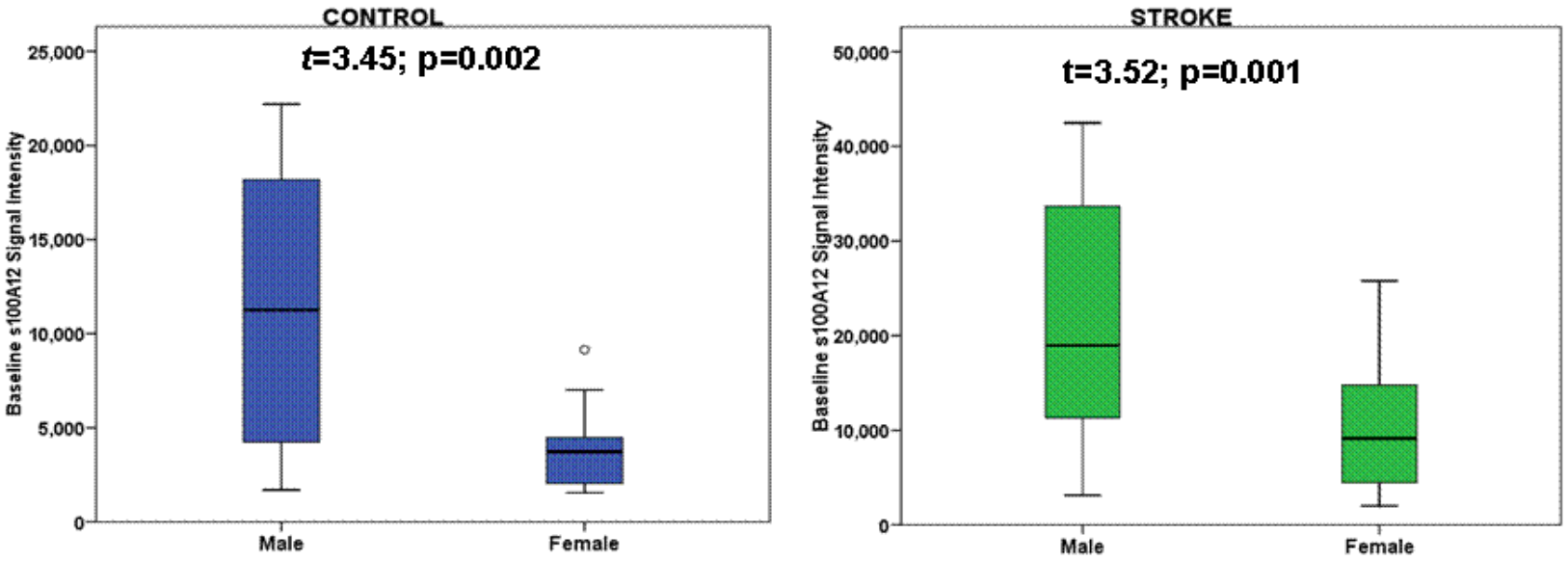


Figure 33. ***S100A12*** **by Gender**

7.4.1.2 Quantitative real-time PCR analysis

Quantitative rt-PCR was used to confirm the results obtained with the Illumina Human ref-8 beadchip. PCR reactions were performed using Taqman probes for the following genes (*ARG1; CCR7: LY96; MMP9*; and *s100A12*) by the 7900HT rt-PCR system (Applied Biosystems).These 5 genes were chosen because four of them were also found to be significant in the first gene expression profiling study for stroke and one of them (*CCR7*) was the only down-regulated gene for stroke found in this study. Rt-PCR validated significant changes in mRNA levels in 4 of the 5 genes. The mRNA level of *ARG1* was 3.5 times higher in stroke patients (t=3.7; p=0.00); *CCR7* was 2.3 times lower in stroke patients (t=-4.3; p=0.00); *LY96* was 1.2 times higher in stroke patients (t=1.94; p=0.06); *MMP9* was 1.8 times higher in stroke patients (t=2.24; p=0.03); and *s100A12* was not validated with rt-PCR (t=1.1; p=0.28). RT-PCR results correlated with microarray signal intensity values for all genes: *ARG1* (r=0.801; p=0.00); *CCR7* (r=0.96; p=0.00); *LY96* (r=0.96; p=0.00); *MMP9* (r=0.59; p=0.00); and *s100A12* (r=-0.47; p=0.000).

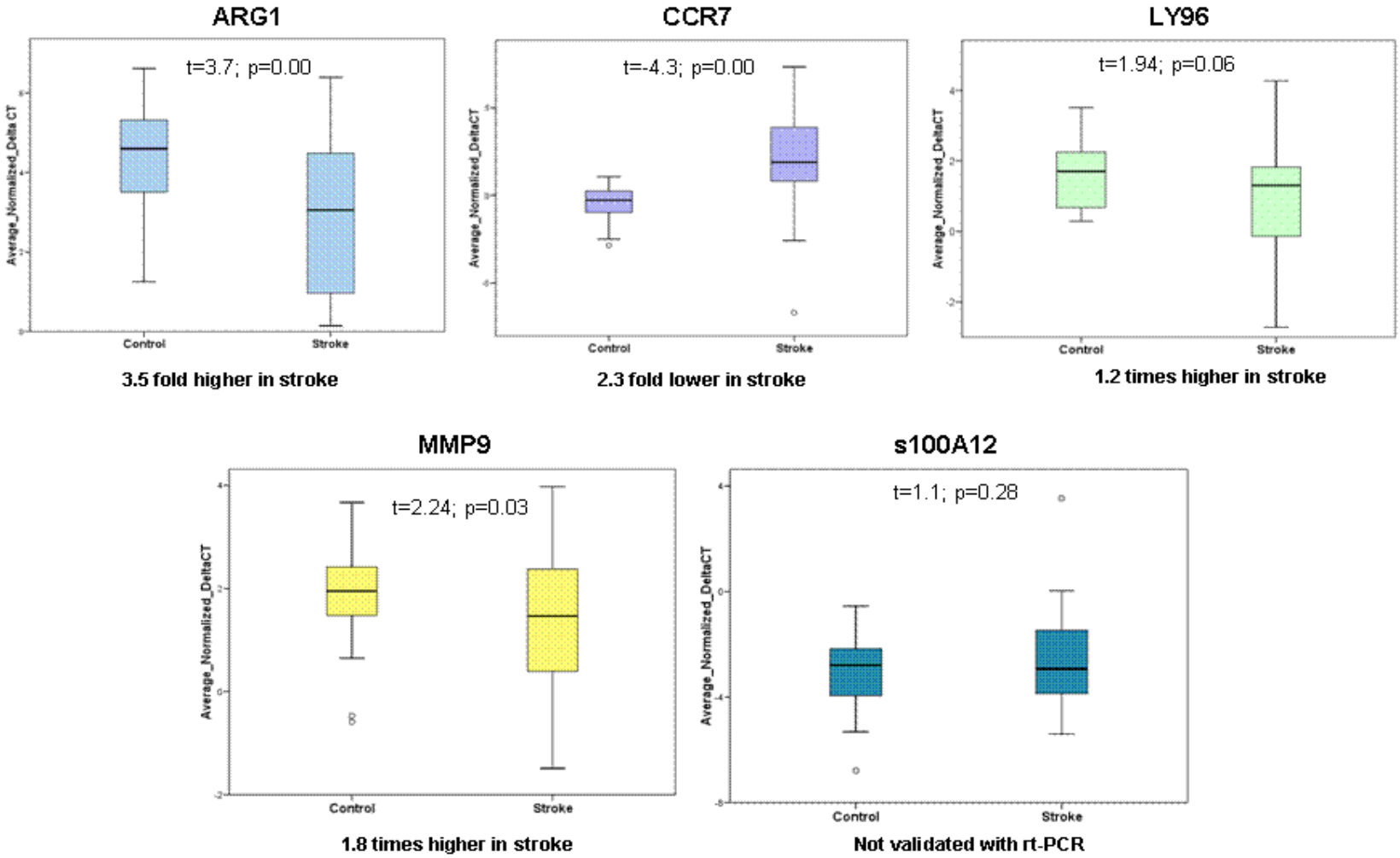


Figure 34. QRT-PCR Results

7.4.1.3 Ingenuity Pathway Analysis

Data were analyzed through the use of Ingenuity Pathway analysis (IPA) (Ingenuity® Systems, www.ingenuity.com). The 1.5 fold gene list (containing gene identifiers and corresponding expression values) obtained in GeneSpring prior to significance analysis of 500 genes was uploaded into the program. Of the 500 genes with 1.5 fold difference between stroke patients and control subjects, 484 genes were mapped to its corresponding gene object in the knowledge database, and 356 genes were eligible for pathway analysis. Canonical pathways analysis identified the pathways from the IPA library that were most significant to the data set. The significance of the association between the data set and the canonical pathway was measured in two ways: 1) a ratio of the number of genes that mapped to the canonical pathway (the number of molecules in a given pathway that meet the 1.5 fold cut off, divided by the total number of molecules that make up that pathway). 2) A right tailed Fisher's exact test to calculate a p-value determining the probability that the association between the genes in the dataset and the canonical pathway is explained by chance alone.

IPA analysis showed that the top-5 most significant canonical pathways in the peripheral blood of AIS patients were associated with CD28 signaling in T-helper cells (p=4.03E00), nuclear factor of activated T cells (NFAT) in regulation of the immune response (p=4.03E00) dendritic cell maturation (p=3.4E00), toll-like receptor signaling (p=3.33E00), and calcium-induced T-lymphocyte apoptosis (p=2.92E00).

There were 12 genes in the dataset involved in CD28 signaling in T-helper cells: *CD247, FOS, ACTR3, CD3E, CDC42, PPP3R1, ITPR3, ARPC5, ZAP70, CD3D, HLA-DRB5,* and *ITK* (p=4.03E00).

However, there were more genes involved in Toll-like receptor signaling within the dataset compared to the total number of genes in the Toll-like receptor pathway with a ratio >2 and p=3.33E00 (*TLR2, TLR1, FOS, LY96, TLR8* (includes *EG:51311*), *IF2AK2, IRAK3*).

The two other most significant pathways associated with stroke in the whole blood are NFAT in the regulation of the immune system ((p=4.03E00) and Dendritic cell maturation (p=3.4E00). There are 15 genes in the dataset involved in NFAT regulation: *FCGR3B, CD247, CD3E, FCGR2A, FCGR1A, CD3D, FOS, PPP3R1, ITPR3, ZAP70, GNA13, FCGR1B, FCGR3A, HLA-DRB5*, and *ITK*). There are 13 genes in the dataset involved in dendritic cell maturation: *FCGR3B, FCGR2A, LTB, CD58, HLA-DRB1, CREB5, FCGR1A, HLA-DQB1, TLR2, CCR7, FCGR3A, HLA-DRB5, and FCGR1B*.

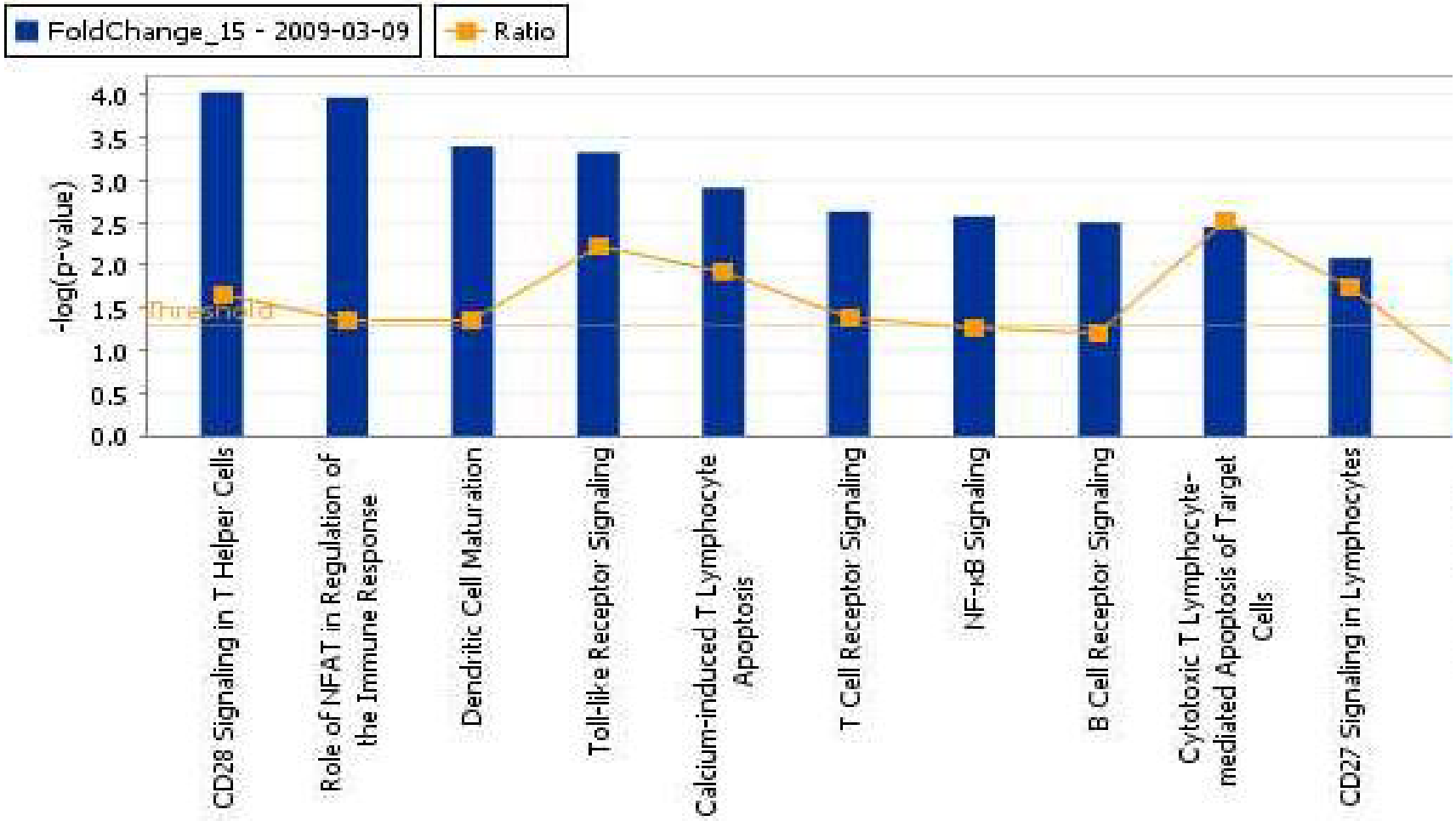


Figure 35. Top 10 Associated Pathways in Peripheral blood of AIS patients

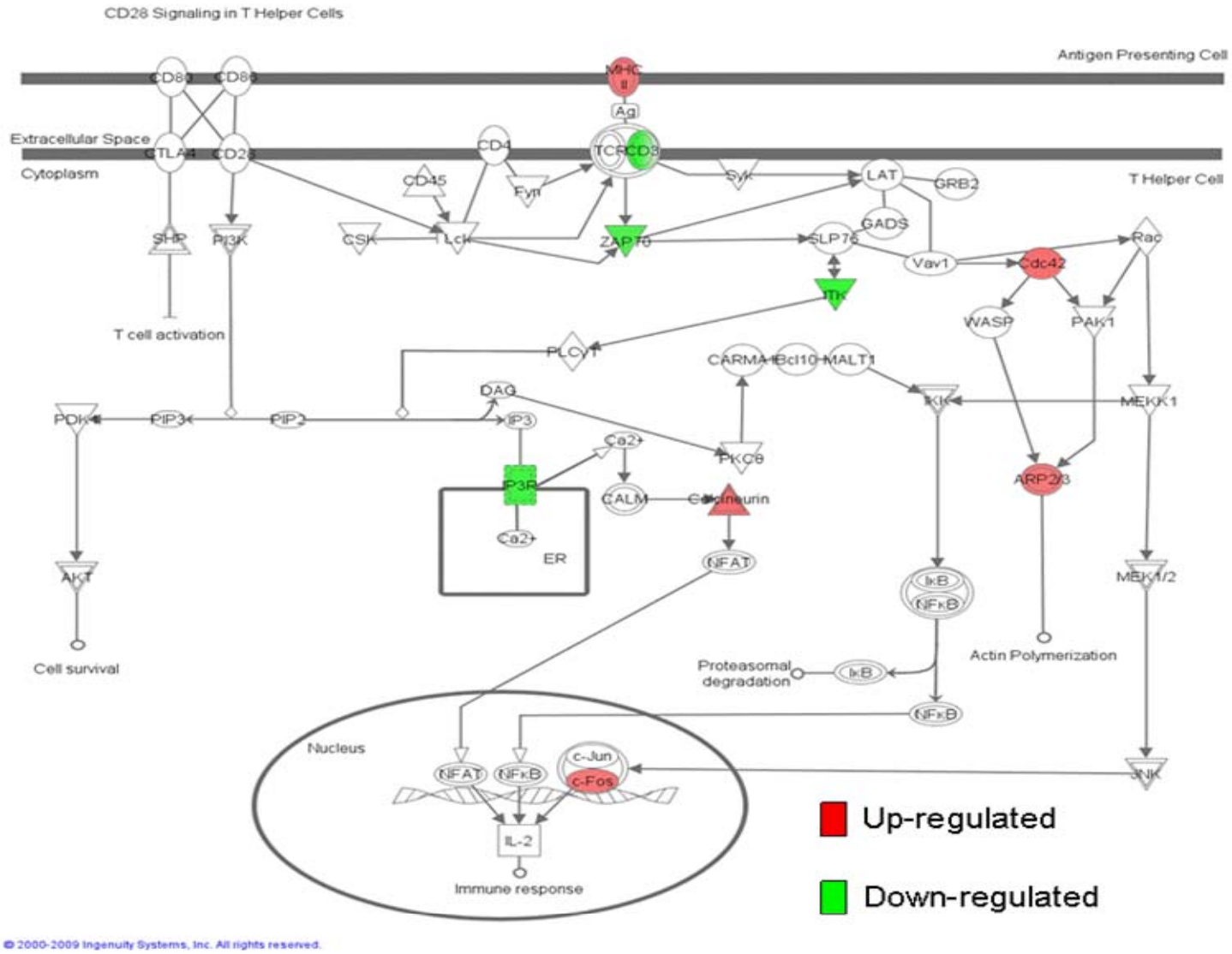


Figure 36. CD28 Signaling in T-Helper cells

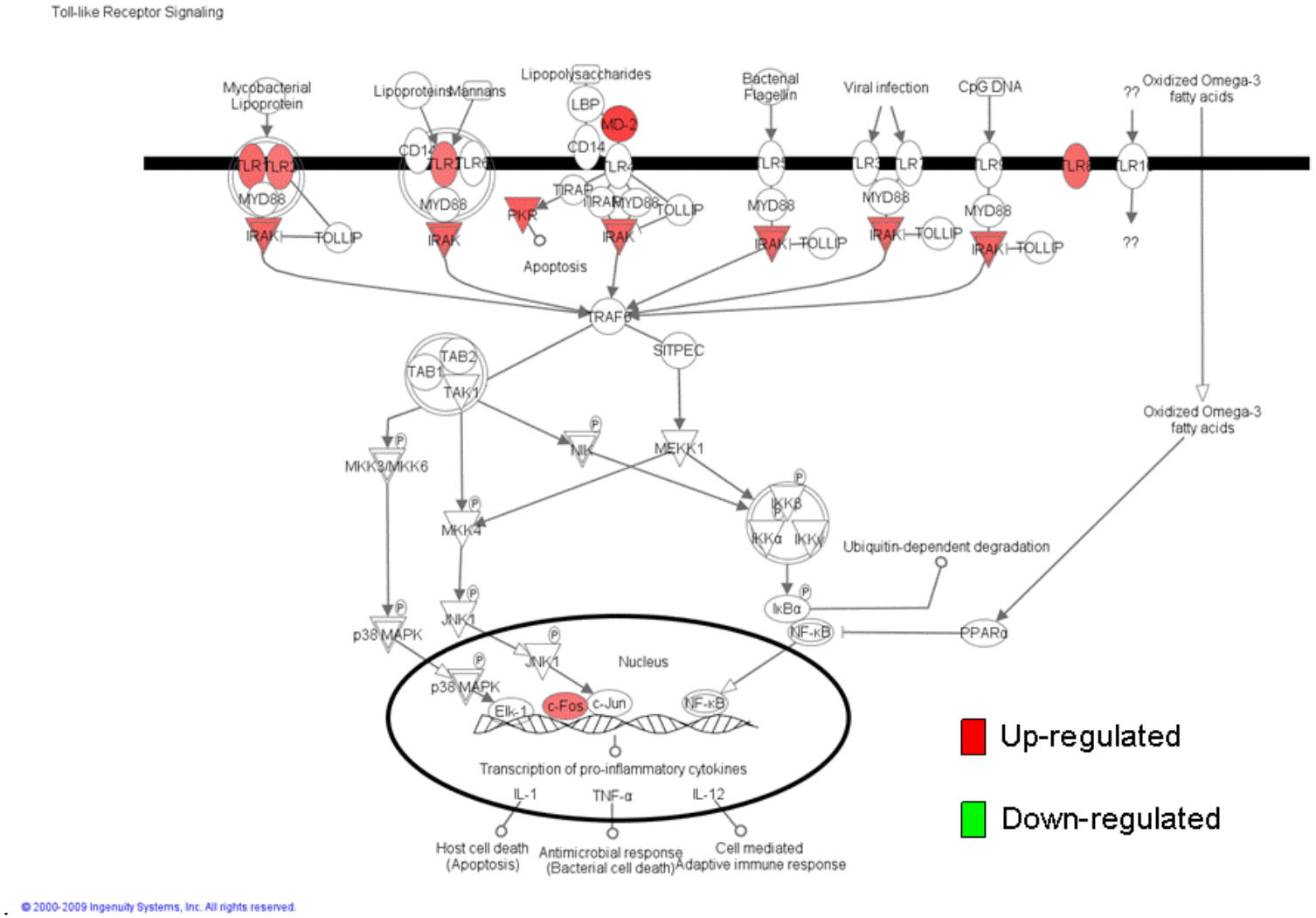


Figure 37. Toll-like Receptor Signaling

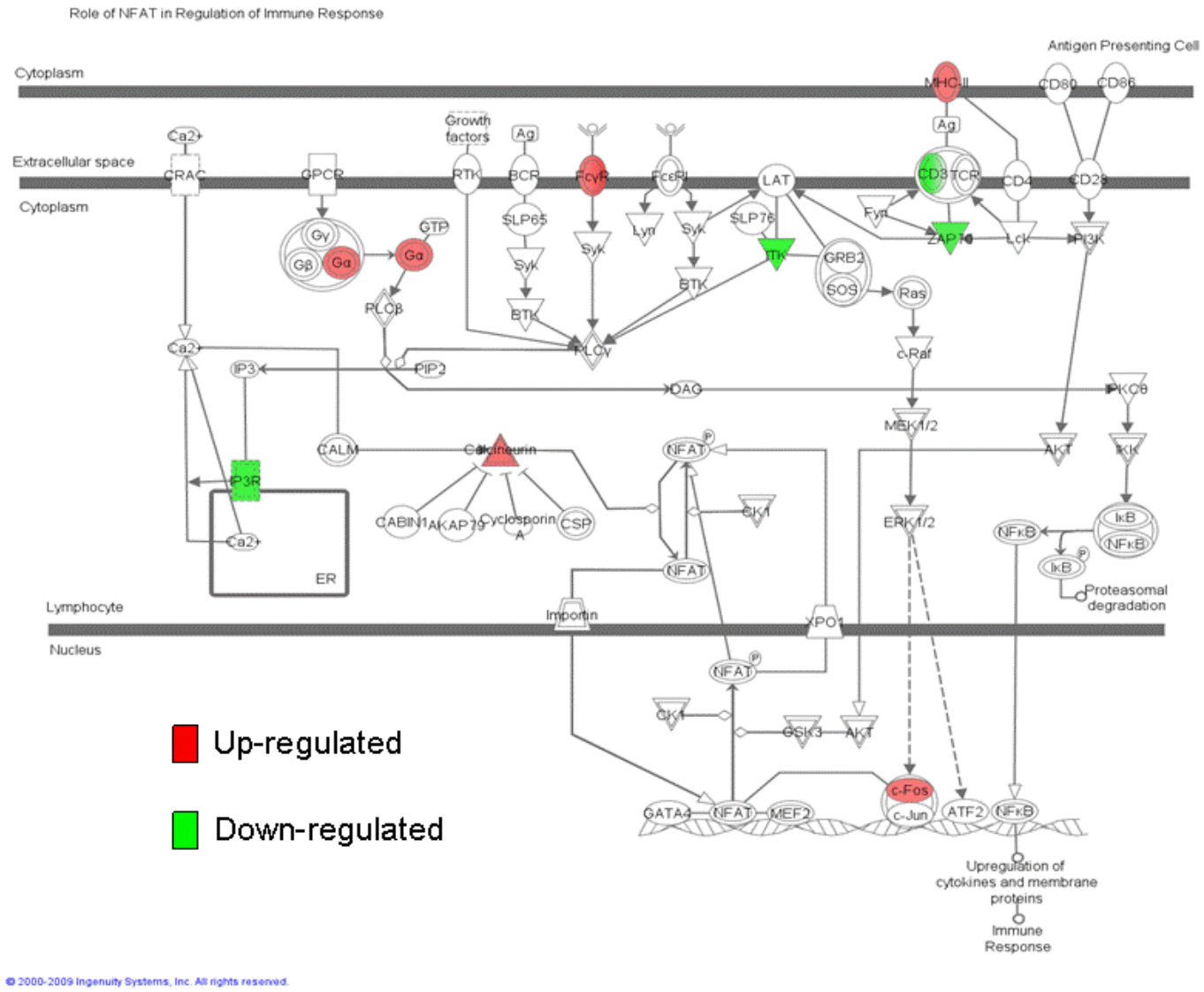


Figure 38. NFAT in Regulation of Immune Response

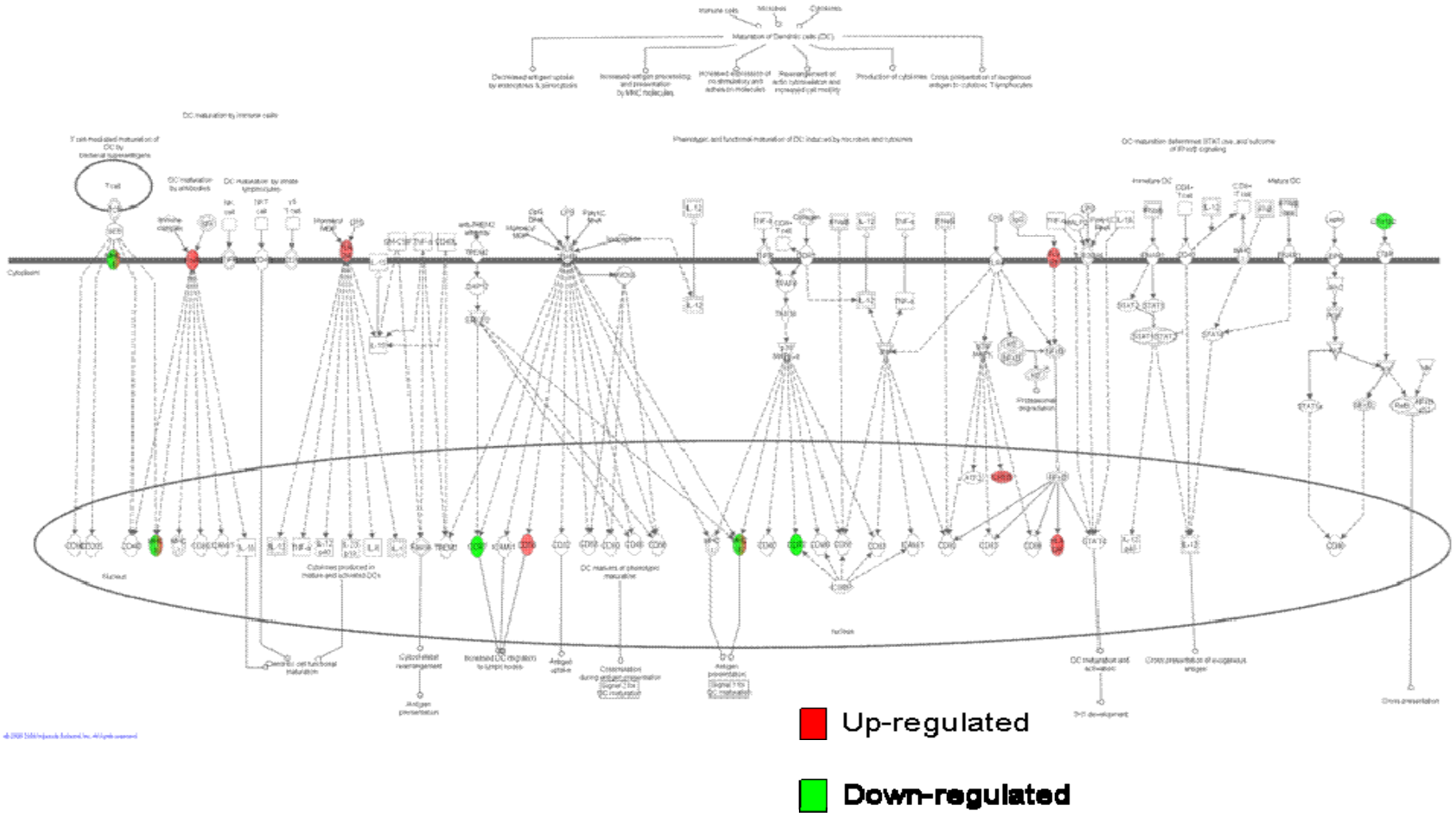


Figure 39. Dendritic cell maturation

7.4.2 Analysis of Secondary Aim

Specific Aim 2: Determine the changes in gene expression that occur in the first 24-48 hours following acute ischemic stroke.

RG2. How will blood gene expression profiles change between the acute phase of ischemic stroke (0-24 hours) and 24-48 hours following onset of symptoms?

7.4.2.1 Change in blood expression profile following stroke

There were only 21 genes with 1.5 fold difference in expression (Bonferroni corrected $p<0.05$) between baseline and 24-48 hour follow up time points in AIS patients (Appendix U). All of these genes were down-regulated at the follow up time point.

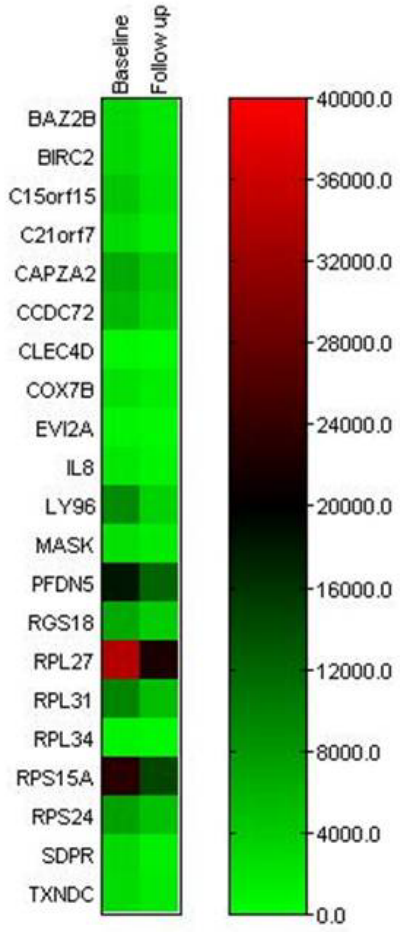


Figure 40. Genes with 1.5 fold difference between baseline and follow up

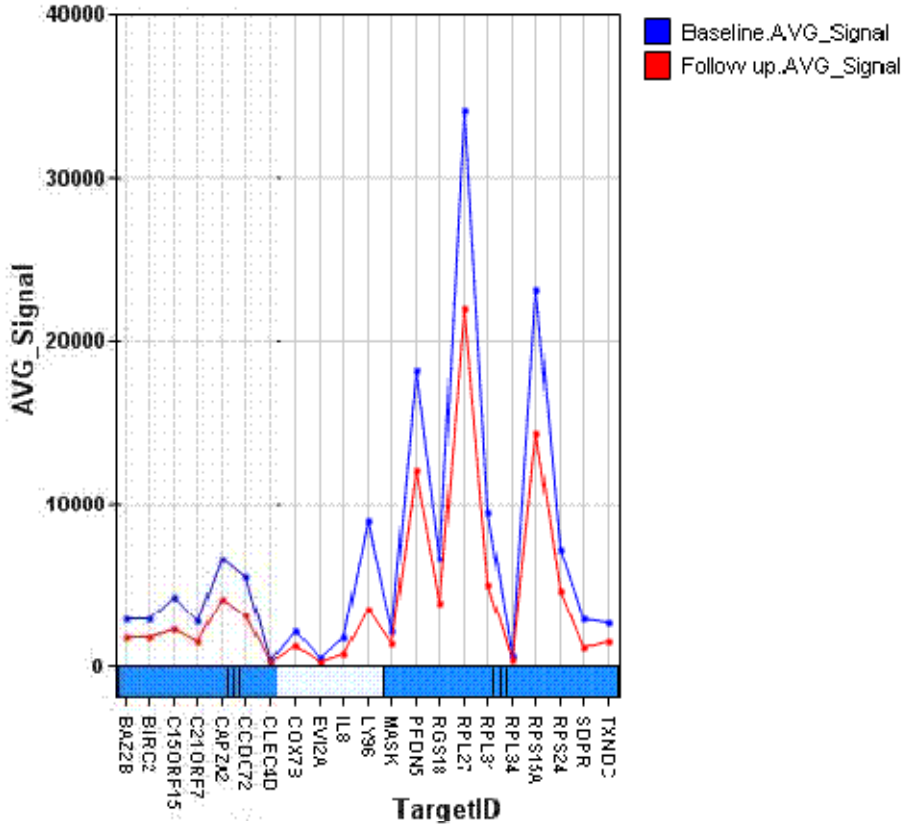


Figure 41. Signal intensity for 21 genes between baseline and follow up

Additionally, only 3 of these genes had a 2 fold difference in expression (Bonferroni corrected $p<0.05$) between baseline and follow up (Appendix V); interleukin-8 (*IL8*), lymphocyte antigen 96 (*LY96*; MD2 protein) and serum deprivation response phosphatidylserine-binding protein (*SDPR*).

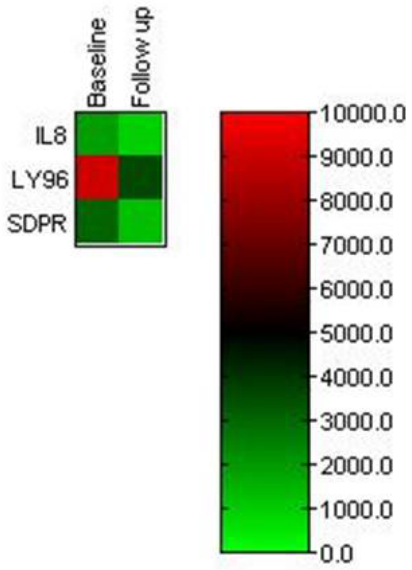


Figure 42..Genes with 2 fold difference between baseline and follow-up

LY96 (MD2 protein) is necessary for the activation of toll like receptor 4 (*TLR4*) as part of the innate immune system. TLRs are pathogen recognition receptors and respond to both pathogen associated molecular patterns (PAMPs) and damage associated molecular patterns (DAMPs) to release inflammatory cytokines. *LY96* modulates *IL8* expression through the activation of *NF-kappaB* (nuclear factor of kappa light polypeptide gene enhancer in B-cells).

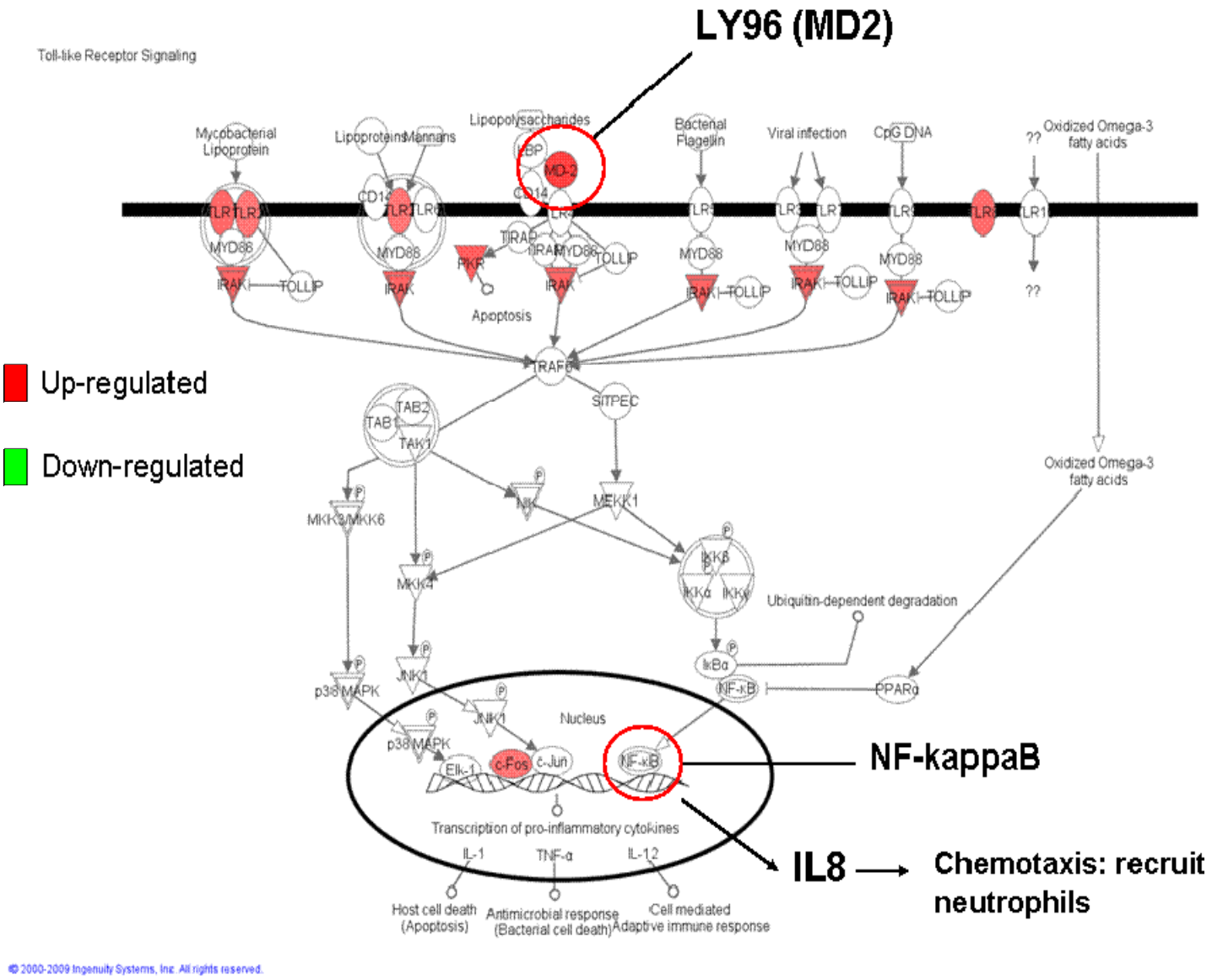


Figure 43. *LY96* and *IL8* in Toll-like receptor pathway

Even though *NF-kappaB* signal intensity was not differentially expressed between baseline and follow up in BeadStudio, the baseline and follow-up signal intensities were significantly different (paired t=-4.4; p=0.000) with the follow up being slightly higher than baseline (average baseline intensity=5482; average follow-up intensity=6560). *LY96* and *NF-kappaB* signal intensity were significantly correlated at baseline (Pearson R -0.44; p=0.009); and follow up (Pearson R -0.563; p=0.001). *IL8* and *NF-kappaB* were also significantly correlated at both baseline (Pearson R -0.39; p=0.022) and follow-up (Pearson R -0.917; p=0.000).

There was not a significant association between *LY96* at baseline (p=0.5) or follow up (p=0.9) with good outcome (MRS 0-1) at 30 days; but there appears to be a trend for a non-linear exponential correlation where higher levels of baseline *LY96* correspond to better outcome at 30 days (r=0.15; p=0.5); with the exception of the two patients who died. Additionally, there was not a significant association between *IL8* at baseline (p=0.9) or follow up (p=0.5) with good outcome (MRS 0-1) at 30 days.

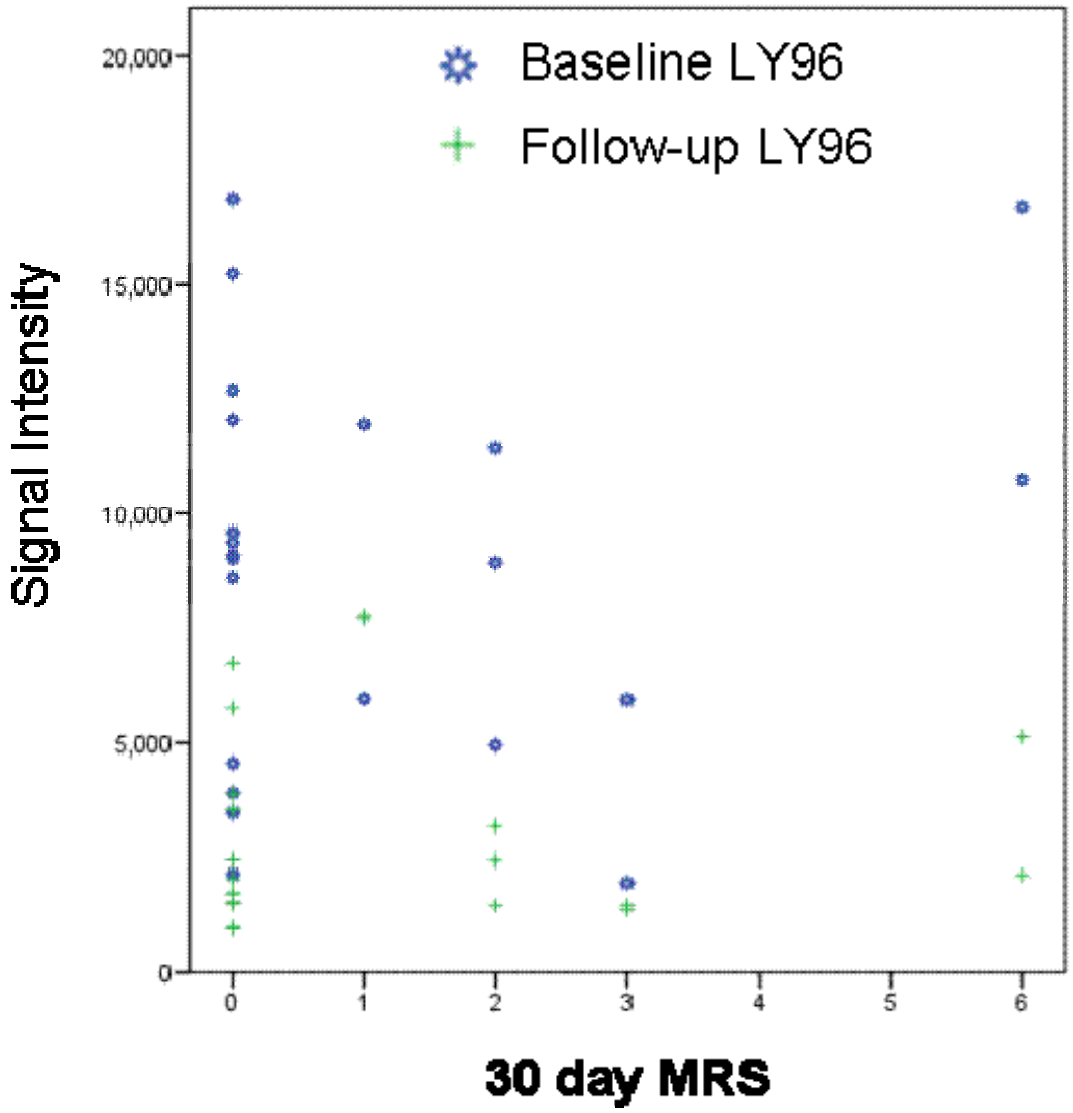


Figure 44. *LY96* and 30 day MRS

Interestingly however, there is a significant correlation between follow up *NF-kappaB* signal intensity and MRS at 30 days post ischemic stroke (Pearson R -0.531; p=0.009), with higher follow-up levels corresponding to better outcome (F=6.05; p=0.003). There is also a correlation between follow-up *s100A12* signal intensity and 30 day MRS; with higher levels being correlated with worse outcome (r=0.59; p0.003). There is no correlation between follow-up *NF-kappaB* and follow up *s100A12* (r=-0.23; p=0.196).

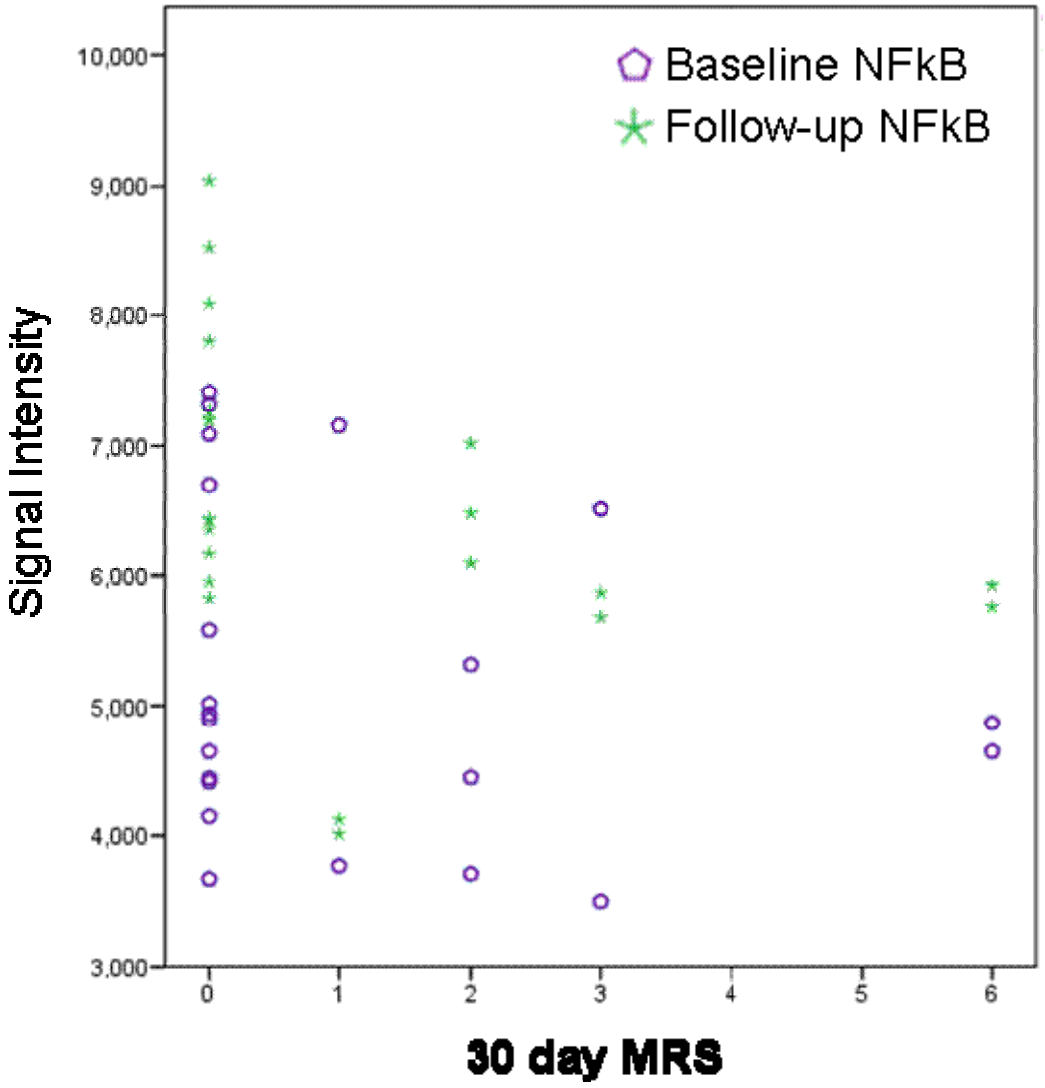


Figure 45. ***NF-kappaB*** **and 30 day MRS**

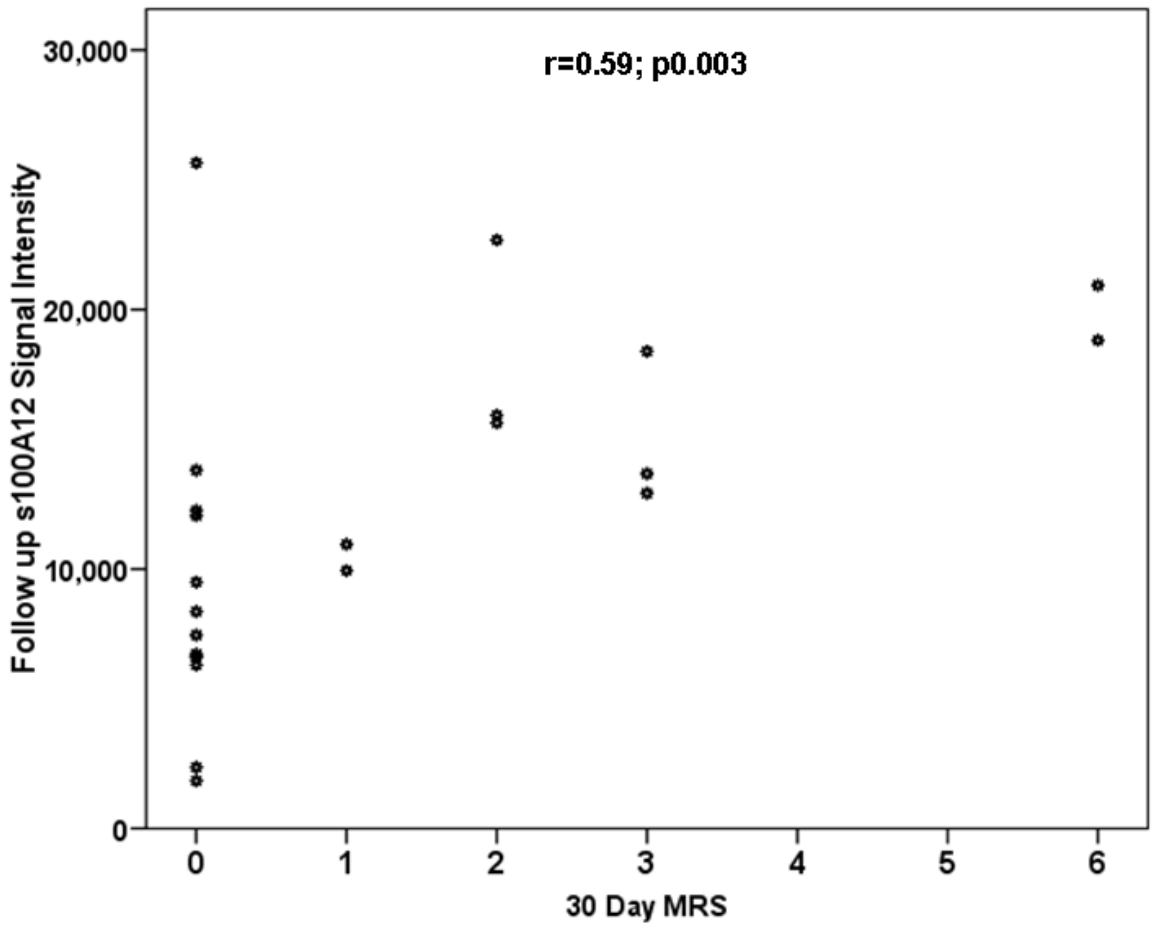


Figure 46. Follow-up *s100A12* and 30 day MRS

In addition, baseline (r=-0.37; p=0.03) and follow-up *NF-kappaB* was found to correlate with age (r=-0.419; p=0.014) with patients who are less than 60 years having significantly higher follow-up *NF-kappaB* signal intensity compared to patients older than 60 years (t=2.49; p=0.018). *s100A12* signal intensity was significantly higher in older patients at both baseline (*t*=-2.7; p=0.012) and follow up (*t*=-1.3; p=0.04).

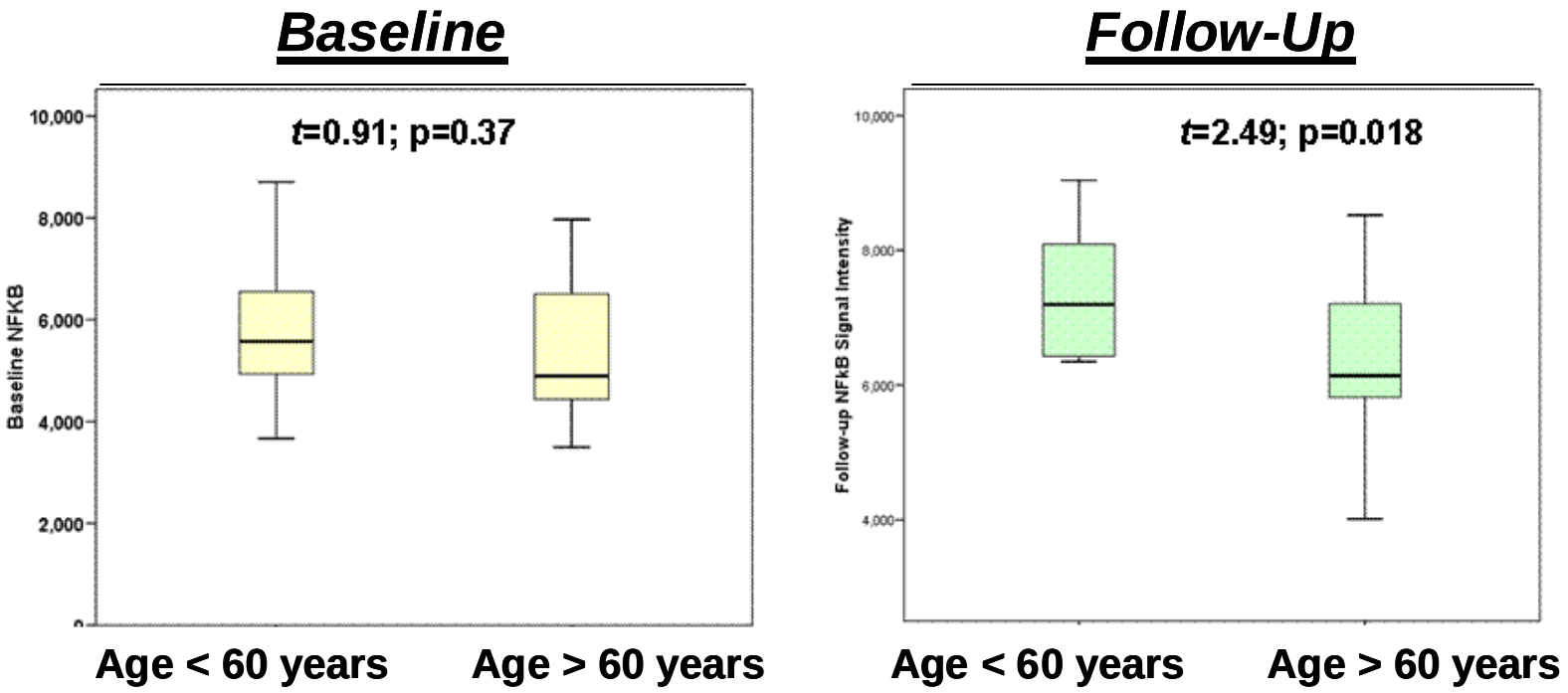


Figure 47. ***NF-kappaB*** **by Age**

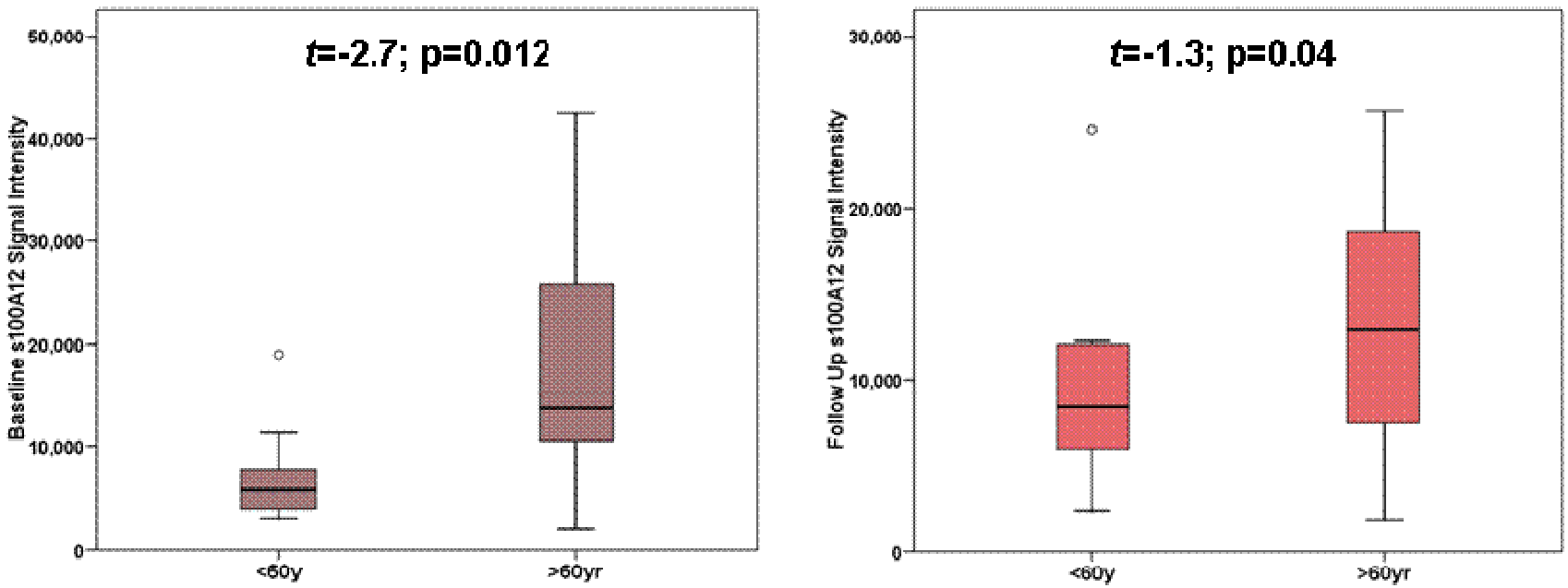


Figure 48. ***s100A12*** **by Age**

7.4.3 Analysis of Tertiary Aim

Specific Aim 3. Determine whether acute ischemic stroke patients with BBB disruption have a specific blood genomic profile compared to AIS patients without BBB disruption.

RQ3. Is there a specific blood genomic profile associated with the development of BBB disruption as HARM on MRI after AIS?

7.4.3.1 Genomic profile for BBB Disruption

Blood brain barrier disruption was characterized as the presence of HARM under two criteria: 1. HARM category-none, mild, moderate, or severe; and 2. Severe HARM-yes or no (binary). Each of these descriptions was then tested independently against two separate models: 1. signal intensities for the 16 genes specific for stroke, using age, gender, rtPA treatment, hypertension, diabetes, hyperlipidemia, and smoking history (previous, current or non-smoker) as covariates; and 2. Signal intensities for the 16 genes specific for stroke, age, gender, and rtPA treatment. This resulted in the testing of four separate models.

For the full model one predicting HARM categories the following covariates were significant predictors of HARM category: *LY96* (p=0.005); *ORM1* (p=0.04); age (p=0.01); and rtPA administration (p=0.01). For the partial model two predicting HARM categories, only *LY96* (p=0.019), age (p=0.018) and rtPA treatment (p=0.013) were significant. For both the full model three and partial model four predicting Severe HARM (yes or no) only *AKAP7* was a significant predictor of Severe HARM (p=0.042 and p=0.034 respectively). (Appendix T)

Patients with any presentation of HARM were significantly older (F=3.36; p=0.034) and over 50% of patients who received rtPA displayed HARM.

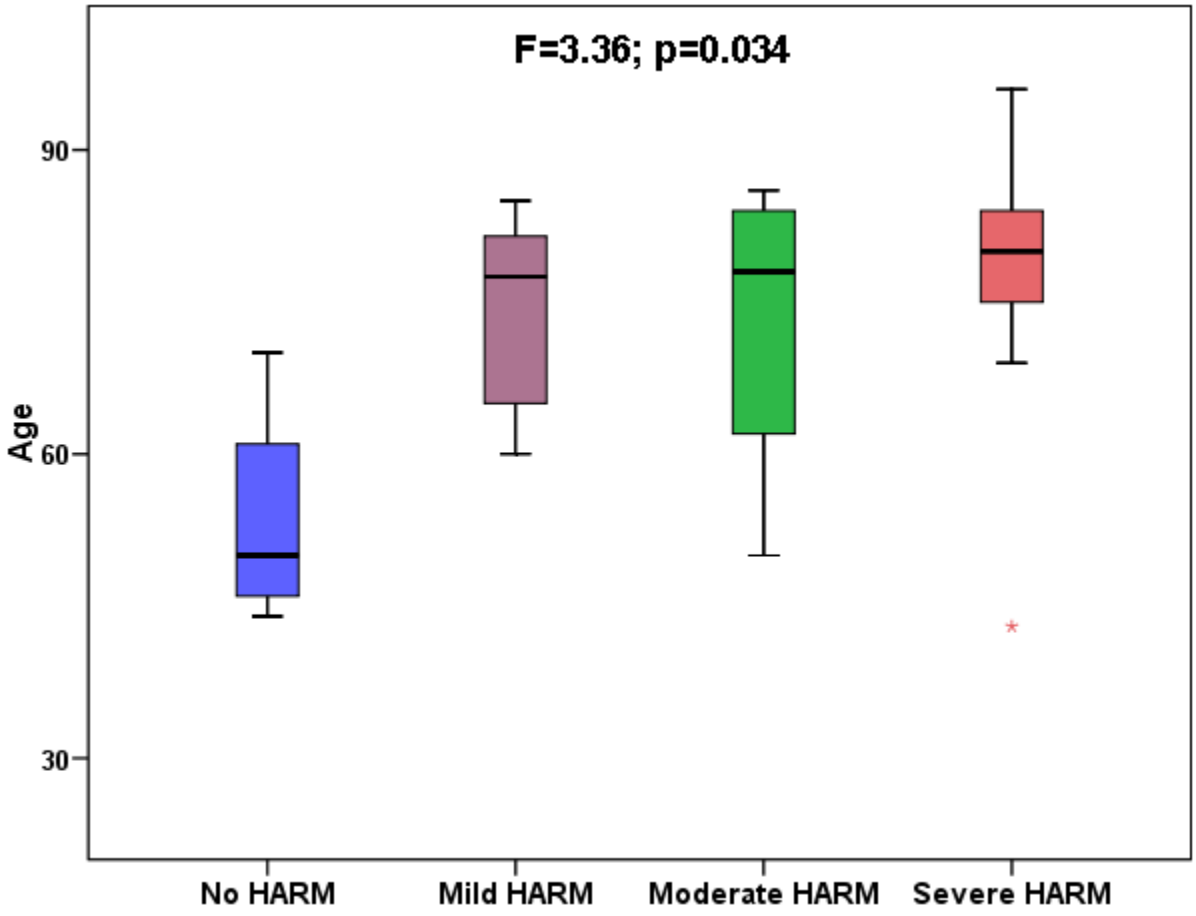


Figure 49. Age and HARM Category

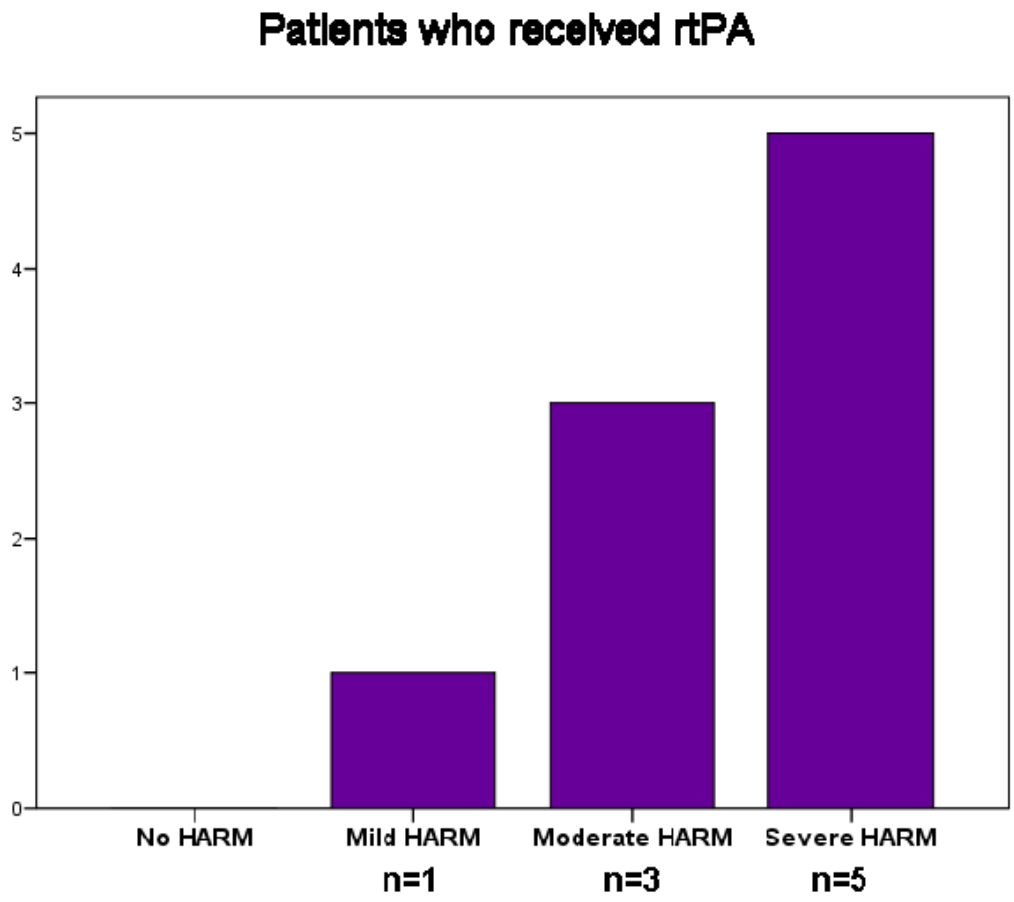


Figure 50. HARM Category and rtPA

Similarly, with the exception of one patient, patients with Severe HARM were significantly older (t=-2.34; p=0.027) and more likely to have received rtPA (χ^2=3.99; p=0.046).

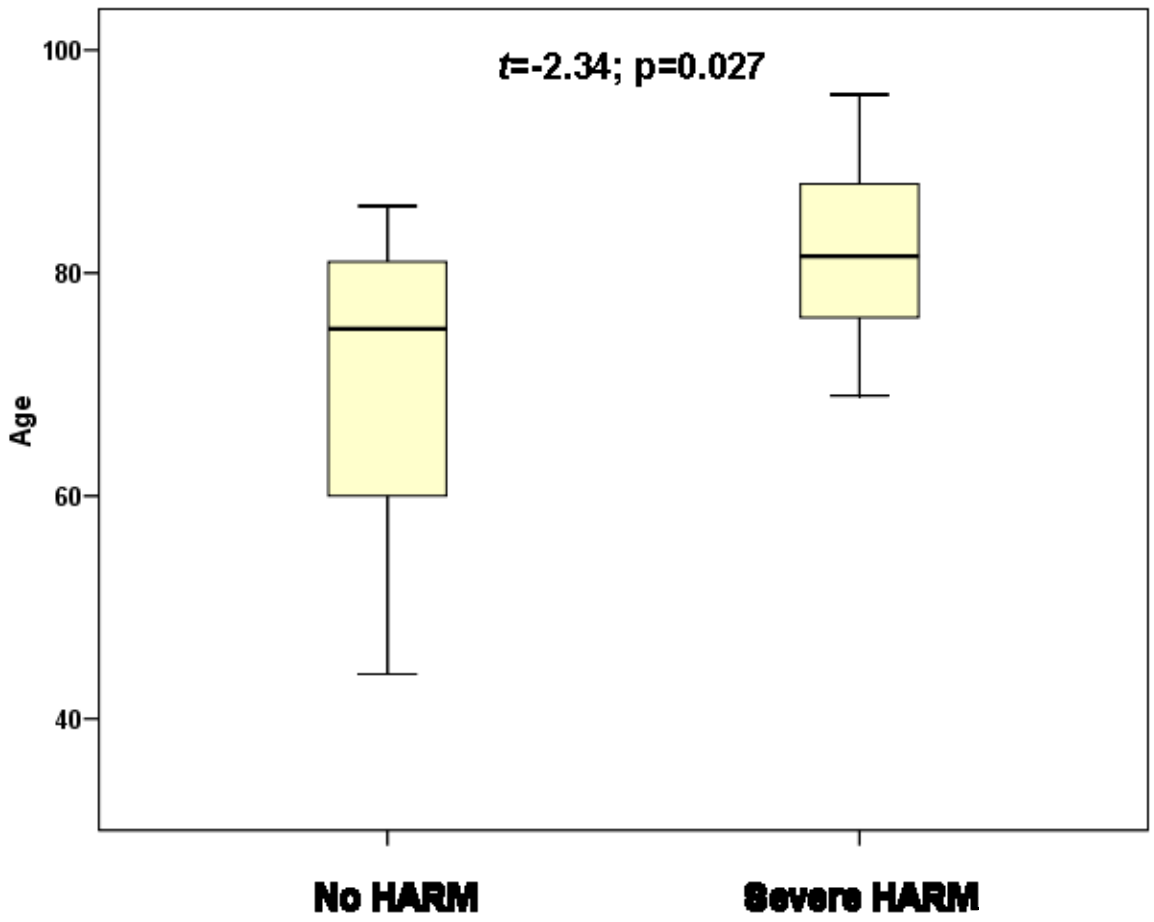


Figure 51. Severe HARM and Age

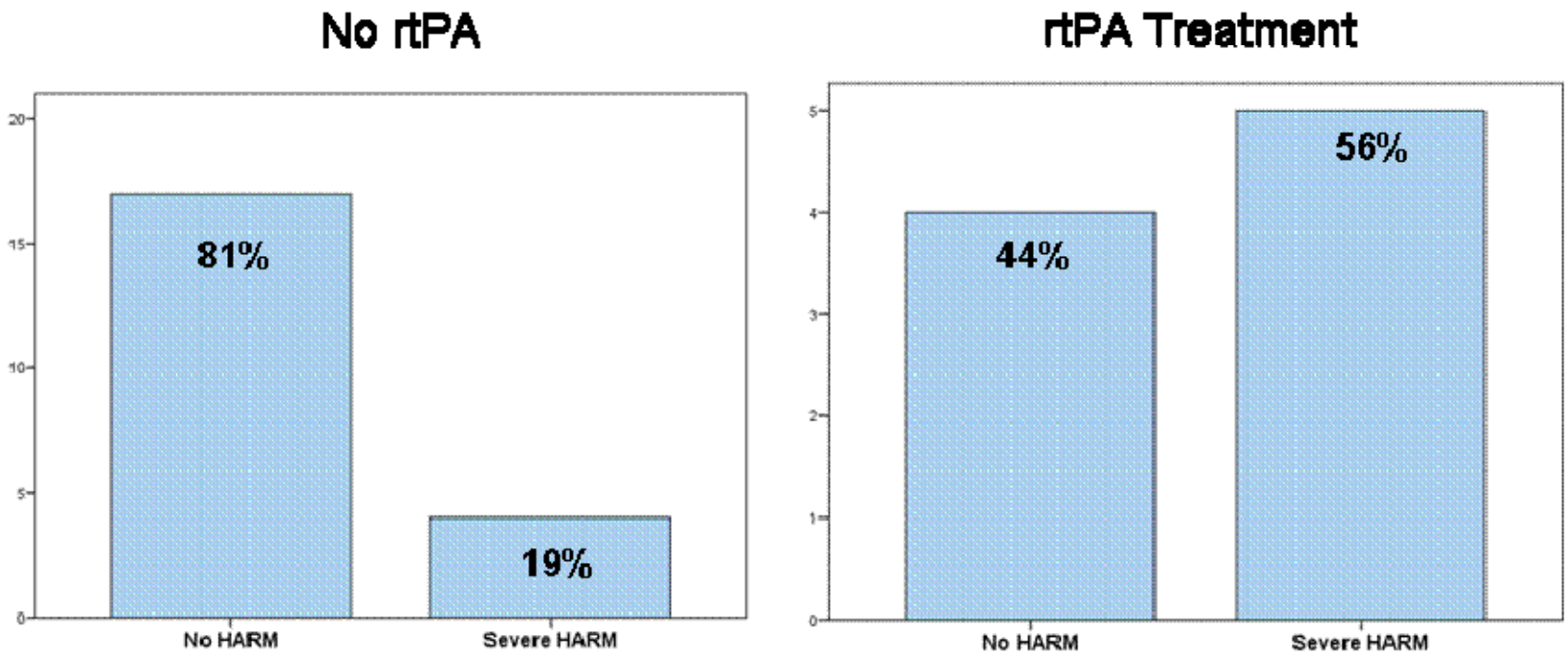


Figure 52. Severe HARM and rtPA

The mean log transformed signal intensity of *LY96* for no Harm (-0.31±1.6), mild Harm (-0.34±1.03), moderate HARM (0.85±0.73), and severe HARM (0.94±0.36) was significantly higher in the moderate and severe HARM groups (F=4.52; p=0.011). *LY96* was not correlated with age (r=0.2; p=0.29), severity of injury (r=0.28; p=0.14), or *MMP9* (r=0.21; p=0.28). However there was a trend for a correlation between *LY96* and *ORM1* (r=0.34; p=0.07).

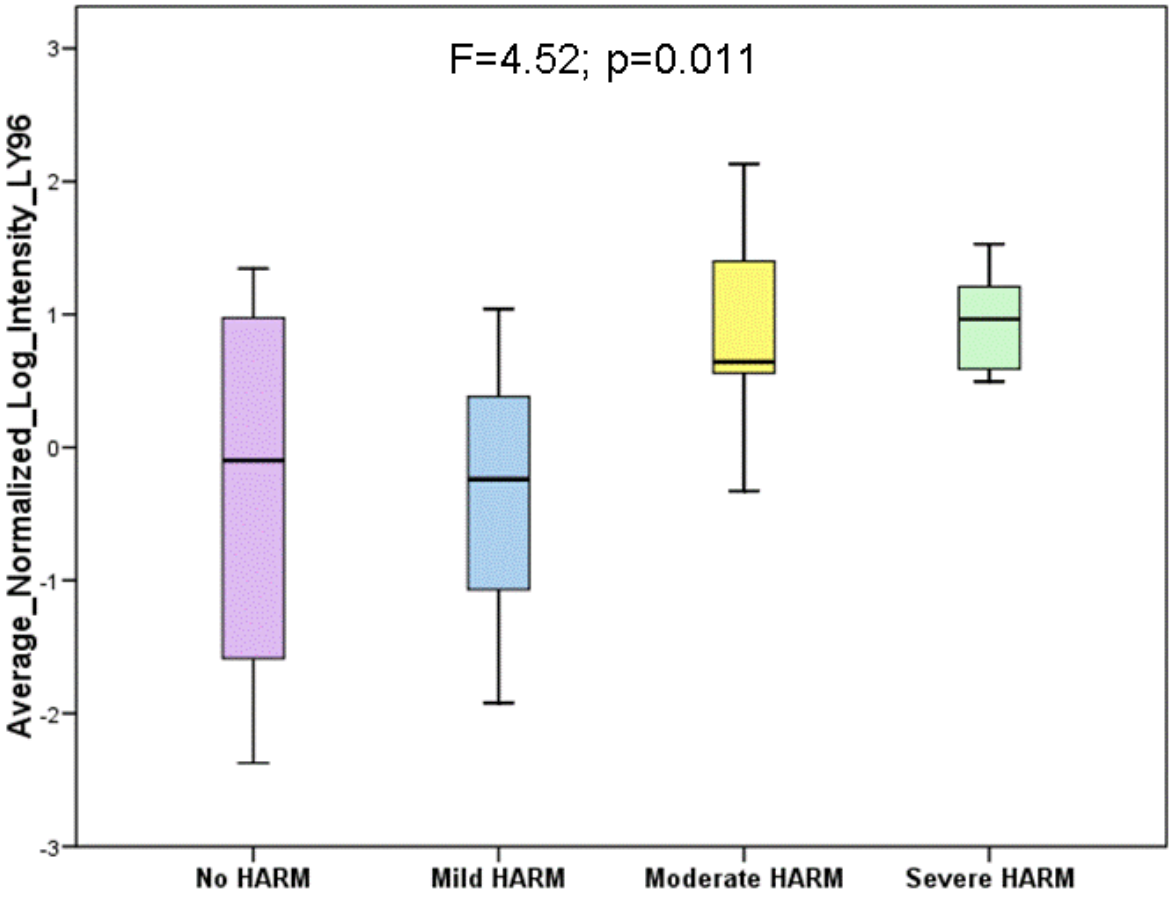


Figure 53. *LY96* and HARM Category

Mean signal intensity of *ORM1* was not significantly different between the categories of HARM (F=0.249; p=0.861); neither was the mean signal intensity for *MMP9* (F=0.49; p=0.69). There was no correlation between *ORM1* with age (r=0.12; p=0.55) or between *MMP9* with age

(r=0.15; p=0.43). Interestingly both *ORM1* (r=0.44; p=0.01) and *MMP9* (r=0.51; p=0.004) were correlated with baseline NIHSS and *ORM1* was significantly correlated with *MMP9* (r=0.72; p=0.000).

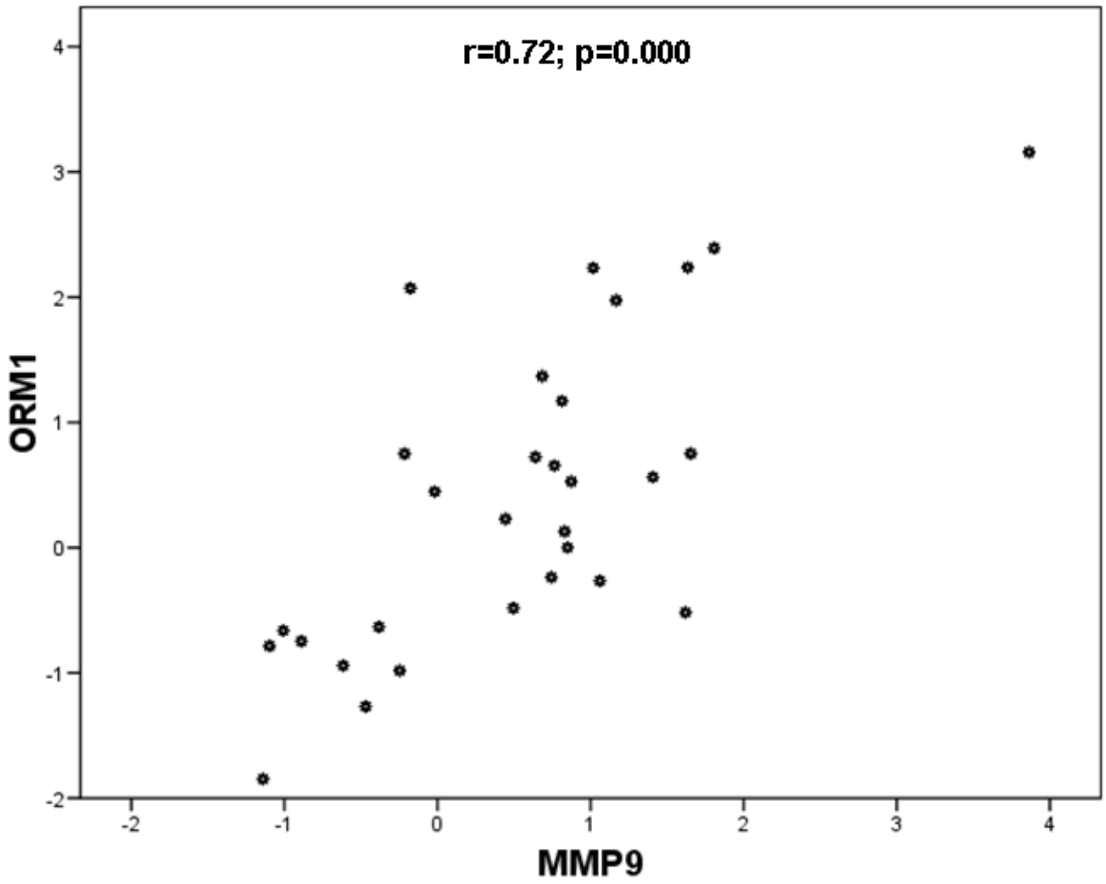


Figure 54. *ORM1* and *MMP9*

The mean log transformed signal intensity of *AKAP7* for Severe HARM (1.53±0.79) was significantly higher then the mean log transformed signal intensity for No HARM (0.35±1.29), t=-2.5; p=0.017. *AKAP7* was not correlated with age (r=0.22; p=0.24), severity of injury (r=0.07; p=0.72), *MMP9* (r=0.1; p=0.79), or *ORM1* (r=0.2; p=0.28); but it was correlated with LY96 (r=0.56; p=0.001). There was also a trend for a significance of *LY96* with severe HARM (t=-1.9; p=0.07).

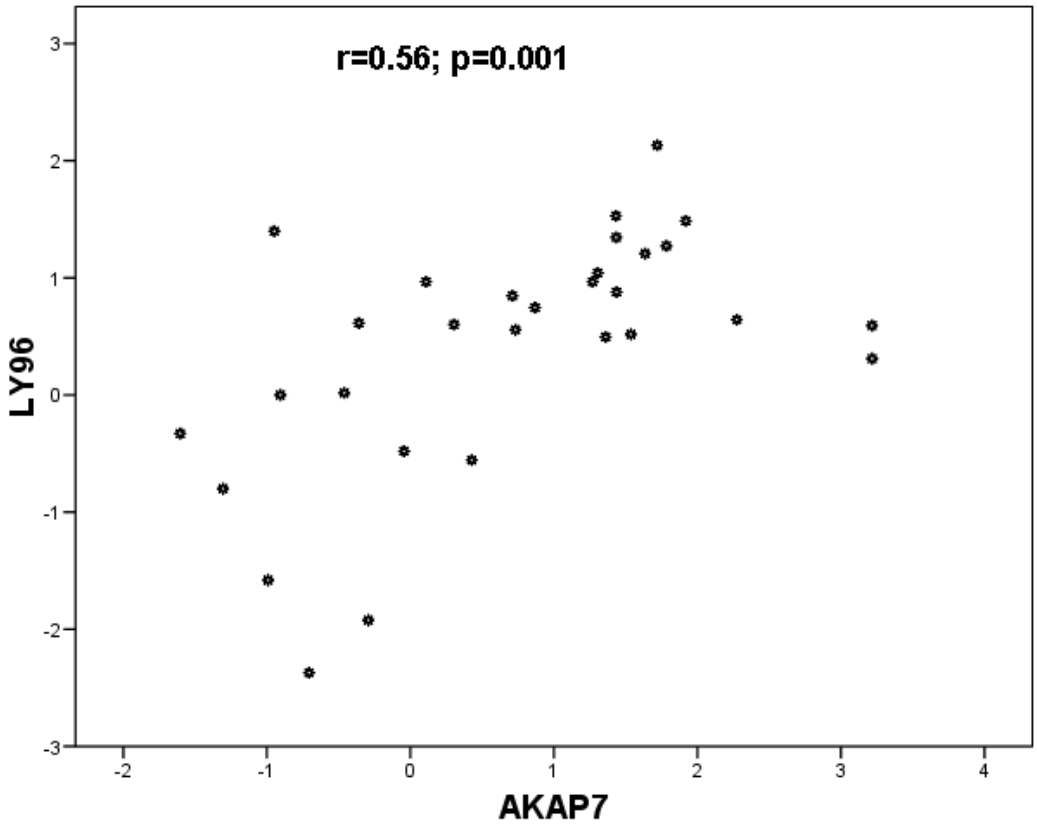


Figure 55. ***AKAP7*** **and** ***LY96***

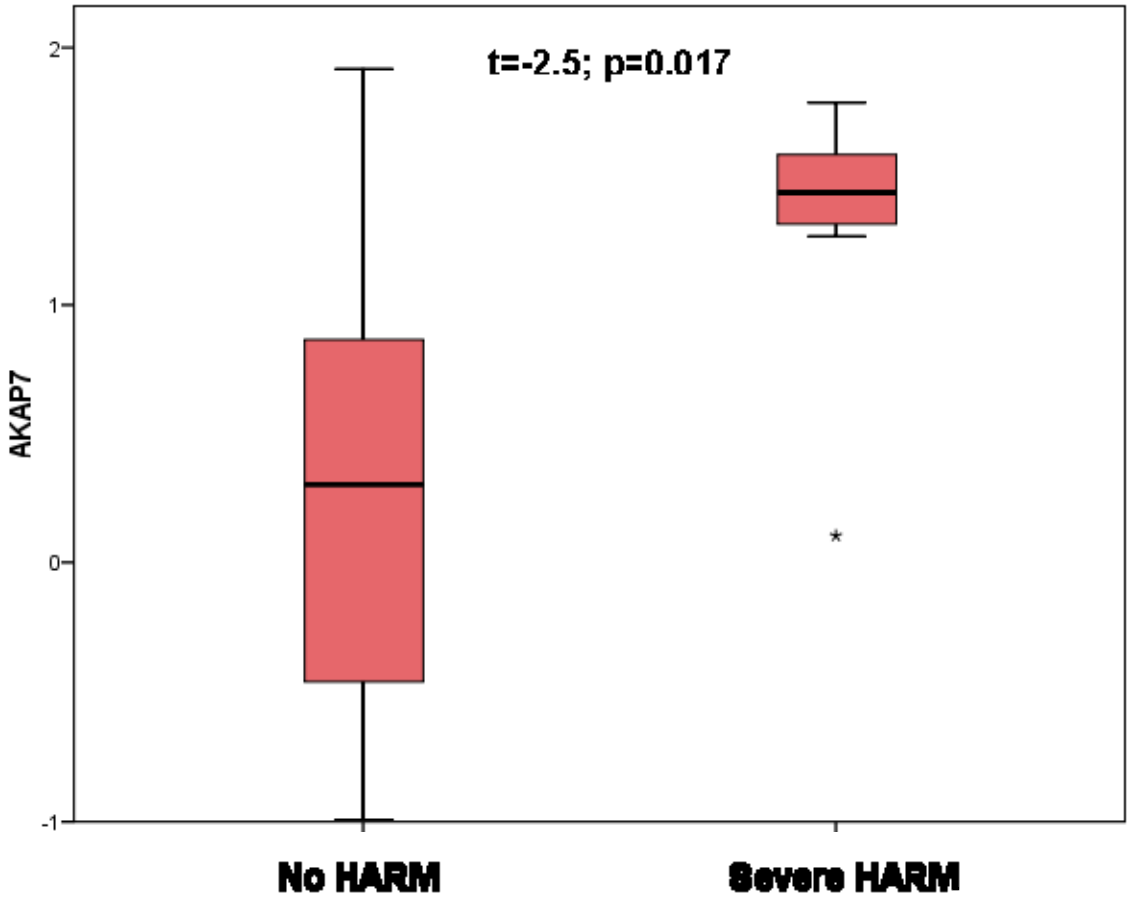


Figure 56. ***AKAP7*** **by HARM**

8.0 SUMMARY OF FINDINGS

8.1 SUMMARY OF AIM ONE FINDINGS

A nine gene profile has been identified for acute ischemic stroke. Five of these nine genes were identified in the first gene expression profiling study. One of the nine genes identified (*s100A12*) was found to be up-regulated with increasing age and is possibly not specific for acute ischemic stroke. However RT-PCR validated 4 of the 5 genes tested from this profile; with the exception of *s100A12*. So the analysis by age for *s100A12* should be interpreted with caution. Pathway analysis revealed a robust inflammatory and immune response, with CD28 signaling in T-helper cells and toll like receptor signaling identified as highly significant canonical pathways present in the peripheral whole blood of acute ischemic stroke patients.

8.2 SUMMARY OF AIM TWO FINDINGS

Only three genes with 2 fold change in expression (down-regulated) and statistically significant at $p<0.05$ were identified as the whole blood expression profile change over time (*LY96*, *IL8,* and *SDPR*). Both *LY96* and *IL8* are part of the toll like receptor pathway, and *LY96* modulates *IL8* expression through *NF-kappaB*. There was a significant difference between baseline *NF-kappaB*

signal intensity and follow-up. *NF-kappaB* was significantly correlated with both *LY96* and *IL8* at both baseline and follow-up. *LY96* (baseline or follow-up) was not correlated with 30 day MRS; however there was a significant association between follow-up *NF-kappaB* and 30-day MRS with higher levels of *NF-kappaB* corresponding to better outcome. This relationship may be mediated by age, as higher follow-up *NF-kappaB* was associated with younger age.

8.3 SUMMARY OF AIM THREE FINDINGS

Acute ischemic stroke patients who presented with HARM were significantly older and more likely to have received rtPA. The mean signal intensity for *LY96* was significantly higher in the moderate and severe HARM groups. *LY96* was not correlated with either age, severity of injury or *MMP9* but there was a trend for a correlation between *ORM1*. *ORM1* was not significantly different between the HARM groups, but it was correlated with baseline NIHSS and *MMP9*. *AKAP7* was significantly predictive of severe HARM, as higher mean signal intensity of *AKAP7* was correlated with the presence of Severe HARM. Logistic regression analysis revealed that *LY96*, *ORM1*, age, and rtPA were significant predictors of HARM category and only *AKAP7* was a significant predictor of severe HARM after controlling for covariates.

9.0 DISCUSSION

The findings of this study support the claim that gene expression profiling of peripheral whole blood can be used to identify diagnostic markers of acute ischemic stroke. This leaves great promise for the use of whole blood gene expression profiling for scientific advancement in other neurological and psychiatric diseases.

9.1 QUALITY ASSESSMENT

All stroke patient and control subject samples were collected under similar situations; all samples were treated the same; and RNA extraction, hybridization, and washing were performed in parallel. With the exception of one patient outlier, all samples group appropriately and all microarrays show uniform intensities across housekeeping probes. Quality control analysis reveals high quality RNA and high quality microarray data. In addition, all analyses were conducted first in Bead Studio and then verified using a different analysis package, Gene Spring. Therefore the results of this study are credible and can be used to design trials to test the identified genes as diagnostic markers for acute ischemic stroke and to further understand the relationship between inflammatory and immune pathways and the response to acute ischemic stroke to design novel therapeutics.

In addition to the high quality of the data, this is the largest gene expression profiling study of acute ischemic stroke in humans and the only to interpret the results through pathway analysis. It is also one of the first to use clinical demographic data as covariates for the identification of genes associated with a phenotype of clinical severity (blood brain barrier disruption) and clinical outcome (modified rankin scale). This model should be used in future gene expression profiling studies to identify clinically useful gene sets for populations of interest taking into consideration clinical phenotypes.

9.2 A GENE PANEL FOR DIAGNOSIS OF ACUTE ISCHEMIC STROKE

A rapid blood test for the diagnosis of acute ischemic stroke would transform stroke care in the US and throughout the world. Most hospitals across the US are not large academic centers where Stroke neurologists are available 24/7 and where an MRI can be used for acute assessment of cerebral ischemic changes prior to the administration of rtPA. The norm however is something quite different. More often than not, an ER physician is assessing, diagnosing and treating acute ischemic stroke through clinical history assessment and CT without the assistance of a stroke-trained neurologist. Recent studies have reinforced that although ER physicians are more than capable of treating stroke patients, they are often reluctant to give rtPA unless the diagnosis is definitive. In addition, there is a shortage of trained emergency personnel and ER nursing staff capable of identifying stroke symptoms or conducting an appropriate stroke assessment. The small percentage of patients who actually receive rtPA (3-5%) and the large numbers of patients who leave the hospital with either a diagnosis of transient ischemic attack (TIA) or stroke of undetermined cause pays testament to the need to identify additional means of stroke diagnosis.

The field of cardiology is quite familiar with the use of peripheral blood markers as diagnostic biomarkers of acute ischemia. Class One A recommendations from the American College of Cardiology and the American Heart Association identify the use of cardiac troponin as the preferred blood biomarker for acute myocardial infarction (MI) diagnosis and CK-MB as the alternative when troponin is unavailable.(Alpert, Thygesen et al. 2000) More than 30 years of research has led to the acceptance of CK-MB and only recently troponin as specific markers of acute MI. (Jaffe 2008) The identification of these markers as specific and sensitive for MI have withstood the test of time, and have therefore become part of the standard of care in Cardiology and have had a tremendous positive impact on patient outcome.

Most would argue that peripheral blood markers specific for brain injury has proven virtually impossible to identify. Some groups have even begun to question the use of blood biomarkers in the study of acute brain injury. Numerous studies over the years have resulted in either insignificant findings or could not be replicated. Traditional methods for the identification of these biomarkers have fallen short of the rigor and sensitivity necessary to identify the troponin or CK-MB for brain injury. I would argue that now, more than ever, with the widespread use and dissemination of novel genomic and proteomic methods is the time that "brain specific" biomarkers can and ultimately will be identified.

The completion of the Human Genome Project in 2003, only six years ago, has already transformed biomedical research as we know it through advanced knowledge of genetic contributions to disease. Through the continued work of deciphering the genome, the International HapMap project, comparative genomics, epigenomics, and refinement of computational biology techniques personalized medicine is becoming a reality. In the near future it will be inevitable to avoid addressing genetic/genomic contributions to disease or response to

treatment when interpreting the clinical utility of novel therapeutics. Even so, no one would argue that there is not going to be a single biomarker for the diagnosis of acute ischemic stroke; but rather a more realistic approach is the identification of a collection of biomarkers that may not be directly specific for brain injury, but respond differently in concert with one another following ischemic stroke.

The results of this study and the previous studies, suggest a panel of genes can be used to diagnosis acute ischemic stroke. No doubt, more work needs to be done and multiple additional studies need to be conducted to test this diagnostic panel, but these results are promising. The 9 genes identified in this study predicted stroke with an accuracy of 95%; which is higher than the diagnostic capability of both MRI (85%) and CT (54%). (Chalela, Kidwell et al. 2007)

The nine gene panel identified in this study is up-regulated for all genes, except for *CCR7* and consists of the following: Arginase 1 (*ARG1*); carbonic anhydrase 4 (*CA4*); chemokine receptor 7 (*CCR7*); chondroitin sulfate proteoglycan 2 (*CSPG2*); IQ motif-containing GTPase activation protein 1 (*IQGAP1*); lymphocyte antigen 96 (*LY96*); matrix metalloproteinase 9 (*MMP9*); orosomucoid 1 (*ORM1*); s100 calcium binding protein A12 (*s100A12*).

9.2.1 Arginase-1

Arginase-1 (*ARG1*) is an enzyme induced by T-helper 2 cytokines that metabolizes L-arginine to ornithine and urea and is a critical regulator of nitric oxide (NO) synthesis.(Durante, Johnson et al. 2007) Inflammatory stimuli (T-helper 1 cytokines) result in an increased expression of inducible NO synthetase (*iNOS*) through L-arginine metabolism. It is possible to determine the type of inflammatory response to injury depending on the relative amount of *ARG1* and *iNOS* since both compete for L-arginine. (Popovic, Zeh et al. 2007) Trauma is associated with an

increase activity of *ARG1* and a decrease in the level of arginine. (Tsuei, Bernard et al. 2001) In addition recent studies suggest activation of the JAK and STAT pathways induces *ARG1* in smooth muscle. (Wei, Jacobs et al. 2000) Since humoral anti-inflammatory cytokines induce *ARG1*, the up-regulation of *ARG1* in this dataset suggests that the response to acute ischemic stroke favors an innate humoral immune response.

9.2.2 Carbonic Anhydrase IV

Carbonic anhydrase IV (*CA4*) is a zinc enzyme that catalyzes the conversion between carbon dioxide and the bicarbonate ion, thus making it crucial for all physiologic processes involved in cellular respiration and transport. *CA4* is a membrane-bound protein found in tissues throughout the body and is found in the brain within the luminal surface of capillary endothelial cells suggesting a role for *CA4* in the blood brain barrier as a regulator of CO2 and bicarbonate homeostasis in the brain. (Ghandour, Langley et al. 1992) The up-regulation of *CA4* in this dataset suggests there is an increase in cellular respiration following acute ischemic stroke that requires an increase in *CA4* to convert CO_2 to $HC0_3$ to maintain pH.

9.2.3 Chemokine Receptor 7

Chemokines are a family of small proteins that regulate leukocyte trafficking. Aside from their role in inflammatory and immune responses there is increasing evidence that they play a significant role in glial cell proliferation and migration as part of the neuro-immune response. (Columba-Cabezas, Serafini et al. 2003) Several chemokines have been identified in both the serum and CSF of stroke patients. *CXCL5, CCL2, CCL3*, and *CXCL8* are significantly increased

following stroke and play a modulatory role of inflammation during the acute phase of ischemia. (Mines, Ding et al. 2007) In addition *CCR8* is expressed in activated microglia on brain sections of ischemic stroke patients. (Trebst, Staugaitis et al. 2003) The down regulation of *CCR7* in this dataset suggests there is decreased glial cell proliferation and migration very early in the acute phase of ischemic stroke; which coincides with the literature that these cytokines become increasingly more active during recovery and repair.

9.2.4 Chondroitin sulfate proteoglycan 2

Chondroitin sulfate proteoglycan 2 (*CSPG2*) also known as versican, was first identified in hyaline cartilage where it provides mechanical support. Recent studies have identified *CSPG2* as a primary component of the extracellular matrix in the CNS. A disaccharide degradation product of *CSPG2* has been shown to stimulate microglia to possess increase phagocytic activity without cytotoxic effects. This suggests a role for *CSPG2* in immune-related neurodengenerative disorders. (Ebert, Schoeberl et al. 2008) In addition, increased CSPGs exhibit growth inhibiting properties and inhibit axonal sprouting within the glial scar. Within the infarct core *CSPG2* expression is dramatically increased, resulting in increased cell death and reactive astrocytosis. Several enzymatic processes cleave *CSPG2*, including the matrix metalloproteinases. (Carmichael, Archibeque et al. 2005) The up-regulation of *CSPG2* in this dataset suggests there is inhibited axonal growth in the acute phase of ischemic stroke.

9.2.5 IQ Motif-containing GTPase activating protein 1

IQ Motif-containing GTPase activating protein 1 (*IQGAP1*) is an evolutionarily conserved molecule that serves as a scaffold protein and plays a fundamental role in cell polarity. It modulates several cellular activities including cytoskeletal architecture, cell-cell adhesion, transcription and signaling (*ERK* signaling). Rho-family GTPases, including *Cdc42* require *IQGAP1* to regulate actin cytoskeleton and produce a gradient of signaling molecules. *Cdc42* and *IQGAP1* it co-localizes with actin filaments throughout the brain. (Fukata, Nakagawa et al. 2003) In addition, increased *Cdc42* activity has been implicated in the breakdown of the blood brain barrier (BBB). (Wojciak-Stothard and Ridley 2003) An up-regulation of *IQGAP1* expression in this dataset suggests there is an increase in cellular signaling and transcription in the acute phase of ischemic stroke and *IQGAP1* may mediate the disruption of the BBB as a means by which signals from the brain enter the periphery to augment cellular recruitment.

9.2.6 Lymphocyte antigen 96

Lymphocyte antigen 96 (*LY96*) also known as MD2 protein, is critical for toll-like receptor 4 (*TLR4*) activation as an innate response to lipopolysaccharide (LPS) which is the main constituent of gram-negative bacteria. (Miyake 2003) *TLR4* activation induces transduction pathways resulting in *NF-kappaB* expression and subsequent release of pro-inflammatory cytokines (e.g. *IL6* and *IL8*). Interestingly, natural selection has shaped the sequence patterns of TLR genes in primate evolution. (Nakajima, Ohtani et al. 2008) However, pathogens and LPS are not the only cause of tissue damage; ischemia is another mechanism. There is accumulating evidence that ischemic tissue damage is recognized at the cell level via receptor-mediated

detection of proteins (alarmins) released by dead cells. Therefore there are exogenous pathogen-associated molecular patterns (PAMPs; such as LPS) and endogenous alarmins that elicit similar responses of the innate immune system known as damage associated molecular patterns (DAMPs). (Bianchi 2007) The upregulation of *LY96* in this dataset suggests that the response to acute ischemic stroke is mediated by the innate immune system and TLR signaling.

9.2.7 Matrix Metalloproteinase 9

Matrix Metalloproteinase 9 (*MMP9*) is a zinc and calcium dependent endopeptidase responsible for regulation of the extracellular matrix (ECM). Ischemia and reperfusion injury results in oxidative stress that mediates BBB disruption through metalloproteinase activation. *MMP9* expression is the result of activated leukocytes (particularly neutrophils), (Gidday, Gasche et al. 2005) and results in *IL1beta* activation (Russo, Siviglia et al. 2007) and initiation of the inflammatory cascade (Kolev, Skopal et al. 2003), further contributing to BBB impairment. Up-regultion of *MMP9* following acute ischemic stroke suggests an increase in proteolytic activity early that may contribute to BBB disruption, which would allow cellular migration and signaling to and throughout the CNS.

9.2.8 Oromucosid 1

Oromucosid 1 (*ORM1*) also known as alpha-1 acid glycoprotein is an acute phase protein and increases 2-5 times during an acute phase response. It has been shown to suppress lymphocyte response to LPS (thereby preventing ongoing tissue damage by neutrophil proteases), decrease platelet aggregation (and thus further platelet recruitment), and enhance cytokine secretion (as

possibly part of a feedback mechanism). (Hochepied, Berger et al. 2003) It exhibits both pro and anit-inflammatory effects and is therefore suggested to play a significant role in immunomodulation. An up-regulation of *ORM1* in this dataset suggests a neuroimmune response in acute ischemic stroke mediated by a balance between pro and anti-inflammatory signaling molecules.

9.2.9 s100 calcium binding protein A12

s100 calcium binding protein A12 (*s100A12*) also known as calgranulin C and EN-RAGE (extracellular newly identified RAGE binding protein) is specifically related to innate immune function. *S100A12* is expressed by phagocytes and released at the site of tissue inflammation. It is an endogenous DAMP that turns pro-inflammatory after a release into the extracellular space following brain injury. (Foell, Wittkowski et al. 2007) The Receptor for Advanced Glycation End Products (*RAGE*) is a member of the immunoglobulin superfamily and is a specific cell surface reaction site for advanced glycation endproducts (*AGEs*) which increase with advancing age. Intreaction between *AGEs* and *RAGE* has been linked to chronic inflammation (Schmidt and Stern 2001) Once engaged *RAGE* interaction in inflammatory and vascular cells results in the increased expression of MMPs. (Clynes, Moser et al. 2007) The up-regulation of *s100A12* supports the claim that the response to acute ischemic stroke is largely driven by innate immunity.

9.3 GENE LIST COMPARISON TO PREVIOUS STUDIES

The first whole blood gene expression profiling study for ischemic stroke identified an 18-gene panel for ischemic stroke.(Tang, Xu et al. 2006) Five of these genes have been replicated in this study, using a different population of patients, a different methodological platform, and a different analysis plan. Therefore when used together the following genes have strong evidence for diagnostic capability in acute ischemic stroke: *ARG1, CA4, LY96, MMP9*, and *s100A12*.

In the study conducted by Tang et al (2006) a group of 15 stroke patients and 8 control subjects were used to identify a gene expression profile for stroke. The stroke group was significantly older (64±14 years) than the control group (49±11) and there were African American patients in the stroke group and only Caucasian patients in the control group. Population stratification is a major issue for genetic studies and should be avoided; therefore even though they did not find large differences in expression based on race, some genes identified may have been non-specific changes regulated by race, not by stroke. In this study there was a much larger population of stroke patients (n=39) and control subjects (n=24). There was a significant difference between stroke patients and control subjects by age (73±14 and 59±9 respectively); however this group was older than the population of the first study and a separate analysis controlling for age was conducted to determine genes regulated by age. *S100A12* was found to associate with increasing age, and therefore may not be specific for acute stroke, but rather a marker of increasing age. In addition, this study was only conducted with Caucasian stroke patients and control subjects, therefore changes in expression cannot be attributed to population stratification.

The very first gene expression profiling study of stroke conducted by Moore et al (2005) recruited patients up to 48 hours following stroke and isolated RNA from one cell type in the

blood (peripheral blood mononuclear cells; monocytes). Tang et al (2006) demonstrated that a large majority of the early gene expression changes are found within polymorphonuclear leukocytes and neutrophils following ischemic stroke; later changes in gene expression are found in monocytes. For these reasons primarily it is difficult to compare the results of this study to the first study conducted by Moore et al (2005), because different cell types were studied. That study chose a specific cell type, whereas the Tang et al study and this study isolated RNA from every cell present in whole blood. Even so, there was one gene, *CSPG2* (Versican), in this study in common with the Moore et al study.

9.4 INNATE IMMUNITY AND RESPONSE TO ISCHEMIC STROKE

The innate immune system has been created by mammalian evolution and is a fast and blunt non-specific endogenous response to cellular stress.(Parham 2003) Adaptive immunity on the other hand is much more specific and can take days to weeks to mount a full response. The balance between these two forces enables an organism to fight disease and survive. Since the innate immune system is not specific, innate immune cells must recognize antigens and stress signals through a predetermined set of encoded receptors. Pathogen associated molecular patterns (PAMPs) are a diverse set of stress and injury-induced molecules which have highly conserved structures that are recognized by pattern recognition receptors (PRRs) such as the Toll like receptor (TLR) pathway. Traditionally the PAMP/TLR activation pathway has only been implicated in tolerance to endotoxins (microbes with lipopolysaccharide chains) that alert an organism to intruding pathogens. However, it is becoming increasingly clear that microbial

invasion is not the only mechanism by which the TLR pathway becomes activated.(Bianchi 2007; Rubartelli and Lotze 2007.) Products of degradation, damaged DNA, fibrinogen and heat shock proteins have emerged as activators of the TLR pathway through a mechanism known as damage associated molecular pattern (DAMPs) recognition. This mechanism of endogenous alarmin activation shares many commonalities with the response generated by TLR activation through exogenous PAMPs.(Kariko, Weissman et al. 2004) This "new" awareness of the similarities between trauma and pathogen evoked innate immune responses is receiving a lot of recent attention, and has tremendous implications for the response of the human body to acute ischemic stroke.

Alarmins are endogenous molecules released following non-programmed cell death that recruit and activate cells of the innate immune system and subsequently promote the restoration of damaged tissue. Putative alarmins include such molecules as high mobility group box 1 (HMGB1), s100s, annexins, heparan sulfate, and heat shock proteins (HSP) and can engage TLRs, IL1 receptors (*IL1R*) or receptors of advanced glycation end products (*RAGE*) making the alarmins critical regulators of downstream inflammatory and immune responses.(Rubartelli and Lotze 2007.; Jordan, Sequra et al. 2008) Engagement of TLRs, *IL1R*s, and *RAGE* receptors all result in the translocation of *NF-kappaB* to the nucleus. *NF-kappB* is the first step of any inflammatory cascade since it begins transcription of inflammatory cytokines which results in subsequent release and activation of chemokines and metalloproteinases. In addition, the polarized response of the adaptive immune system may be modulated by the presence of DAMPs and PAMPs. (Rubartelli and Lotze 2007.) Thus it is becoming increasingly clear why innate immunity may play a critical role in understanding the response to human stroke.

Inflammation is necessary to recruit immune cells to the site of local injury. It is therefore a beneficial part of the response to acute ischemic stroke; however when the inflammatory process is prolonged it will exacerbate cell death and negatively impact clinical outcome. Within the brain this process of pro and anti-inflammatory signals, known as immunomodulation, is mediated primarily by signals from astrocytes and microglia and requires constant communication between cytokines and other immune cells to tailor the response as necessary.(Jordan, Sequra et al. 2008) There is evidence that peripherally derived cytokines are involved in brain inflammation as well and this process is facilitated by blood brain barrier (BBB) disruption. When the BBB becomes disrupted (as part of the endogenous response to ischemia) the peripheral immune system comes into contact with the central nervous system and adds to the local inflammatory process and propagates the changes of gene expression identified in this study.

TLR4 activation has recently been implicated as a negative effector of the innate immune response.(Kilic, Kilic et al. 2008) *TLR4* receptors are present on microglia, astrocytes and neurons and are activated by alarmins in the brain following acute ischemia. Both the activation of *TLR4* and *TLR2* result in activation of *IRAK1* through interaction with *MyD88*. This leads to engagement of *TRAF6*, which is a member of the TNF receptor family. Then through the *IkappB* kinase pathway, *NF-kappaB* translocates from the cytoplasm to the nucleus where it stimulates transcription of both pro-inflammatory and anti-inflammatory cytokines and chemokines (e.g. pro-inflammatory *IL6* and *IL8*; anti-inflammatory *IL10* and *TNFα*). Simultaneous with *NF-kappaB* activation, engagement of *TRAF6* also results in: 1) the stimulation of the JNK pathway and activation of immediate early response genes *cJun* and *cFos* which come together to form the *AP1* early response transcription factor which increases the transcription of neurotrophic

factors, such as brain derived neurotrophic factor (*BDNF*); and 2) the activation of the *MAPK* signaling cascade with subsequent *ELK1* activation with binding to the serum response factor (*SRF*) in the promoter of the *cFos* proto-oncogene. Therefore, within the *TLR4* pathway there are both pro-inflammatory mechanisms of engagement (*NF-kappaB*) and anti-inflammatory and neurotrophic pathways of regeneration (*BDNF* and *SRF*).(Bianchi 2007; Rubartelli and Lotze 2007.; Jordan, Sequra et al. 2008)

There are only a few studies that have studied *TLR4* activation in regard to ischemic stroke and all have found that a down regulation of *TLR4* results in a decrease in final infarct volume and better outcome in mice MCAO models. *TLR4* deficient mice seem to be protected by ischemic injury through a down-regulation of *iNOS* production.(Ziegler, Harhausen et al. 2007; Caso, Pradillo et al. 2007.) In addition, mice who are *TLR4* deficient also have better behavior following MCAO that is preceded by significant psychological stress.(Caso, Pradillo et al. 2008) Human studies have added to this theory of *TLR4* mediated negative outcome and have identified that increased activation of *TLR4* following ischemic stroke corresponds to worse clinical outcome.(Yang, Li et al. 2008; Urra, Cervera et al. 2009)

Given the data some have postulated that deactivation of the innate immune response targeting *TLR4* is a novel therapeutic approach for treatment of acute ischemic stroke and mediation of the acute inflammatory/immune response.(Kilic, Kilic et al. 2008) However, given that the *TLR4* pathway is highly conserved across species and has survived natural selection this raises a completely different perspective. Is it possible that the strongest effect of *TLR4* activation is not neurodegeneration; but rather mediation of the immune response through its role as a neurotropic generator? The positive effects of *TLR4* activation could be the explanation as to why older patients have worse clinical outcomes following stroke when compared to their

younger counterparts. To understand this line of thought it is first essential to address the idea of ischemic tolerance.

Ischemic tolerance (IT) or ischemic preconditioning (IP) was first characterized by Janoff in 1964 as the protective response of an organism to potentially recurring challenges.(Janoff 1964.) Thus the notion of IT/IP has become increasingly prominent in the stroke literature over time and resides in the fact that the severity of post-ischemic injury can be manipulated through small and short bursts of ischemia prior to the severe ischemic event. This sub-lethal stress primes cells for subsequent ischemic events through exposure to *IL1β* and *TNFα*.(Kariko, Weissman et al. 2004) Several noxious events can serve as stimuli capable of inducing IT/IP: global and focal cerebral ischemia; cortical spreading depression; and even epilepsy.(Dirnagl, Iadecola et al. 1999) Preconditioning shifts subsequent responses to ischemia from TLR4 activation to increased natural killer (NK) cell activity (*TNFα*. and *IFNβ*). Therefore immune tolerance may be through a protective mechanism of NK cell activity.(Marsh, Stevens et al. 2009)

Given the argument that ischemic tolerance induces a hyporesponsive state to subsequent ischemic insults, then it can be assumed that the human body becomes able to tolerate such insults over time (in the presence of mild ischemia through hypertension, possibly carotid artery blockage, and even small vessel disease secondary to diabetic complications) and that this results in a dampened activation of the TLR4 pathway upon significant insults, such as ischemic stroke. A down-regulation of TLR4 leads to a less profound inflammatory and immune response, but also results in the production of less neurotrophic factors and subsequently less opportunity for regeneration and regrowth within the brain. In this study it was found that increased expression of *NF-kappaB* 24 hours following acute ischemic stroke correlated with better clinical outcome

on the 30 day MRS; however this relationship was mediated by age. Younger patients had higher levels of *NF-kappaB* and better outcome. Aging provides a brain environment that is already "primed" to a peripheral immune challenge and is one of the biggest predictors of clinical outcome following acute ischemic stroke. Is this because of a dampened response of the *TLR4* pathway? Evidence to support this theory lies in the fact that patients who have experienced a transient ischemic attack (TIA) very soon before their stroke have better clinical outcomes than patients with first ever ischemic stroke.(Weih, Kallenberg et al. 1999; Moncayo, de Freitas et al. 2000.; Sitzer, Foerch et al. 2004) It is plausible that this pathway of repair was activated with the first insult (TIA) and is continuing while the second more severe insult (ischemic stroke) occurs. The previous human studies postulating that *TLR4* activation correlates with worse clinical outcome did not address what made the patients with worse clinical outcome so different; was it because they had a previous stroke; or had a history of hypertension complicated by diabetes; or were they just older?

In addition to finding that older patients had lower expression of *NF-kappaB*, we found the expression of *s100A12* was significantly increased with age. S100 is an endogenous alarmin and the increased expression of *s100A12* with age implies an environment of increased stress. Therefore the downregulation of *NF-kappaB* with the simultaneous upregulation of *s100A12* with age implies the response of the *TLR4* pathway is dampended with age.

To add to this argument, recent experience with anti-inflammtory approaches in the clinical setting has been largely unsuccessful. Enlimomab (a murine monoclonal anti–human *ICAM-1* antibody)(2001), monoclonal antibody to *CD18* (antibody Hu23F2G or leukarrest)(Becker 2002), and even arguably Minocycline(Lampl, Boaz et al. 2007; Schabitz, Schneider et al. 2008) have either been detrimental or shown to have no clinical benefit when

used in a human ischemic stroke population. This is proof that the mechanisms of neuro-immunomodulation are still poorly understood. It is possible that the identified positive effects of ischemic preconditioning are not just in its ability to harness the inflammatory process; but rather through a different mechanism of action. If preconditioning afforded protection from inflammation at only the level of *NF-kappaB* translocation, discrimination between detrimental and neuroprotective mechanisms would be impossible. There is data suggesting that gene expression changes of IT/IP only affect transcription of genes for which trans-activation depends on *p300* interaction with *NF-kappaB*. (Ginis, Jaiswal et al. 2002) Therefore it is possible there is an effector of *NF-kappaB* that regulates NF-kappaB and therefore the transcription of inflammatory mediators once activated following preconditioning.

A plausible case for innate immunity through the activation of *TLR4* as a mediator of response to ischemic stroke has been made from the results of this study. There is some evidence present in this dataset for a strong relationship between innate immunity and age, as increased 24 hour *NF-kappaB* expression in younger patients was correlated with better functional outcome at 30 days post-stroke. Younger patients had a stronger *TLR4* receptor activation response than older patients (>60years); *s100A12* expression increased with age as *NF-kappaB* expression decreased with age. A further look at the relationship between age and *TLR4* activation is required to assess the possibility that ischemic tolerance developed over time with aging results in a dampened neurotrophic response of *TLR4* activation.

9.5 AKAP7 AND BLOOD BRAIN BARRIER DISRUPTION

Messages from the extracellular space influence intracellular processes via activation of signal transduction pathways that involve protein kinase A (*PKA*) and protein kinase C (*PKC*).(Fraser, Tavalin et al. 1998) Fluctuations in intracellular Ca^{+2} and cAMP influence a series of cellular processes: ion channel regulation; cellular metabolis; growth and differentiation; and cell trafficking.(Trotter, Fraser et al. 1999) These signals are propagated via *PKA* phosphorylation. *PKA* localizes close to L-type Ca^{+2} channels in order to facilitate rapid and efficient channel phosphorylation. There are about 2,000 kinases present in the human genome and up to 25 different A-kinase anchor proteins (*AKAPs*).(Edwards and Scott 2000) *AKAPs* are functionally related and characterized by their interaction with either type 1 or type 2 regulatory subunits (RI and RII) of *PKA*.(Edwards and Scott 2000) Specificity of this process is mediated at the molecular level by a second motif unique to each *AKAP* allowing it to sequester *PKA* or *PKC* to specific intracellular regions.(Fraser, Tavalin et al. 1998)

A-kinase anchor protein-7 (*AKAP7*) also known as *AKAP18* and *AKAP15* (in rat)(Fraser, Tavalin et al. 1998) directs the activity of protein kinase A (*PKA*) and protein kinase C (*PKC*) to calcium and sodium channels in the brain, skeletal muscle, heart, and other organs. *AKAP7* targets PKA to the cytoplasmic face of the plasma membrane, where it enhances cAMP responsive calcium (Ca^{+2}) currents and is therefore the physiologic partner of the Ca^{+2} channel and fundamental to specific hormone mediated responses that utilize cyclic adenosine monophosphate (cAMP).(Fraser, Tavalin et al. 1998) The gene for *AKAP7* is alternatively spliced resulting in four splice variants all with common functions.(Trotter, Fraser et al. 1999)

AKAP15, the rat homolog of *AKAP7/18*, also interacts with neuronal sodium (Na^{+}) channels.(Tibbs, Gray et al. 1998; Few, Scheuer et al. 2007) Protein phosphorylation of Na^{+}

channels in the brain serves as a process of neuromodulation and is important for neuronal plasticity which decreases overall excitability within the hippocampus.(Few, Scheuer et al. 2007) Dopamine signaling modulates this Na^+ current through *PKA* and requires *AKAP15* to anchor *PKA* to the Na^+ channel via a leucine zipper-like motif.(Few, Scheuer et al. 2007) Normal dopamine signaling is only achieved when *PKA* is anchored directly to the channel via *AKAP15*. Recent evidence suggests that *AKAP15* can regulate Na^+ channels via a novel PKA-independent pathway as well which suggests *AKAP7/18* may be generally distributed throughout the plasma membrane and could participate in a wide variety of transmembrane signaling pathways.(Bengrine, Li et al. 2007) *PKC* also decreases Na^+ current within the brain and enhances the *PKA* response. The maximum modulation of this effect requires phosphorylation of four distinct sites within the Na^+ channel. This suggests that signals from multiple pathways can converge and become integrated at the cellular level within the brain and makes the process of deciphering these signals extremely complex.(Cantrell, Tibbs et al. 2002)

Alterations of the *PKA* and *PKC* pathways occur during anoxic brain injury. (Grammas, Moore et al. 1998) Engagement of *ICAM1* as part of the inflammatory process following ischemic stroke induces signals across the BBB via *TNFalpha* secretion and activation of PKA.(Etienne-Manneville, Chaverot et al. 1999) *TNFalpha* has been implicated in BBB opening in multiple sclerosis(Sharief, Noori et al. 1993), and therefore *ICAM1* binding on astrocytes may be a stimulus for BBB opening following ischemic stroke as well.

Brain endothelial cells exposed to oxygen deprivation show an increase in *PKC* signaling with a simultaneous decreased in *PKA* signaling.(Grammas, Moore et al. 1998) Over activation of *PKC* increases the influx of fluid (via Na+ entry into the intracellular space) and macromolecules across the microvascular wall. *PKC* inhibitors subsequently decrease this

permeability(Qi, Inagaki et al. 2008); therefore *PKC* has been suggested as a mediator of microvascular permeability. Neuroprotection via *PKC* inhibition prevents BBB opening even in the presence of sustained high blood pressure. In addition, brain samples of patients with hypertension have increased staining of *PKC* in arteriolar endothelial cells and astrocytes.(Qi, Inagaki et al. 2008) *PKC* signaling also regulates the morphology of tight junction proteins within the BBB.(Krizbai and Deli 2003) *PKC* is involved in both the activation of *ERK* and *PKA* signaling, and *ERK* signaling has been shown to increase matrix metalloproteinases.(Tyagi, Gillespie et al. 2009)

In addition to its connection with ERK signaling, the *PKC* pathway also seems to be mediated by the RhoA/RhoKinase pathway. Following ischemia there are two canonical Wnt signaling pathways: 1. Wnt/Ca+2 (PKC driven) and 2. Wnt/PCP (RhoA/RhoKinase) driven.(Li, Chong et al. 2005) Pro-inflammatory stimuli cause an increase in BBB permeability by inducing endothelial cell retraction, dependent upon the phosphorylation state of myosin light chains (MLCs). RhoA and its downstream target RhoKinase increases phosphorylation of MLCs and therefore enhance contractility of brain endothelial cells.(Stamatovic, Dimitrijevic et al. 2006) *PKC* also affects phosphorylation of BEC, specifically the tight junctions (TJs). The chemokine monocyte chemoattractant protein-1 (*CCL2*) mediates this process by redistributing the TJ proteins dependent on RhoA/RhoKinase activation.(Stamatovic, Keep et al. 2003) A recent study found that *PKC* activation via *CCL2* is highly dependent on the co-activation of RhoA.(Stamatovic, Dimitrijevic et al. 2006) The interaction between RhoA and *PKC* activates the transcription factor *AP1*(Chang, Pratt et al. 1998) and has been shown to increases stress fiber formation.(Coghlan, Chou et al. 2000) In addition there is high affinity binding between *PKC* and RhoA and RhoA can specifically regulate *PKC* activation.(Slater, Seiz et al. 2001;

Schmitz, Lorberg et al. 2002) Strong evidence exists that these two pathways interact to contribute blood brain barrier disruption following acute ischemic stroke.

The finding that *AKAP7* expression was higher in Severe HARM (severe blood brain barrier (BBB) disruption patients suggests that there is increased *PKA* and *PKC* signaling following acute ischemic stroke that is associated with BBB disruption. In support of this finding is the association of *IQGAP1* with *Cdc42*, a small Rho GTPase. *IQGAP1* was identified as one of the significant genes for stroke in this study. *IQGAP1* colocalizes with *Cdc42* in the brain and contributes to BBB disruption. Further work should be conducted to determine the relationship between *PKC* (mediated by *AKAP7/18*) and RhoA/RhoKinase signaling in the contribution of BBB disruption in acute ischemic stroke.

9.6 LIMITATIONS

There are several limitations to address with this dissertation. The original goal of this study was to compare stroke patients to healthy control subjects matched on age, to eliminate any potential bias age may have on identifying a gene expression profile for stroke. This study however ended up with a significantly older stroke population. Therefore age had to be used as a covariate in a subsequent analysis, where *s100A12* was identified as being associated with old age. This secondary analysis results in decreased power to make the assumption that *s100A12* was solely associated with age. Future studies should aim to have groups with similar ages. In addition, the generalizability of the results is limited given the group studied. All of the patients in this study were Caucasian, so the next step would be to test this gene expression profile in other racial and ethnic groups to determine its utility in the clinical setting.

Another important consideration is the time of the baseline blood draw for the RNA profile. For these genes to be used as acute diagnostic markers the baseline blood draw needs to conducted within 3 hours from onset of injury. The mean time from onset to baseline blood draw in this study was 10 hours. Tang et al (2006) determined that gene expression profiles don't change significantly during the first 24 hours from onset of symptoms; in fact, most of the genes that were differentially expressed at 3 hours from onset, were also expressed at 5 and 24 hours from onset. Even so, this does not rule out the possibility that some genes identified in this study may not have been differentially expressed at an earlier time point.

The final consideration of the study is the diagnostics capability of the findings. Even though this study identified 5 genes in common with the first whole blood expression profiling study of stroke, the results need to be validated and replicated in a larger cohort and tested in the acute clinical setting for their diagnostic capability. It will be essential to determine a gene profile that can rule out stroke from stroke mimic; therefore following studies need to add a "TIA" group for comparison to stroke and control to determine the specificity of the gene profile identified in this study for stroke.

9.7 CONCLUSIONS

The findings of this study support the claim that gene expression profiling of peripheral whole blood can be used to identify diagnostic markers of acute ischemic stroke. Nine genes were identified in this study as being differentially expressed between stroke patients and control subjects: Arginase 1 (*ARG1*); carbonic anhydrase 4 (*CA4*); chemokine receptor 7 (*CCR7*); chondroitin sulfate proteoglycan 2 (*CSPG2*); IG motif-containing GTPase activation protein 1

(*IQGAP1*); lymphocyte antigen 96 (*LY96*); matrix metalloproteinase 9 (*MMP9*); orosomucoid 1 (*ORM1*); s100 calcium binding protein A12 (*s100A12*).

This is the first whole blood expression profiling of human stroke to analyze the data through pathway analysis. A plausible case for innate immunity through the activation of *TLR4* as a mediator of response to ischemic stroke has been made from the results of this study. It was found that increased expression of *NF-kappaB* 24 hours following acute ischemic stroke correlated with better clinical outcome on the 30 day MRS; however this relationship was mediated by age. Younger patients had higher levels of *NF-kappaB* and better outcome. Younger patients had a stronger *TLR4* receptor activation response than older patients (>60years); *s100A12* expression increased with age as *NF-kappaB* expression decreased with age. A further look at the relationship between age and *TLR4* activation is required to assess the possibility that ischemic tolerance developed over time with aging results in a dampened neurotrophic response of *TLR4* activation.

The finding that *AKAP7* expression was higher in Severe HARM (severe BBB disruption) patients suggests that there is increased *PKA* and *PKC* signaling following acute ischemic stroke that is associated with BBB disruption. Further work should be conducted to determine the relationship between *PKC* (mediated by AKAP7/18) and RhoA/RhoKinase signaling in the contribution of BBB disruption in acute ischemic stroke.

It is believed that delayed secondary pathologic responses and neuroprotective changes lie in the genomic response of the neuron to the initial insult.(Marciano, Eberwine et al. 2002) Given the broad based and at times non-specific response of genes and pathways following brain injury and neurological disease it may be futile to search for specific pathways to target therapeutic strategies (von Gertten, Flores Morales et al. 2005). The ability to assess multiple

genes and pathways simultaneously using microarray has led to a paradigm shift regarding the acknowledgement of the complexity of the genomic response following brain. Expression profiling is essential to understanding these processes and formulating rationale approaches to treatment. This study and those conducted by Moore et al (2005) and Tang et al (2006) provide the foundation of data that support the use of peripheral whole blood for future blood profiling studies of neurological disease; which significantly opens the door of opportunity.

9.8 FUTURE DIRECTIONS

This study was important in identifying the utility of peripheral whole blood gene expression profiling in human acute ischemic stroke. Five of the 9 genes identified were replicated from the first whole blood profiling study. In addition, this study is one of the first to raise the question as to whether mediators of the innate immune response may have a positive influence on clinical outcome and therefore should not be deactivated but augmented to target neurotrophic properties. Future directions in original research or secondary analyses should address the following areas of interest:

1. The capability of the diagnostic gene panel identified in this study for its ability to differentiate stroke from stroke mimic.
2. The generalizability of the diagnostic gene panel to other races and ethnic categories.
3. The relationship between age and innate immunity, specifically toll like receptor signaling and neurotrophic response.
4. The relationship between *NF-kappaB*, *LY96*, and *IL8* mediated by age, on clinical outcome following acute ischemic stroke.

5. The relationship between *AKAP7*, *PKC* and RhoA/RhoKinase in the development of blood brain barrier disruption in acute ischemic stroke.
6. The proteomic profiles of the 9 genes identified in this study following acute ischemic stroke.

APPENDIX A

CLASSIFICATION OF ACUTE ISCHEMIC CEREBROVASCULAR SYNDROME (AICS)

Category	Definition	Examples
Definite AICS	Acute onset of neurologic dysfunction of any severity consistent with focal brain ischemia AND imaging/ laboratory CONFIRMATION of an acute vascular ischemic pathology.	1. Sudden onset of right hemiparesis and aphasia persisting for 3 hours with DWI showing acute ischemic changes. 2. Twenty-minute episode of left hemisensory loss, which resolved, with acute right thalamic ischemic lesion confirmed on DWI.
Probable AICS	Acute onset of neurologic dysfunction of any severity suggestive of focal brain ischemic syndrome but WITHOUT imaging/laboratory CONFIRMATION of acute ischemic pathology* (diagnostic studies were negative but INSENSITIVE for ischemic pathology of the given duration, severity and location). Imaging, laboratory, and clinical data studies do not suggest nonischemic etiology: possible alternative etiologies ARE ruled out.	1. Sudden onset of pure motor hemiplegia that persists with normal CT at 12 hours after onset. MRI was not performed. 2. Ten-minute episode of aphasia and right hemiparesis in a patient with atrial fibrillation and subtherapeutic INR. MRI, including DWI, was negative.
Possible AICS	Acute neurologic dysfunction of any duration or severity possibly consistent with focal brain ischemia WITHOUT imaging/laboratory CONFIRMATION of acute ischemic pathology* (diagnostic studies were not performed or were negative and SENSITIVE for ischemic pathology of the given duration, severity and location). Possible alternative etiologies are NOT ruled out. Symptoms may be nonfocal or difficult to localize.	1. Two-hour episode of isolated vertigo and headache in a 50-year-old man with a history of hypertension; symptoms resolved at time of imaging. MRI including DWI was negative. 2. Twenty-minute episode of isolated word finding difficulty in 85-year-old woman with a history of dementia and coronary artery disease. Head CT was negative, and MRI was not performed.
Not AICS	Acute onset of neurologic dysfunction with imaging/laboratory CONFIRMATION of NONISCHEMIC pathology (including normal . imaging/laboratory studies that are highly sensitive for ischemic pathology of the given duration, severity, and location) as the cause of the neurologic syndrome	1. Sudden onset of left hemiparesis and hemineglect. MRI showed right frontoparietal intracerebral hemorrhage. 2. Thirty-year-old man with known seizure disorder found with altered mental status and right hemiplegia. Normal diffusion, perfusion-weighted MRI, and MR angiography (MRA) were acquired while symptoms were still present. EEG showed left temporal spikes.

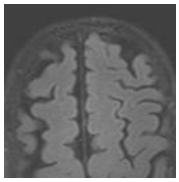
Kidwell, CS., Warach, S. Acute Ischemic Cerebrovascular Syndrome: Diagnostic Criteria Stroke2003;34;2995-2998;

APPENDIX B

NINDS STROKE TEAM-HARM RATING SCALE

SULCAL HARM

NONE

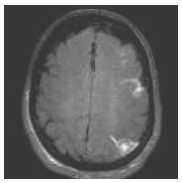


-black CSF in sulci

OR

-one or two point-like regions not contiguous across slices

MILD to MODERATE

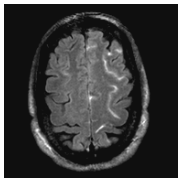


-numerous point-like regions

OR

- linear regions confined to 10 or fewer contiguous slices

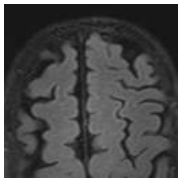
SEVERE



- linear regions
-contiguous across greater than 10 slices

BACKGROUND HARM

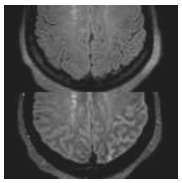
NONE



-black CSF in sulci

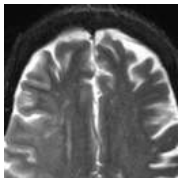
-uniform and bilateral

MILD to MODERATE



- dirty CSF in sulci
- dark gray to light grey sulcal space
- less than or iso-intense compared to cortex
- uniform and bilateral

SEVERE



- bright CSF in sulci

-white sulcal space

--hyper-intense compared to cortex

-uniform and bilateral

Developed by investigators in the Section on Stroke Diagnostics and Therapeutics, NINDS/NIH

APPENDIX C

GENE LIST OF FIRST HUMAN EXPRESSION PROFILING STUDY OF ISCHEMIC STROKE

Gene	*Description*
CD163	Hemoglobin scavenger receptor
Hypothetical protein FLJ22662	Laminin A motif (probably adhesion)
Amyloid ß(A4) precursor-like protein 2	Beta-amyloid protein
n-acetylneuraminate pyruvate lysase	Converts sialic acid to acylmannosamines and pyruvate
v-fos FBJ murine osteosarcoma	Human oncogene c-fos
Toll-like receptor 2	Innate non-specific immune response
Ectonucleoside triphosphate diphosphohydrolase 1	Present on endothelial cells and inhibits platelet aggregation
Chondroitin sulfate proteoglycan 2	Interact with hyaluronan and form large supramolecular complexes
Interleukin 13 receptor, a1	Secreted by T cells
CD14 antigen	Monocyte differentiation antigen
Bone marrow stromal cell antigen 1/CD157	B lymphocyte development
Complement component 1, q subcomponent, receptor 1	Complement receptor
Paired immunoglobulin-like type 2 receptor a	Leukocyte immunoglobulin recpetor
Fc fragment of IgG, high-affinity Ia, receptor for CD64	Tumor progression
Adrenomedullin	Hypotensive peptide
Dual-specificity phosphatase-1	Inactivates mitogen-activated protein
Cytochrome b-245, ß polypeptide	chronic granulomatous disease
Leukotriene A4 hydrolase	Inflammatory response mediator
v-ets Erythroblastosis virus E26 oncogene homolog 2 (avian)	Involved in cell senescence and death
CD36 antigen (thrombospondin receptor)	Leukocyte differentiation antigen
Baculoviral IAP repeat-containing protein 1	Neuronal apoptosis inhibitory protein
KIAA0146 protein	Hypothetical

Moore, D.F., et al., Using peripheral blood mononuclear cells to determine a gene expression profile of acute ischemic stroke: a pilot investigation. Circulation, 2005. 111(2):p.212-21.

APPENDIX D

GENE LIST OF SECOND HUMAN EXPRESSION PROFILING STUDY OF ISCHEMIC STROKE

Gene	*Description*
Hox 1.11	Clone IMAGE:5019307, mRNA, homologous to mouse homebox protein (Hox-1.11) gene
CKAP4	Cytoskeleton-associated protein 4
S100A9	S100 calcium binding protein A9 (calgranulin B)
MMP9	Matrix metalloproteinase 9 (gelatinase B, 92 kDa gelatinase, 92kDa type IV collagenase)
S100P	S100 calcium binding protein P
F5	Coagulation factor V (proaccelerin, labile factor)
FPR1	Human N-formylpeptide receptor (fMLP-R98) mRNA, complete cds
S100A12	S100 calcium binding protein A12 (calgranulin C)
RNASE2	Ribonuclease, RNase A family 2 (liver eosinophil-derived neurotoxin)
ARG1	Arginase I
CA4	Carbonic anhydrase 4
LY96	Lymphocyte antigen 96
SLC16A6	Solute carrier family 16 (monocarboxylic acid transporters), 6
HIST2H2AA	Histone 2, H2aa
Ets-2	v-ets erythroblastosis virus E26 oncogene homolog 2 (avian)
BCL6	B-cell CLL/lymphoma 6
PYGL	Glycogen phosphorylation
NPL	N-acetylneuraminate pyruvate lyase (dihydrodipicolinate synthase)

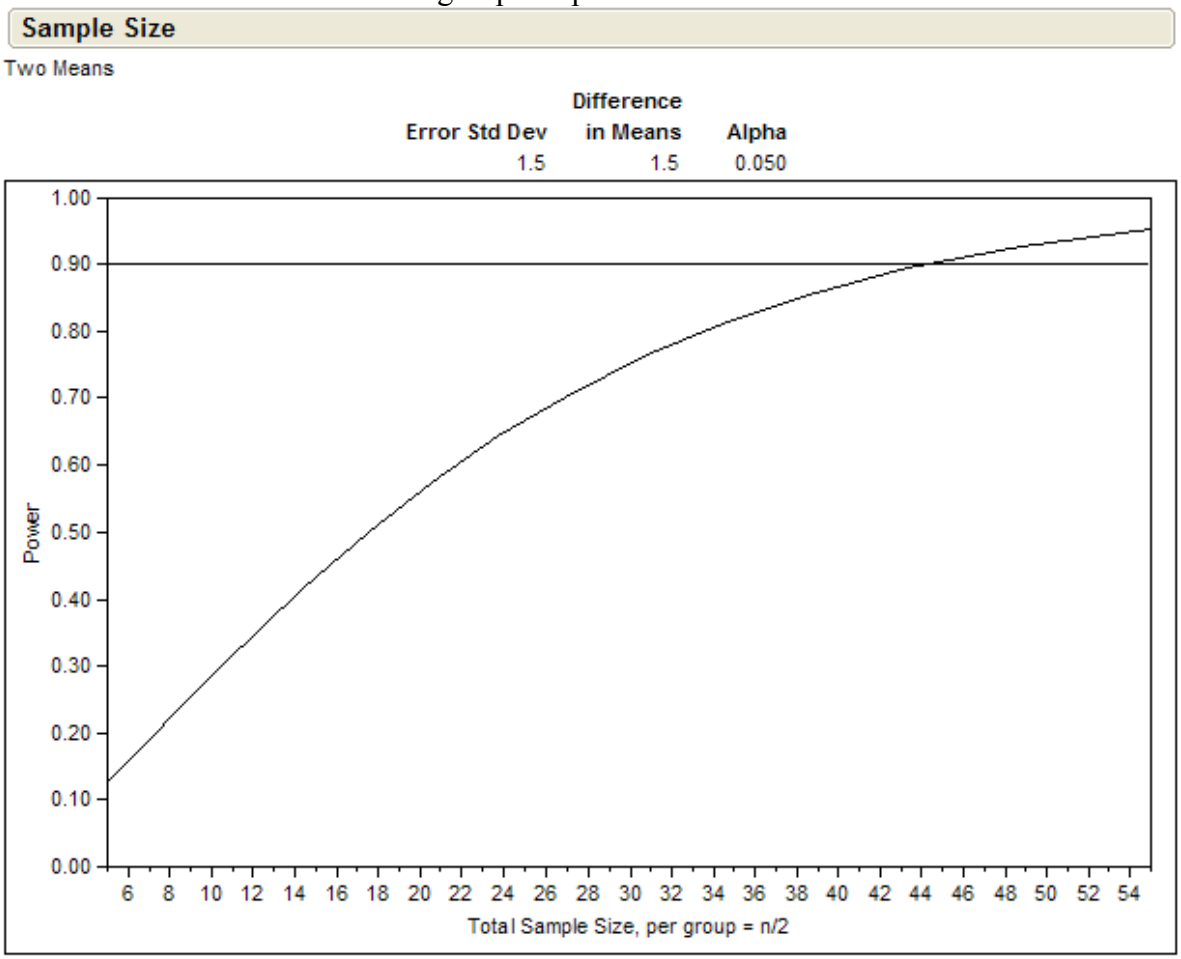
Tang, Y., et al., Gene expression in blood changes rapidly in neutrophils and monocytes after ischemic stroke in humans: a microarray study. J Cereb Blood Flow Metab, 2006. **26**(8):p.1089-102.

APPENDIX E

SAMPLE SIZE CALCULATIONS

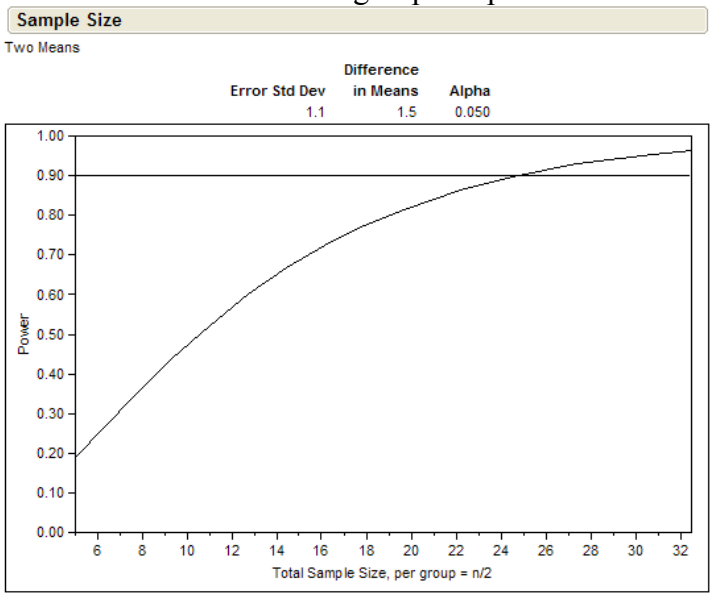
1. Proposed model for study aims

With α=0.05, Error Std Dev = 1.5, difference to detect = 1.5, power = 0.9,
Per group sample size = 44/2 ≈ 22

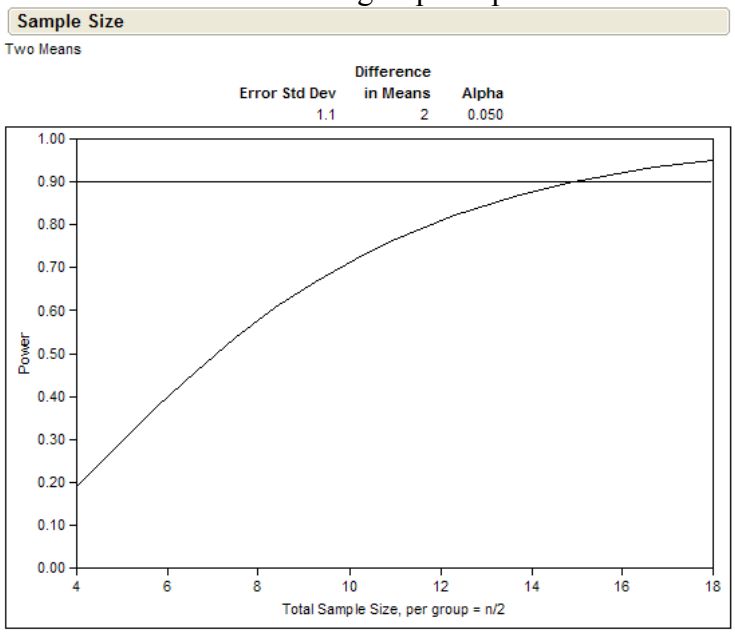


2. Additional Models

With α=0.05, **Error Std Dev** = 1.1, difference to detect = 1.5, power = 0.9,
Per group sample size = 25/2 ≈ 13



With α=0.05, **Error Std Dev** = 1.1, difference to detect = 2.0, power = 0.9,
Per group sample size = 15/2 ≈ 8



APPENDIX F

CASE REPORT FORMS

Medical Record #_____ **Blood Draw ID**

SPOTRIAS Barcode
(if applicable)

Natural History Blood Tracking
Baseline/Acute Blood Draw

Collectors Initials _____

Date/Time of Blood Draw __ __ / __ __ / __ __ __ __ (mm/dd/yyyy) __ __:__ __ (24hr)

Centrifuge Date/Time __ __ / __ __ / __ __ __ __ (mm/dd/yyyy) __ __:__ __ (24hr)

Date and Time of Freeze __ __ / __ __ / __ __ __ __ (mm/dd/yyyy) __ __:__ __ (24hr)

Number of 4ml citrate cryovials	Number of 4ml EDTA cryovials	Number of 1.8ml citrate cryovials	Number of 1.8ml EDTA cryovials	Number of 1.8ml Serum cryovials	Number of Paxgene RNA tubes	Number of 10ml yellow top tubes

Was the Blood: □ Lipemic (milky white or viscous plasma) □ Hemolyzed (pink/red plasma) □ Neither

From where was the blood obtained?

□ Venipuncture □ Peripheral line □ Central line □ Mediport

□ PICC Line □ Other (specify)_____

If the blood was drawn from an IV line (peripheral, central, etc.) was there anything running through the line?

□ No
*Was it a □ Heparin or □ Saline Lock?

□ Yes (please explain) _____
*Was the IV fluid stopped during the blood draw? □ Yes □ No

Were the blood tubes placed on ice?

□ No □ Immediately □ Within One Hour □ Other (specify)_____

Blood Pressure at Time of Blood Draw __ __ __/__ __ __

Data Collected By: □ Neurologist □ Physician\Not-Neurologist □ Research Coordinator\RN

□ Self Report\Subject

Comments:_____
_____
_____
_____

Medical Record #_____ **SPOTRIAS Barcode On plasma/DNA sample** (if applicable)

Natural History Demographic and Clinical Variables

Collectors Initials _____

Date/Time Last Known Normal __ __ / __ __ / __ __ __ __ (mm/dd/yyyy) __ __:__ __ (24hr)

Date/Time Baseline MRI/CT __ __ / __ __ / __ __ __ __ (mm/dd/yyyy) __ __:__ __ (24hr)

Patient received IV TPA □ Yes □ No

Date/Time of IV TPA Start__ __ / __ __ / __ __ __ __ (mm/dd/yyyy) __ __:__ __ (24hr)

NIHSS on Admission_____

Patient's Zip Code (1st 3 digits) __ __ __ **Country of Origin**_____

Other Family Members in Coriell Repository? □ Unknown □ Yes □ No

Smoking History □ Never □ Previous □ Current Years Smoking_____

Family History of Stroke (blood relatives only)
□ Yes □ No □ Unknown (adopted)

If yes, then list all affected including type (e.g. brother-ischemic stroke) _____
_____

Family History of Cerebral Aneurysm (blood relatives only)
□ Yes □ No □ Unknown (adopted)

If yes, then list all affected _____
_____

Primary Clinical Diagnosis	Present	Absent		Present	Absent
Silent cerebral infarction	□	□	Intracerebral hemorrhage	□	□
Transient ischemic attack	□	□	Aneurysmal SAH	□	□
Unruptured intracranial aneurysm	□	□	Vascular cognitive impairment	□	□
Symptomatic ischemic stroke	□	□	AVM	□	□

Other (specify) _____

Medical History	Present	Absent
Hypertension	□	□
Diabetes mellitus	□	□
Atrial fibrillation	□	□
Myocardial infarction	□	□
Dyslipidemia	□	□

Other (specify) _____

Medication History: List medications and dosages
_____
_____
_____
_____
_____

Other Diagnoses	Present	Absent		Present	Absent
Parkinson's Disease	□	□	Epilepsy	□	□
Alzheimer's Disease	□	□	ALS	□	□
Dementia	□	□	Other (specify) _____		

Was a Plasma specimen collected from the patient for SPOTRIAS? □ Yes □ No

Was a DNA specimen collected from the patient for SPOTRIAS? □ Yes □ No
Date of DNA sample collection __ __ / __ __ / __ __ __ __ (mm/dd/yyyy)

Medical Record #_____ **Blood Draw ID**

SPOTRIAS Barcode
(if applicable)

Natural History Blood Tracking
Follow-up/24hr Blood Draw

Collectors Initials _____

Date/Time of Blood Draw __ __ / __ __ / __ __ __ __ (mm/dd/yyyy) __ __:__ __ (24hr)

Centrifuge Date/Time __ __ / __ __ / __ __ __ __ (mm/dd/yyyy) __ __:__ __ (24hr)

Date and Time of Freeze __ __ / __ __ / __ __ __ __ (mm/dd/yyyy) __ __:__ __ (24hr)

Number of 4ml citrate cryovials	Number of 4ml EDTA cryovials	Number of 1.8ml citrate cryovials	Number of 1.8ml EDTA cryovials	Number of 1.8ml Serum cryovials	Number of Paxgene RNA tubes	Number of 10ml yellow top tubes

Was the Blood? □ Lipemic (milky white and viscous) □ Hemolyzed (pink/red color to plasma) □ Neither

From where was the blood obtained?

□ Venipuncture □ Peripheral line □ Central line □ Mediport □ PICC Line

□ Other_____

If the blood was drawn from an IV line (peripheral, central, etc.) was there anything running through the line?

□ No
*Was it a □ Heparin or □ Saline Lock?

□ Yes (please explain) _____
*Was the IV fluid stopped during the blood draw? □ Yes □ No

Were the blood tubes placed on ice?

□ No □ Immediately □ Within One Hour □ Other (specify)_____

Blood Pressure at Time of Blood Draw __ __ __/__ __ __

Data Collected By: □ Neurologist □ Physician\Not-Neurologist □ Research Coordinator\RN

□ Self Report\Subject

Medications during admission: List medications and dosages_____
_____
_____

Comments:_____
_____
_____

APPENDIX G

RNA EXTRACTION

Paxgene Blood RNA Kit Handbook Procedure

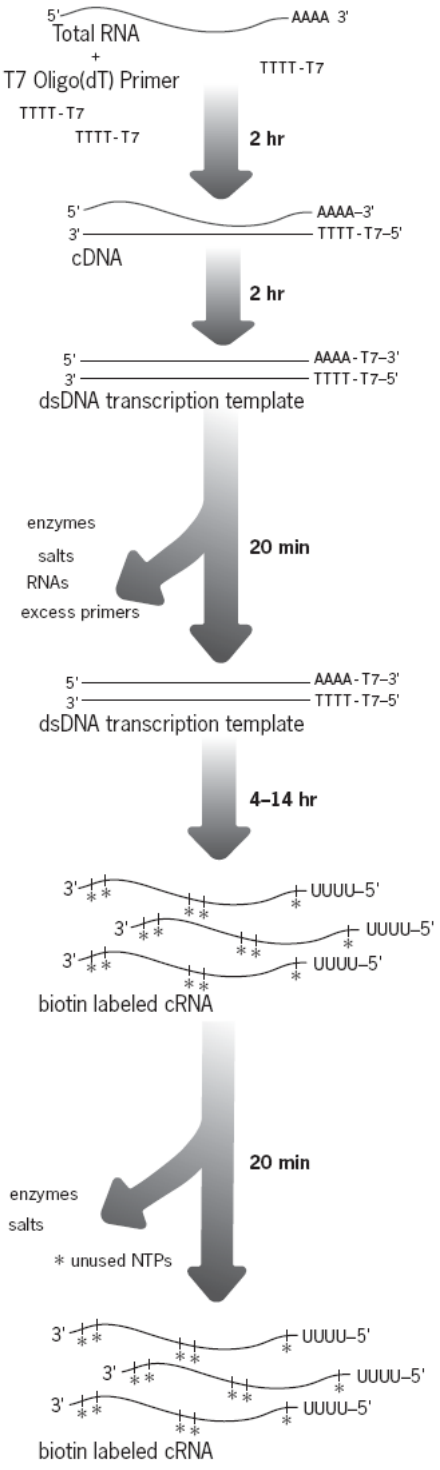
1. Centrifuge the PAXgene Blood RNA Tube for 10 minutes at 3000–5000 *x g* using a swing-out rotor.
2. Remove the supernatant by decanting or pipetting. Add 4 ml RNase-free water to the pellet, and close the tube using a fresh secondary Hemogard closure. If the supernatant is decanted, take care not to disturb the pellet, and dry the rim of the tube with a clean paper towel.
3. Vortex until the pellet is visibly dissolved, and centrifuge for 10 minutes at 3000–5000 x *g* using a swing-out rotor. Remove and discard the entire supernatant.
4. Add 350 µl Buffer BR1 and vortex until the pellet is visibly dissolved.
5. Pipet the sample into a 1.5 ml microcentrifuge tube. Add 300 µl Buffer BR2 and 40 µl proteinase K. Mix by vortexing for 5 seconds, and incubate for 10 minutes at 55°C using a shaker–incubator at 400–1400 rpm.
6. Pipet the lysate directly into a PAXgene Shredder spin column (lilac) placed in a 2 ml processing tube, and centrifuge for 3 minutes at maximum speed (but not to exceed 20,000 x *g*).
7. Carefully transfer the entire supernatant of the flow-through fraction to a fresh 1.5 ml microcentrifuge tube without disturbing the pellet in the processing tube.
8. Add 350 µl ethanol (96–100%, purity grade p.a.). Mix by vortexing, and centrifuge briefly (1–2 seconds at 500–1000 x *g*) to remove drops from the inside of the tube lid.
9. Pipet 700 µl sample into the PAXgene RNA spin column (red) placed in a 2 ml processing tube, and centrifuge for 1 minute at 8000–20,000 x *g*. Place the spin column in a new 2 ml processing tube, and discard the old processing tube containing flow-through.
10. Pipet the remaining sample into the PAXgene RNA spin column, and centrifuge for 1 minute at 8000–20,000 x *g*. Place the spin column in a new 2 ml processing tube, and discard the old processing tube containing flow-through.
11. Pipet 350 µl Buffer BR3 into the PAXgene RNA spin column. Centrifuge for 1 minute at 8000–20,000 x *g*. Place the spin column in a new 2 ml processing tube, and discard the old processing tube containing flow-through.
12. Add 10 µl DNase I stock solution to 70 µl Buffer RDD in a 1.5 ml microcentrifuge tube. Mix by gently flicking the tube, and centrifuge briefly to collect residual liquid from the sides of the tube.

13. Pipet the DNase I incubation mix (80 µl) directly onto the PAXgene RNA spin column membrane, and place on the benchtop (20–30°C) for 15 minutes.
14. Pipet 350 µl Buffer BR3 into the PAXgene RNA spin column, and centrifuge for 1 minute at 8000–20,000 x *g*. Place the spin column in a new 2 ml processing tube, and discard the old processing tube containing flow-through.
15. Pipet 500 µl Buffer BR4 to the PAXgene RNA spin column, and centrifuge for 1 minute at 8000–20,000 x *g*. Place the spin column in a new 2 ml processing tube, and discard the old processing tube containing flow-through.
16. Add another 500 µl Buffer BR4 to the PAXgene RNA spin column. Centrifuge for 3 minutes at 8000–20,000 x *g*.
17. Discard the tube containing the flow-through, and place the PAXgene RNA spin column in a new 2 ml processing tube. Centrifuge for 1 minute at 8000–20,000 x *g*.
18. Discard the tube containing the flow-through. Place the PAXgene RNA spin column in a 1.5 ml microcentrifuge tube, and pipet 40 µl Buffer BR5 directly onto the PAXgene RNA spin column membrane. Centrifuge for 1 minute at 8000–20,000x *g* to elute the RNA.
19. Repeat the elution step (step 18) as described, using 40 µl Buffer BR5 and the same microcentrifuge tube.
20. Incubate the eluate for 5 minutes at 65°C in the shaker–incubator (from step 5) without shaking. After incubation, chill immediately on ice.
21. If the RNA samples will not be used immediately, store at –20°C or –70°C. Since the RNA remains denatured after repeated freezing and thawing, it is not necessary to repeat the incubation at 65°C. If using the RNA samples in a diagnostic assay, follow the instructions supplied by the manufacturer.

PreAnalytix Paxgene Blood RNA Kit Handbook (cat#762164)

APPENDIX H

RNA AMPLIFICATION



II.C. Reverse Transcription to Synthesize First Strai

1. Bring RNA samples to 11 µl with Nuclease-free Water
2. Add 9 µl of Reverse Transcription Master Mix and place at 42°
3. Incubate for 2 hr at 42°C

II.D. Second Strand cDNA Synthesis

1. Add 80 µl Second Strand Master Mix to each sample
2. Incubate for 2 hr at 16°C

Potential stopping point

II.E. cDNA Purification

Preheat Nuclease-free Water to 50–55°C

1. Add 250 µl cDNA Binding Buffer to each sample cDNA Binding to each sample
2. Pass the mixture through a cDNA Filter Cartridge
3. Wash with 500 µl Wash Buffer
4. Elute cDNA with a total of 19 µl 50–55°C Nuclease-free Wate

Potential stopping point

II.F. In Vitro Transcription to Synthesize cRNA

1. Add 7.5 µl of IVT Master Mix to each cDNA sample, and mix
2. Incubate for 4–14 hr at 37°C
3. Add 75 µl Nuclease-free Water to each sample

Potential stopping point

II.G. cRNA Purification

Preheat Nuclease-free Water to 50–60°C

Assemble cRNA Filter Cartridges and tubes

1. Add 350 µl cRNA Binding Buffer to each sample
2. Add 250 µl 100% ethanol and pipet 3 times to mix
3. Pass samples through a cRNA Filter Cartridge(s)
4. Wash with 650 µl Wash Buffer
5. Elute cRNA with 100 µl preheated Nuclease-free Water

Potential stopping point

Illumina Total Prep RNA amplification kit (cat# IL1791)

APPENDIX I

ARRAY HYBRIDIZATION

Preparation

1. Preheat the oven (with rocking platform) to 58°C.
2. Prepare cRNA samples dried down, if necessary to achieve required concentration.
3. Add RNase-free water to cRNA and mix:
•For the 6-Sample chip, bring 1.5 µg of cRNA up to a total volume of 10 µl using RNase-free water and mix.
•For the 8-Sample or 12-Sample chip, bring 750 ng up to a total volume of 5 µl using RNase-free water and mix.
4. Leave at room temperature (~22°C) for 10 minutes to resuspend cRNA.
5. Place the HYB and HCB tubes in the 58°C oven for 10 minutes to dissolve any salts that may have precipitated in storage. Inspect the solution; if any salts remain undissolved, incubate at 58°C for another 10 minutes. After cooling to room temperature, mix thoroughly before using.
6. Add HYB to each cRNA sample:
•For the 6-Sample chip, add 20 µl HYB to each cRNA sample.
•For the 8-Sample or 12-Sample chip, add 10 µl HYB to each cRNA sample.
7. Place the Illumina Hyb Chamber gaskets into the BeadChip Hyb Chamber.
8. Dispense 200 µl HCB into the humidifying buffer reservoirs. Only add buffer to reservoirs next to loaded BeadChips.
9. Seal the Hyb Chamber with lid and keep on bench at room temperature until ready to load BeadChips into the Hyb Chamber.
10. Remove all BeadChips from their packages.

11. Holding the BeadChip by the coverseal tab with tweezers or with powder-free gloved hands, slide the BeadChip into the Hyb Chamber insert so that the barcode lines up with barcode symbol on the insert.
12. Preheat the assay sample at 65°C for 5 minutes.
13. Briefly vortex, then briefly centrifuge to collect the liquid in the bottom of the tube. Allow sample to cool to room temperature before using. Pipette sample immediately after cooling to room temp.
14. Load the Hyb Chamber inserts containing BeadChips into the Hyb Chamber.
15. Dispense assay sample onto the large sample port of each array:
•For the 6-Sample chip, add 30 µl.
•For the 8-Sample or 12-Sample chip, add 15 µl.
16. Seal lid onto the Hyb Chamber carefully to avoid dislodging the Hyb Chamber insert(s).
17. Incubate for 16-20 hours at 58°C with rocker speed at 5.

Wash and Stain

Prepare for High-Temp Wash & Overnight Incubation
1. Prepare 1X High-Temp Wash buffer (add 50 ml 10X stock to 450 ml RNase-free water).
2. Place waterbath insert into heat block, and add 500 ml prepared 1X High-Temp Wash buffer.
3. Set heat block temp to 55°C and pre-warm High-Temp Wash buffer to that temperature.
4. Close heat block lid and leave overnight.

Next Day

1. Make the Wash E1BC solution (add 6 ml E1BC buffer to 2 L RNase-free water).
Pre-warm Block E1 buffer (4 ml/chip) to room temperature.
2. Prepare Block E1 buffer (2 ml/chip) with streptavidin-Cy3 (2 µl of 1 mg/ml stock per chip). Use a single conical tube for all BeadChips. Store in dark until detection step.
Place 1 L of diluted Wash E1BC buffer in a Pyrex No. 3140 beaker.

Seal Removal

1. Remove the Hyb Chamber from the oven and disassemble.
If you are processing multiple chambers, remove them from the oven and process the BeadChips one at a time. Process all BeadChips in the first chamber as described in steps 2–5 below, then remove second chamber from the oven, process all of its BeadChips, and so on until all chambers are processed.
2. Using powder-free gloved hands, remove all BeadChips from the Hyb Chamber and submerge them face up at the bottom of the beaker.
3. Using powder-free gloved hands, remove the coverseal from the first BeadChip. Ensure that the entire BeadChip remains submerged during removal.
4. Using tweezers or powder-free gloved hands, transfer the peeled BeadChip into the slide rack submerged in the staining dish containing 250 ml Wash E1BC solution.
Do not empty the staining dish containing the 250 ml Wash E1BC solution after using because it will be used again in the 1st Room-Temp Wash below.
5. Repeat steps 3 and 4 for all BeadChips from the same Hyb Chamber.
6. Use the slide rack handle to transfer the rack into the Hybex Waterbath insert containing High-Temp Wash buffer.

High-Temp Wash

Incubate static for 10 minutes with the Hybex lid closed.

1st Room-Temp Wash

1. After the 10-minute High-Temp Wash buffer incubation is complete, immediately transfer the slide rack back into the staining dish containing the Wash E1BC used in step 4 of the Seal Removal steps.
2. Briefly agitate using rack, then shake on orbital shaker for 5 minutes at the highest speed possible without allowing solution to splash out of dish.

Ethanol Wash

1. Transfer rack to a clean staining dish containing 250 ml 100% Ethanol (use fresh from Ethanol source bottle).
2. Briefly agitate using rack handle, then shake on orbital shaker for 10 minutes.

2nd Room-Temp Wash

1. Transfer rack to a clean staining dish containing fresh 250 ml Wash E1BC solution.
2. Briefly agitate using rack handle, then shake on orbital shaker for 2 minutes.

Block

1. Pipette 4 ml Block E1 buffer into the wash tray(s).
2. Transfer the BeadChip, face up, into BeadChip wash tray(s) on rocker.
3. Rock at medium speed for 10 minutes.

Detect

1. Pipette 2 ml Block E1 buffer + streptavidin-Cy3 into fresh wash tray(s).
2. Transfer the BeadChip, face up, into wash tray(s) on rocker.
3. Place cover on tray and rock at medium speed for 10 minutes.

3rd Room-Temp Wash

1. Add 250 ml of Wash E1BC solution to a clean staining dish.
2. Transfer the BeadChip to the slide rack submerged in the staining dish.
3. Briefly agitate using rack, and then shake at room temperature on orbital shaker for 5 minutes.

Dry

1. Prepare centrifuge with plateholders, paper towels, and balance rack. Set speed to 275 relative centrifugal force.
2. Centrifuge the rack of BeadChips at room temperature for 4 minutes. If processing only one slide rack, redistribute the BeadChips between two racks, or counterbalance it with another rack loaded with an equal number of used BeadChips to maintain centrifuge balance.
3. Store dry chips in slide box until scanned.

Illumina Whole-Genome Gene Expression Direct Hybridization Assay Experienced User Card

APPENDIX J

CDNA SYNTHESIS

cDNA synthesis Protocol

- A total of 1ug of total RNA was the input for all samples, therefore the specific ul of RNA added to the following reaction depended on the starting ng/ul of each specific sample.

1. In a 0.5ml tube the following were combined:

50 um Oligo DT	1ul
RNA	xul
10mMdNTP mix	1ul
Rnase-free water	xul
FINAL VOLUME	10ul

2. The samples were heated at 65°C for 5 minutes to denature RNA and primer and placed on ice for at least 1 minute.

3. Prepare cDNA synthesis Master Mix: vortex the 10x RT Buffer for 5 seconds.

10x RT Buffer	2ul
25mM MgCl2	4ul
0.1 M DTT	2ul
RNase OUT	1ul
SuperScript III RT	1ul
FINAL VOLUME	10ul

4. Add 10ul of master mix to each RNA/primer mixture; mix gently and collect by brief centrfiguation. Incubate at 50°C for 50 minutes.

5. Terminate the reaction at 85°C for 5 minutes. Chill on ice.

6. Store cDNA at -20°C or use immediately for PCR.

TaqMan Gene Expression PCR Protocol

1. In a 384 well PCR plate the following were combined for each target gene and housekeeping gene separately (to be run in triplicate):

20X TaqMan Gene expression assay	1ul
cDNA template + RNase-free water	9ul
2x TaqMan Gene expression master mix	10ul
FINAL VOLUME	20ul

2. Centrifuge the plate briefly and load into the ABI 7900 HT.

3. The reaction was run under the following conditions for a standard 384 well plate:

Stage	**Temp °C**	**Time**
Hold	50	2 min
Hold	95	10 min
Cycle	95	15 sec
(40 cycles)	60	1 min

Superscript III First Strand Synthesis Systems for RT-PCR systems; Invitrogen (SKU 18080-051)

APPENDIX K

CORIELL CONTROL CLINICAL DATA ELEMENTS

Control Clinical Data Elements

Principal Investigator Responsible for Accuracy of Data (Name): **Subject ID number**

Subject Zip Code (1st 3 digits): _____ **Country** _____
Other family members or spouse in repository? Unknown ☐ Yes ☐ No ☐ If Yes, list subject ID: _____

Year of birth: _____ **Gender**: Male ☐ Female ☐

Ethnic Category (as reported by subject)-Check one: Hispanic or Latino ☐ Not Hispanic or Latino ☐

Racial Categories (as reported by subject) Check all that apply:
American Indian/Alaska Native ☐ Asian ☐ Native Hawaiian/ Other Pacific Islander ☐
Black/African American ☐ White ☐ Other ☐ specify:_____ Unknown ☐

Data collected by: Physician ☐ Research Coordinator/RN ☐ self reported (subject) ☐

Type of control Unaffected spouse ☐ Population control ☐ Related to an affected individual ☐ (REQUIRES SPECIAL APPROVAL) ID number _____

Medical History:

	Present	Absent		Present	Absent
Alzheimer's	☐	☐	Heart disease	☐	☐
Amyotrophic lateral sclerosis	☐	☐	Hypertension	☐	☐
Ataxia	☐	☐	Memory loss	☐	☐
Autism	☐	☐	Migraine	☐	☐
Bipolar	☐	☐	Multiple sclerosis	☐	☐
Brain aneurysm	☐	☐	Muscle disease	☐	☐
Cancer	☐	☐	Obsessive Compulsive	☐	☐
Dementia	☐	☐	Parkinson's disease	☐	☐
Depression	☐	☐	Schizophrenia	☐	☐
Diabetes	☐	☐	Stroke	☐	☐
Dystonia	☐	☐	Suicide/attempt	☐	☐
Epilepsy	☐	☐	Tourettes	☐	☐

Other (specify): _____

Family History (attach pedigree):	Present	Absent	List Relatives		Present	Absent	List Relatives
Alzheimer's	☐	☐		Heart disease	☐	☐	
Amyotrophic lateral sclerosis	☐	☐		Hypertension	☐	☐	
Ataxia	☐	☐		Memory loss	☐	☐	
Autism	☐	☐		Migraine	☐	☐	
Bipolar	☐	☐		Multiple sclerosis	☐	☐	
Brain aneurysm	☐	☐		Muscle disease	☐	☐	
Cancer	☐	☐		Obsessive Compulsive	☐	☐	
Dementia	☐	☐		Parkinson's disease	☐	☐	
Depression	☐	☐		Schizophrenia	☐	☐	
Diabetes	☐	☐		Stroke	☐	☐	
Dystonia	☐	☐		Suicide/attempt	☐	☐	
Epilepsy	☐	☐		Tourettes	☐	☐	

Other (specify): _____

Optional Data:
Smoking History Current ☐ Previous ☐ Never ☐ Years smoking _____
Mini-Mental status score _____ Neurological exam completed Yes ☐ No ☐
Handedness Left ☐ Right ☐ Ambidextrous ☐

I hereby verify that the clinical data are complete and the diagnosis is correct as indicated. Appropriate IRB approved informed consent has been obtained and unsigned copy of this consent is attached or previously submitted. No biopsies or cell cultures submitted to the repository were obtained from a live fetus, defined by the presence of pulse, circulation, and other vital signs. All publications regarding this sample or kindred have or will cite the NINDS repository and the NINDS repository will be notified thereof.

Submitter (please print)_____Signature_____ Date ____/____/____

APPENDIX L

ILLUMINA CUSTOM ALGORITHM

This model assumes that target signal intensity (l) is normally distributed among replicates of a biological condition (stroke patient or control subject). Variation stems from three components: sequence specific biological variation (σ_{bio}); nonspecific biological variation (σ_{neg}); and technical error (σ_{tech}).

Equation 1. Variance

$$I = N(\mu,)\sigma$$
$$\sigma = \sqrt{(\sigma^2 tech + \sigma^2 neg + \sigma^2 bio)}$$
$$\sigma_{tech} = a + b < I >,$$

Variation of nonspecific signal (σ_{neg}) is estimated from the signal intensity of negative control sequences using the median absolute deviation. For $\sigma_{tech,}$ two sets of parameters (a_{ref} and b_{ref}) and (a_{cond} and b_{cond}) are estimated for reference and condition groups respectively. σ_{tech} is then estimated using iterative robust least squares fit, which reduces the influence of highly variable genes, assuming the majority of genes do not have high biological variation amongst replicates. When this assumption does not hold, technical error is over-estimated by some averaged biological variation.

When groups contain biological replicates, p-values are produced based on the following approach:

Equation 2. P-value determination

$$S_{ref} = (\max(s_{ref}, a_{ref} + b_{ref} I_{ref}))$$
$$S_{cond} = (\max(s_{cond}, a_{cond} + b_{cond} I_{cond}))$$

$$P = z \{ | I_{cond} - I_{ref} | \div (\sqrt{(S^2_{ref} + S^2_{neg(ref)}/N_{ref}) + (S^2_{cond} + S^2_{neg(cond)}/N_{cond})}),$$

*where S_{ref} and S_{cond} are standard deviations of probe signals from the reference and control group respectively; and N_{ref} and N_{cond} denote the number of samples in each group.

It is considered that standard deviations exceeding σ_{tech} reflect biological variation. However it is assumed that estimates smaller than σ_{tech} are caused by random error. There the larger of the two estimates is used to regulate low abundance transcripts. Z is the two sided tail probability of the standard normal distribution.

A diff score for all probes is then computed based on the p-value of significance:

Equation 3. Diff Score determination

$$DiffScore = (10_{sgn}(I_{cond}-I_{ref})\log_{10}(p))$$

For each gene, diff scores of corresponding probes are averaged and concordance between the probes is reported.

For a p-value of 0.05, Diff Score = ±13
For a p-value of 0.01, Diff Score = ± 20
For a p-value of 0.001, Diff Score = ±30

APPENDIX M

T-TEST ANALYSIS IN GENESPRING

The t-test approach in GeneSpring is a traditional one-way test to compare conditions defined by a single parameter on the Null Hypothesis of equal mean expression between two groups.

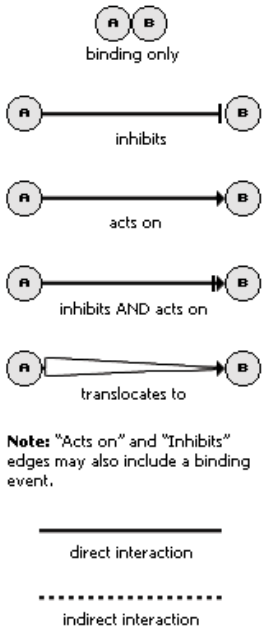
$$t = \overline{X}1-\overline{X}2 \div \sqrt{(s21 +s22)/n)}$$

APPENDIX N

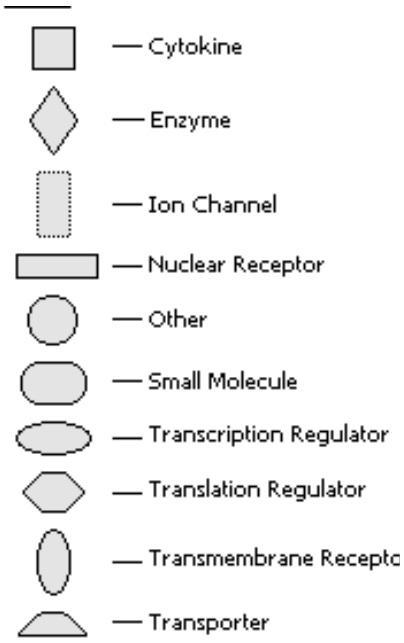
PATHWAY LEGEND

- ↑, red color – up-regulated
- ↓, green color – down-regulated

Edges in Pathway



Nodes in Pathway



APPENDIX O

BEAD STUDIO 1.5 FOLD, P<0.05 GENE LIST, 344 GENES

	TargetID	Signal_Control	Signal_Stroke	DetectionPval_Control	DetectionPval_Stroke	Diff_Score
1	ABCA1	1596.4	2840	0	0	53.7854
2	ABHD5	983.9	1686.6	0	0	77.4183
3	ACOX1	339.3	515.3	0	0	119.5554
4	ACSL1	3986.8	7866.2	0	0	38.8788
5	ADA	1351.1	898.9	0	0	-37.4839
6	ADM	4615.7	7266.2	0	0	44.2196
7	AES	2909.1	1817.1	0	0	-37.3061
8	AGTPBP1	377.4	713.5	0	0	16.7387
9	ALDH1A1	466.6	849.3	0	0	36.2014
10	ANKRD22	365	634.2	0	0	17.5708
11	ANKRD33	836.1	1347.9	0	0	41.5799
12	ANTXR2	1661	2755.9	0	0	108.5084
13	ANXA3	393	748	0	0	26.1211
14	APOBEC3A	1506.9	2942.8	0	0	16.461
15	APRT	2497.4	1478.3	0	0	-105.035
16	AQP9	17286.2	25991.6	0	0	52.855
17	ARG1	370.7	1584.3	0	0	44.1284
18	ATP6V0E2L	3244.5	1894.4	0	0	-107.946
19	AXIN2	583.1	360.9	0	0	-50.6605
20	B3GNT5	259.2	402.1	0	0	23.6942
21	B4GALT5	2403.5	3922	0	0	64.3467
22	BANF1	3516.5	2176.9	0	0	-60.3227
23	BASP1	15501.3	23858.7	0	0	41.2831
24	BAZ2B	1714.6	3018.9	0	0	107.9458
25	BCL6	4435.5	7321.1	0	0	33.7781
26	BIN1	3591.6	2136.9	0	0	-94.3404
27	BIRC2	1934.3	3054.3	0	0	71.6924
28	BNIP3L	722.9	1465.2	0	0	25.6773
29	BPI	333.7	600.9	0	0	13.9413
30	C11ORF2	5923.1	3721.6	0	0	-49.4056
31	C12ORF57	5734.4	3578.7	0	0	-44.2676
32	C15ORF29	420	642.7	0	0	17.748
33	C16ORF30	852.4	496	0	0	-63.587
34	C17ORF49	2779.7	1791.9	0	0	-46.9947
35	C20ORF3	1573.8	2380.5	0	0	59.9599
36	C5AR1	2903.5	5151.8	0	0	97.4469
37	C6ORF48	4769.5	3169.3	0.0006	0	-16.0556
38	C9ORF142	2770.9	1728.5	0	0	-60.7955
39	C9ORF45	700.2	453.2	0	0	-59.0956
40	CA4	1214.9	2651.5	0	0	40.4265
41	CAMP	3760.2	7458.7	0	0	13.2241
42	CARD12	428.6	658.4	0	0	96.1255
43	CCPG1	883.1	1390.5	0	0	74.4016
44	CCR7	6923.4	3331.2	0	0	-71.3653
45	CD163	394	775.9	0	0	62.0098
46	CD19	927.8	583	0	0	-26.1922
47	CD2	6981.5	4071.7	0	0	-79.2269
48	CD247	15897.2	9923.7	0	0	-66.3071

49	CD3D	11348.7	6155.6	0	0	-96.9847
50	CD55	2640.2	4253.1	0	0	55.3114
51	CD58	1398.3	2132.7	0	0	49.8504
52	CD6	4663.7	2440.1	0	0	-114.163
53	CD79A	1692.4	1054.6	0	0	-16.985
54	CD79B	3006.5	1677	0	0	-56.3897
55	CD81	13594.1	8818.9	0	0	-68.2672
56	CD8A	3247.8	1984.6	0	0	-20.2773
57	CD8B	491.6	306.5	0	0	-48.7896
58	CD96	2075.2	1278.2	0	0	-79.2264
59	CDC25B	3421.1	2201.2	0	0	-65.8457
60	CEACAM6	264	538.8	0	0	16.8023
61	CEACAM8	451.8	1080.7	0	0	15.9986
62	CKAP4	2220.2	4121.4	0	0	76.707
63	CLC	14875.7	8835.3	0	0	-20.739
64	CLEC4D	317.1	588.2	0	0	59.614
65	CLEC4E	373	635.7	0	0	30.896
66	CORO1C	909.5	1589.5	0	0	110.8775
67	CPD	1201.9	2264.6	0	0	85.3606
68	CREBBP	939	1501.1	0	0	69.0741
69	CRISPLD2	1962.2	3916.5	0	0	52.855
70	CSPG2	3272.3	6559.7	0	0	53.2479
71	CTNNA1	739.6	1126.1	0	0	74.061
72	CTNNAL1	251.9	380.8	0	0	25.758
73	CTSS	2698.2	4501.4	0	0	106.6598
74	CTSZ	1133.6	1929.1	0	0	96.6401
75	CUTA	2709.4	1724.7	0	0	-57.0073
76	CXCL16	2513.6	3934.4	0	0	51.761
77	CYBRD1	1078.6	1814.4	0	0	73.225
78	CYP1B1	762	1476.5	0	0	62.4409
79	D4S234E	1131.9	713.2	0	0	-24.4686
80	DAAM2	238	380.3	0	0	24.276
81	DEXI	1768.3	1152.3	0	0	-85.2882
82	DNAJA3	1998.9	1309.7	0	0	-82.8754
83	DOCK8	2120.2	3559.7	0	0	106.98
84	DPYD	737.9	1318.4	0	0	88.1617
85	DSC2	479.5	807.9	0	0	29.4951
86	DUSP1	9655.2	14748.7	0	0	38.111
87	DUSP6	1238.9	1870.9	0	0	13.3443
88	DYRK2	2626.8	1651.5	0	0	-42.4577
89	DYSF	2506.9	4556.9	0	0	39.1409
90	EBI2	1601.8	1052.7	0	0	-39.5908
91	ECHDC2	3016.5	1689.6	0	0	-115.751
92	ECHDC3	445.7	965	0	0	17.8497
93	EEF1G	23718.3	14296.5	0	0	-88.8905
94	EGFL5	364.8	578.9	0	0	57.6906
95	EIF2AK2	2428	4297.4	0	0	44.0172
96	EIF3S5	10976.3	6764.2	0	0	-90.1658

97	EMR3	1401.9	2370.7	0	0	67.1934
98	EVL	8039.3	4602.8	0	0	-103.094
99	F13A1	893	1579.3	0	0	28.8585
100	F2RL1	776.3	1221.5	0	0	37.7668
101	F5	518.2	898.3	0	0	56.9211
102	FAIM3	6400.3	3525.7	0	0	-70.7354
103	FAM102A	983.9	642.4	0	0	-53.6603
104	FAM108A2	936.9	595	0	0	-37.9544
105	FAM113B	3898.5	2265.1	0	0	-66.6676
106	FAM63A	1871.5	2929.4	0	0	40.2719
107	FBL	4255.9	2709.3	0	0	-79.395
108	FCGR1A	574.5	1000.9	0	0	31.0612
109	FCGR1B	1023.1	1555.6	0	0	13.1141
110	FCGR3B	5082.3	9627.3	0	0	13.3409
111	FCHO2	398	624.9	0	0	34.6538
112	FCRLM1	1571.4	900.6	0	0	-33.6132
113	FECH	486.9	817.7	0	0	15.4236
114	FKBP5	1634.1	3278.4	0	0	15.106
115	FLJ10357	353.7	630.1	0	0	56.6357
116	FLJ11151	997.3	1699	0	0	17.743
117	FLJ11305	3582.6	2302.7	0	0	-69.0264
118	FLJ20273	1062.1	1998.8	0	0	42.81
119	FLJ42957	415.1	640.6	0	0	44.0067
120	FLT3LG	1048.6	620.8	0	0	-102.123
121	FNBP4	4132	2643.2	0	0	-78.6628
122	FOS	1160	1758	0	0	54.1648
123	FPRL1	2153.9	3368.9	0	0	38.7286
124	FTHL12	14086	23072.9	0	0	18.4383
125	GIMAP5	5407.8	3013.8	0	0	-62.8844
126	GIMAP6	1972.1	1262.4	0	0	-46.3559
127	GLTSCR2	34194.4	21457.6	0	0	-83.6438
128	GNLY	6803	4224.2	0	0	-13.0998
129	GOLGA8B	4199	2505.3	0	0	-48.3234
130	GPR160	485.7	782.7	0	0	50.432
131	GPR177	629.4	1062.6	0	0	38.1491
132	GPR18	1134.3	723	0	0	-81.9171
133	GPR97	490.2	811.1	0	0	49.6932
134	GRAP	2317.5	1264.7	0	0	-76.4163
135	GZMK	4894.3	2741.5	0	0	-28.9085
136	GZMM	1251	800.2	0	0	-43.7455
137	HAL	969.6	1709	0	0	85.2882
138	HDAC4	772.4	1213.1	0	0	33.8785
139	HECW2	309.8	532.5	0	0	60.3227
140	HIF1A	612.6	1064.5	0	0	33.2376
141	HIGD2A	5710.4	3583.5	0	0	-88.8903
142	HIP1	362.9	549.6	0	0	42.9591
143	HIST1H4C	23769.7	15121.7	0	0	-63.3885
144	HIST1H4H	546	954.5	0	0	30.4851
145	HIST2H2AA3	3583.8	5858.4	0	0	48.1938
146	HIST2H2AC	1969.6	2957.9	0	0	38.0212

147	HIST2H2BE	818.2	1406.3	0	0	96.3813
148	HK2	637.2	1210.6	0	0	38.7445
149	HLA-DQB1	1017.2	575.3	0	0	-31.1697
150	HLX1	603.3	905.3	0	0	66.6871
151	HMFN0839	1024.7	1900.3	0	0	96.8593
152	HMGB2	2890.7	4898	0	0	17.1087
153	HMGCR	1040	1622.3	0	0	94.661
154	HP	294.8	517.8	0	0	28.5996
155	HPSE	419.6	629.5	0	0	41.7312
156	HSDL2	701.2	1173.9	0	0	97.8487
157	HSPA1A	4384.2	7383.1	0	0	35.0406
158	HSPA8	13024.6	8638.2	0	0	-68.4789
159	IBRDC2	1044.5	1585.9	0	0	39.1861
160	ICAM2	6620.8	4002.8	0	0	-98.447
161	ID3	1116.6	616.7	0	0	-114.411
162	IFIT2	6820.1	10666.7	0	0	17.2781
163	IGF2BP2	1302.6	2214.1	0	0	14.851
164	IGF2R	5478.4	9220	0	0	57.6982
165	IL17R	1160.3	1756.3	0	0	52.4376
166	IL18R1	724.9	1164.9	0	0	15.6523
167	IL18RAP	4561.4	7327.5	0	0	22.0562
168	IL2RB	3202.4	1996.3	0	0	-50.8265
169	IL32	2560.4	1507.4	0	0	-66.8553
170	IL7R	10745.1	5630.9	0	0	-61.53
171	IQGAP1	1467.3	2953.3	0	0	70.9521
172	IRAK3	920.9	1673.7	0	0	57.871
173	IRS2	785.8	1450.7	0	0	39.1066
174	ITGA2B	312.8	484.9	0	0	16.9578
175	ITGAM	1710.4	2741.5	0	0	83.6438
176	ITK	4025.1	2220.3	0	0	-107.35
177	ITPK1	895.9	1397.7	0	0	52.2192
178	ITPR3	2360.7	1377.1	0	0	-107.946
179	JMJD1C	1283.5	1949.3	0	0	61.7315
180	KBTBD7	840.7	1399.2	0	0	32.3664
181	KCNJ15	479.6	846.4	0	0	55.9495
182	KCTD12	302	626.3	0	0	14.9858
183	KIAA1147	1956	1293.7	0	0	-74.3197
184	KIAA1434	821.2	1265.6	0	0	70.4031
185	KIAA1600	1434.5	2213.3	0	0	85.3606
186	KIAA1754	2148.6	3229.7	0	0	72.6622
187	KIF1B	429.2	719.4	0	0	134.8221
188	KLF4	623.8	969.1	0	0	48.5948
189	KLHL2	625.2	1142.7	0	0	35.1923
190	KLHL3	773.8	506	0	0	-93.2913
191	KLRB1	10335.7	6144.6	0	0	-58.3631
192	KRT10	2130.6	1335.5	0	0	-92.2052
193	KRTCAP2	6660.4	4402.5	0	0	-58.6918
194	LAMP2	2857.5	4772.9	0	0	100.8472
195	LDHB	5693.6	3714.8	0	0	-21.177
196	LEF1	3410.8	1942.9	0	0	-49.9729

197	LGALS2	523.4	810.4	0	0	35.1958
198	LHFPL2	480.3	791.4	0	0	78.5833
199	LIME1	7971	4688.5	0	0	-96.1255
200	LMNB1	672.5	1069.8	0	0	41.2334
201	LOC441268	702.7	1068.3	0	0	27.8489
202	LOC646200	21328.6	13931.3	0	0	-72.3671
203	LRRFIP1	5341.1	8046.9	0	0	42.0347
204	LRRK2	620.5	1040.7	0	0	55.6544
205	LTB	13867.7	8913.3	0	0	-79.4956
206	LY96	4606.7	9419.7	0	0	49.6941
207	MAFB	1225.7	1906.8	0	0	35.9358
208	MAL	5565.6	2795.5	0	0	-76.463
209	MANSC1	493.3	900.5	0	0	68.4597
210	MAP3K2	941.8	1538.1	0	0	46.1578
211	MARCKS	4264	7287	0	0	95.3097
212	MATK	1421.2	941.3	0	0	-28.8905
213	MCEMP1	2121.8	4143.4	0	0	37.0225
214	MEGF9	645.2	1119.2	0	0	26.0697
215	MFGE8	1284.4	782.3	0	0	-55.6268
216	MGC13057	826.5	1369.4	0	0	14.5403
217	MGC17330	5563.2	3353.6	0	0	-46.5673
218	MGC2463	1866.1	1124.1	0	0	-68.8701
219	MIR16	789.4	1186.7	0	0	45.9295
220	MLSTD1	698.6	1225.4	0	0	74.5372
221	MME	1063.9	1846.9	0.0029	0.0003	17.4911
222	MMP9	2334.7	7022.2	0	0	30.009
223	MOSC1	1165.7	2031.3	0	0	36.2001
224	MOSPD2	391.9	617	0	0	31.3968
225	MPO	310.1	553.7	0	0	13.9675
226	MT1X	2299.4	1408.7	0	0	-73.3436
227	MTMR6	891.6	1403	0	0	40.8722
228	NCR3	813	500.1	0	0	-79.2269
229	NDST1	360.2	574.2	0	0	43.7633
230	NDUFA12	3768.3	2469.1	0	0	-71.2322
231	NDUFB11	5369.7	3529.1	0	0	-73.6551
232	NDUFS8	3612.7	2357.5	0	0	-78.3684
233	NEDD9	510.8	799.3	0	0	16.7301
234	NELL2	1416.3	849.8	0	0	-32.1822
235	NFIL3	766.9	1320.7	0	0	73.6669
236	NKG7	19294	12128.1	0	0	-30.822
237	NOSIP	6710.5	4314.4	0	0	-67.0961
238	NOV	491.2	768.6	0	0	63.1656
239	NPL	2441	3666.3	0	0	71.4921
240	NT5C2	2768.2	4208.3	0	0	64.3988
241	OCIAD2	1885.7	1251.8	0	0	-38.0765
242	OLFM4	425.6	1127.1	0	0	18.9712
243	OLR1	271.6	497.2	0	0	15.0239
244	ORF1-FL49	1206.3	1878.3	0	0	34.8729
245	ORM1	812.1	2438.8	0	0	17.0037
246	OSBPL8	1013.1	1630.9	0	0	35.9259

247	PADI4	6462.1	12128.8	0	0	49.8453
248	PARP9	545.9	890	0	0	28.6994
249	PASK	910.5	538.4	0	0	-38.3819
250	PDK4	470.8	990.6	0	0	102.7114
251	PGLYRP1	1745.5	3171	0	0	16.983
252	PIK3AP1	940.3	1540.1	0	0	15.0014
253	PLEK2	578.6	944.3	0	0	15.4851
254	PLEKHA1	2099.6	1335.4	0	0	-61.1927
255	PLXDC2	629.6	1086.2	0	0	338.2282
256	PLXNC1	586.6	936.1	0	0	60.3227
257	PPBP	1902.7	3165.6	0	0	17.4116
258	PPP3CA	455.4	734.8	0	0	19.0882
259	PPP4R1	1641.2	2608.9	0	0	72.7681
260	PRAM1	916.6	1375.2	0	0	62.1266
261	PREX1	707.8	1107.1	0	0	85.3606
262	PROS1	313.2	515.1	0	0	41.4417
263	PTGS2	1851.7	2780.3	0	0	35.9257
264	PTPRCAP	2542.9	1450	0	0	-69.1964
265	PYGL	2307.9	4096.7	0	0	52.1436
266	RAB33B	902.2	1577	0	0	17.8916
267	RBP7	916	1531.9	0	0	68.4154
268	REPS2	507.4	830.6	0.0001	0.0001	51.0499
269	RETN	369.6	574.4	0	0	19.4434
270	RGC32	5891.7	3739.1	0	0	-39.5908
271	RGS2	22350.1	39626.8	0	0	98.3674
272	RIS1	967.8	1799.6	0	0	14.4304
273	RNF13	821.9	1289	0	0	66.7021
274	RNF149	1579.5	2400.8	0	0	24.9521
275	ROCK1	340.6	518.7	0	0	43.1
276	RPAIN	3322.5	2203.9	0	0	-67.0894
277	RPL13A	10151.4	6327.3	0	0	-60.3227
278	RPL4	19401.7	12366.5	0	0	-55.5316
279	RPS15	31980.7	21230.3	0	0	-64.9007
280	RPS23	3607.2	2386.4	0	0	-24.5268
281	RPS4X	20532.1	11904.4	0	0	-74.1174
282	RRAGD	1150.7	1897.3	0	0	68.1093
283	RTN3	1351.9	2139	0.0005	0.0003	30.1272
284	S100A12	6684.7	14239.4	0	0	39.5291
285	SAP30	653.6	1212	0	0	45.9964
286	SCAP1	3748.7	2394	0	0	-65.8538
287	SDCBP	4662.2	7471.4	0	0	86.0235
288	SDPR	1465.8	3018.9	0	0	41.3317
289	SESN3	1633.8	2864.3	0	0	25.8016
290	SH3BGRL2	594.2	995	0	0	23.4456
291	SIGLEC5	702.7	1147.2	0	0	35.7806
292	SIPA1L2	681.4	1176.3	0	0	31.4506
293	SIRPB1	567.5	925.5	0	0	80.1607
294	SKAP2	957.6	1810.5	0	0	38.628
295	SLAMF1	798	528	0	0	-82.1932
296	SLAMF6	4813.4	3158	0	0	-61.0676

297	SLC11A1	3749	6175.2	0	0	34.1734
298	SLC22A15	505.1	763.1	0	0	78.1126
299	SLC22A4	1038.7	1728.8	0	0	72.8324
300	SLC25A42	1936.6	1222.5	0	0	-96.1255
301	SLC2A14	354	596.4	0	0	47.5236
302	SLC40A1	1865.9	2809.2	0	0	54.6594
303	SNRPF	4816.2	2934.1	0	0	-91.2539
304	SNURF	1327	836.4	0	0	-50.3731
305	SNX10	1356.2	2439.3	0	0	39.1366
306	SORL1	5187.2	7819.4	0	0	42.7491
307	SPOCK2	8735.7	5218.6	0	0	-58.6718
308	SRP14	11153.5	7407.8	0	0	-68.3183
309	SRPK1	1652.4	2781.8	0	0	84.4405
310	STK3	404.8	666.5	0	0	338.2282
311	STX2	956.9	627.4	0	0	-58.259
312	SUSD3	1768.2	1089.3	0	0	-107.876
313	SVIL	847.1	1369.9	0.0003	0	116.0819
314	TAOK1	379.6	732.2	0	0	65.0848
315	TCEA3	741.2	479	0	0	-41.4719
316	TCN1	688.1	1321	0	0	24.71
317	TGM3	445.2	698.2	0	0	31.9939
318	THBS1	301.6	546	0	0	43.5021
319	TIMM22	1818.8	1201.9	0	0	-80.9857
320	TLN1	1151.5	1947.2	0	0	57.6894
321	TLR2	397.7	661.5	0	0	44.963
322	TLR4	921.7	1395.6	0	0	49.8996
323	TMEM55A	709	1193	0	0	93.9985
324	TNFRSF25	1118	677	0	0	-72.8108
325	TNFRSF4	873	569.8	0	0	-22.832
326	TNFRSF7	1157.9	752.2	0	0	-80.1097
327	TNFSF10	4761.3	7880	0	0	77.2802
328	TNFSF13B	4280.7	6759.5	0	0	66.6517
329	TPST1	390.6	793.6	0	0	26.8336
330	TRIB2	1175.3	779.9	0	0	-69.5436
331	TSC22D3	3196.7	4919.5	0.0004	0.0001	29.7609
332	TSPAN2	303.2	471.7	0	0	16.0218
333	VASP	507.9	832.3	0	0	42.3093
334	VMD2	844.5	1281.2	0	0	57.1551
335	VNN3	706.2	1251.9	0	0	81.1909
336	VPREB3	984.7	561.6	0	0	-30.3742
337	WBSCR18	1260.4	784.4	0	0	-85.3606
338	WSB1	1764.1	2739.6	0	0	43.397
339	XK	414.8	725	0	0	14.3033
340	YY1	1602.6	2565.9	0	0	14.3766
341	ZCCHC6	503.4	806.2	0	0	65.822
342	ZFP36L1	4514.4	7373.3	0	0	19.4089
343	ZNF337	1263.1	785.5	0	0	-103.544
344	ZNF537	493.3	743	0	0	74.3928

APPENDIX P

BEAD STUDIO 2 FOLD, P<0.05 GENE LIST, 19 GENES

	TargetID	Signal_Control	Signal_Stroke	DetectionPval_Control	DetectionPval_Stroke	Diff_Score
1	ARG1	370.7	1584.3	0	0	44.1284
2	BNIP3L	722.9	1465.2	0	0	25.6773
3	CA4	1214.9	2651.5	0	0	40.4265
4	CCR7	6923.4	3331.2	0	0	-71.3653
5	CEACAM6	264	538.8	0	0	16.8023
6	CEACAM8	451.8	1080.7	0	0	15.9986
7	CSPG2	3272.3	6559.7	0	0	53.2479
8	ECHDC3	445.7	965	0	0	17.8497
9	FKBP5	1634.1	3278.4	0	0	15.106
10	IQGAP1	1467.3	2953.3	0	0	70.9521
11	KCTD12	302	626.3	0	0	14.9858
12	LY96	4606.7	9419.7	0	0	49.6941
13	MMP9	2334.7	7022.2	0	0	30.009
14	OLFM4	425.6	1127.1	0	0	18.9712
15	ORM1	812.1	2438.8	0	0	17.0037
16	PDK4	470.8	990.6	0	0	102.7114
17	S100A12	6684.7	14239.4	0	0	39.5291
18	SDPR	1465.8	3018.9	0	0	41.3317
19	TPST1	390.6	793.6	0	0	26.8336

APPENDIX Q

GENE SPRING 1.5 FOLD, P<0.05, GENE LIST 355 GENES

	Symbol	Corrected p-value	Fold change in Stroke	Regulation in Stroke	Control_Norm_Intensity	Stroke_Norm_intensity
1	ABCA1	8.96E-04	1.8123047	up	-0.45283636	0.4049892
2	ABHD5	2.51E-05	1.7271396	up	-0.4730409	0.31534383
3	ABTB1	0.001192165	1.5105206	up	-0.43969214	0.15535371
4	ACOX1	1.72E-08	1.7499906	up	-0.39899746	0.40834972
5	ACSL1	0.010596965	2.1238022	up	-0.7834313	0.30321807
6	ACSL4	0.002242988	1.5745142	up	-0.31663528	0.33827147
7	ACTN1	0.042329837	1.6357639	up	-0.3341905	0.37577406
8	ADM	1.19E-04	1.7796249	up	-0.5932397	0.23833346
9	AES	8.82E-04	1.618435	down	0.33804038	-0.35655907
10	AES	0.012148798	1.5252695	down	0.28743228	-0.32163197
11	AKAP7	0.027960693	2.1777713	up	-0.44277832	0.68007416
12	ALOX5	0.005035329	1.5656404	up	-0.35596168	0.2907913
13	ALPK1	0.004968409	1.5049171	up	-0.32445824	0.26522574
14	ANKRD13	9.21E-07	1.538632	up	-0.34640583	0.27524242
15	ANKRD22	0.024183083	1.6826864	up	-0.36122015	0.38954622
16	ANKRD33	0.0343414	1.5372375	up	-0.29694462	0.32339543
17	ANTXR2	1.41E-11	1.7635393	up	-0.49011627	0.32835746
18	APAF1	8.24E-05	1.5452136	up	-0.33461007	0.2931962
19	APRT	9.48E-07	1.6393174	down	0.48864126	-0.224454
20	APRT	1.46E-06	1.5625386	down	0.38686624	-0.25702557
21	AQP9	1.76E-04	1.6632756	up	-0.49637404	0.23765317
22	ARG1	7.46E-06	3.1748993	up	-0.62863874	1.0380721
23	ARHGAP9	5.86E-07	1.5350637	up	-0.41039085	0.20790768
24	ARID3A	0.008217917	1.5044478	up	-0.25097618	0.33825788
25	ARPC5	0.031313017	1.5089691	up	-0.4154583	0.17810494
26	ATP6V0E2L	2.03E-04	1.6451572	down	0.28906953	-0.42915595
27	ATP6V1A	1.17E-06	1.5905582	up	-0.4467425	0.22279061
28	ATP6V1B2	7.05E-05	1.503222	up	-0.32041124	0.26764688
29	ATP8B2	1.40E-06	1.5161607	down	0.32480156	-0.27562112
30	AXIN2	2.35E-04	1.5935856	down	0.41863	-0.25364652
31	B4GALT5	5.62E-05	1.7408099	up	-0.3609185	0.43884018
32	BANF1	0.001854022	1.5270411	down	0.35846767	-0.25227118
33	BASP1	0.001656124	1.692908	up	-0.40485874	0.35464483
34	BAZ1A	3.32E-06	1.624906	up	-0.3937578	0.3065984
35	BAZ2B	1.04E-07	1.8645284	up	-0.49394333	0.40486747
36	BCAS4	3.53E-04	1.522654	down	0.31792894	-0.2886593
37	BCAS4	0.002069403	1.625156	down	0.34984484	-0.35073334
38	BCL11B	6.72E-04	1.6628457	down	0.44529155	-0.28836277
39	BCL6	2.38E-04	1.8440986	up	-0.48973742	0.39317834
40	BIN1	1.62E-04	1.5449402	down	0.37067512	-0.25687593
41	BIN1	6.68E-06	1.6605223	down	0.37887493	-0.35276216
42	BIRC2	3.82E-06	1.6563947	up	-0.38282505	0.3452215
43	BLR1	0.022906728	1.6622154	down	0.40923765	-0.3238697
44	C16orf30	3.22E-05	1.6956762	down	0.51157886	-0.25028187
45	C20orf3	9.49E-05	1.586716	up	-0.31725883	0.3487852
46	C5AR1	2.64E-08	1.9257851	up	-0.48803505	0.45741165
47	C9orf142	0.002057869	1.5200473	down	0.37669584	-0.22742042

48	C9orf45	6.71E-04	1.5882019	down	0.39246467	-0.27492967
49	CA4	0.005262172	2.1216044	up	-0.54479116	0.5403645
50	CARD12	2.59E-06	1.5208824	up	-0.4216908	0.18321778
51	CCPG1	1.05E-06	1.6663233	up	-0.4289535	0.30771482
52	CCR7	0.001150122	2.0941548	down	0.40388396	-0.66248417
53	CD163	1.94E-06	1.977347	up	-0.44644794	0.5371181
54	CD163	2.83E-04	1.5054584	up	-0.260286	0.32991675
55	CD19	0.025516115	1.6571147	down	0.47358933	-0.25508416
56	CD2	5.24E-04	1.6109037	down	0.39074025	-0.29713
57	CD247	0.01357406	1.5364294	down	0.14497347	-0.47460806
58	CD3D	8.57E-05	1.7770516	down	0.3560257	-0.47345987
59	CD3D	0.001207411	1.6545517	down	0.40761673	-0.3188237
60	CD55	2.31E-04	1.7383883	up	-0.42369995	0.3740504
61	CD58	6.86E-04	1.5795712	up	-0.38774312	0.27178988
62	CD6	8.63E-06	1.8855989	down	0.4859031	-0.4291197
63	CD79A	0.03709512	1.6709588	down	0.57601184	-0.16466431
64	CD79B	0.003114411	1.7321764	down	0.49508777	-0.2974981
65	CD79B	8.90E-04	1.6874268	down	0.5566309	-0.198194
66	CD79B	9.41E-06	1.9654621	down	0.6683068	-0.30656168
67	CD8B	0.001805257	1.5227647	down	0.43020198	-0.176491
68	CD96	3.64E-06	1.6501937	down	0.45680213	-0.26583323
69	CD96	0.015361225	1.5224718	down	0.24109836	-0.36531714
70	CD97	7.91E-04	1.5206416	up	-0.2835666	0.32111362
71	CDC25B	0.002287968	1.5018301	down	0.37478307	-0.21193856
72	CDC42	0.01226788	1.6299903	up	-0.43720877	0.26765466
73	CEACAM1	0.008439163	1.5251158	up	-0.24410792	0.3648109
74	CEBPD	3.88E-04	1.642421	up	-0.45097446	0.2648495
75	CENTD3	0.010357025	1.5383601	up	-0.31860456	0.30278873
76	CKAP4	9.24E-07	1.9349477	up	-0.457984	0.49431062
77	CLEC2D	3.21E-04	1.5738429	down	0.40625414	-0.24803734
78	CLEC4D	1.77E-04	1.6824795	up	-0.33771107	0.4128778
79	CLEC4E	0.008569909	1.5838113	up	-0.39539754	0.26800284
80	CORO1C	2.92E-07	1.7304131	up	-0.47520924	0.31590727
81	CPD	1.12E-06	1.8952067	up	-0.48466924	0.43768594
82	CREB5	0.006681411	1.5786003	up	-0.46433613	0.19430982
83	CREBBP	1.55E-09	1.7318016	up	-0.4753668	0.31690684
84	CRISPLD2	1.25E-04	2.0627022	up	-0.562452	0.48208353
85	CSPG2	9.08E-04	2.0874317	up	-0.52938193	0.53234696
86	CTBS	1.10E-04	1.5915315	up	-0.37712824	0.29328746
87	CTNNA1	4.94E-05	1.5275059	up	-0.4146488	0.1965292
88	CTSS	8.76E-11	1.8215016	up	-0.53922606	0.3259022
89	CTSZ	5.66E-07	1.7908455	up	-0.54706603	0.29357484
90	CUTA	1.66E-06	1.5084157	down	0.4256169	-0.16741714
91	CXCL16	6.42E-04	1.6656028	up	-0.36483136	0.37121302
92	CYBRD1	6.43E-06	1.698955	up	-0.454548	0.31009966
93	CYP1B1	3.36E-04	1.8498029	up	-0.47369754	0.41367403
94	DOCK8	4.54E-09	1.8103185	up	-0.5535638	0.3026797

95	DPYD	9.35E-06	1.7590023	up	-0.40481353	0.40994382
96	DSC2	0.013481921	1.7159183	up	-0.36346447	0.41551638
97	DUSP1	3.80E-04	1.682358	up	-0.3743663	0.37611845
98	DYRK2	0.002165404	1.5440761	down	0.27149758	-0.3552462
99	DYSF	0.001368416	1.8924569	up	-0.35435605	0.5659044
100	E2F3	4.42E-04	1.5338274	up	-0.36689016	0.25024593
101	EBI2	0.02487179	1.5259324	down	0.3679166	-0.24177445
102	ECHDC2	4.46E-05	1.716245	down	0.33941677	-0.4398388
103	EEF1G	0.001656069	1.5083916	down	0.2436264	-0.34938464
104	EIF2AK2	4.74E-04	1.8460835	up	-0.35851967	0.52594817
105	ELOVL5	0.001900409	1.6279141	up	-0.39230406	0.31072047
106	EMR3	0.004741052	1.7467172	up	-0.533542	0.27110407
107	EMR3	7.42E-04	1.6231146	up	-0.4570636	0.24170125
108	ERGIC1	5.13E-05	1.7122784	up	-0.41896558	0.35695165
109	ETS2	4.67E-05	1.5149037	up	-0.37972677	0.21949929
110	EVL	3.34E-05	1.6366651	down	0.23423262	-0.4765265
111	F13A1	0.009173424	1.6828539	up	-0.36125728	0.3896526
112	F5	8.70E-04	1.6404358	up	-0.31013	0.4039491
113	FAIM3	2.21E-04	1.753313	down	0.49295402	-0.31712955
114	FAM102A	6.35E-05	1.5720942	down	0.39831316	-0.2543745
115	FAM108A2	5.86E-04	1.6873633	down	0.3757974	-0.37897322
116	FAM113B	8.18E-04	1.6465008	down	0.41296184	-0.30644146
117	FAM63A	4.44E-04	1.7099757	up	-0.5002774	0.27369848
118	FCGR1A	0.004699358	1.6864612	up	-0.3136489	0.44035026
119	FCGR2A	0.02978616	1.5598056	up	-0.3418266	0.29953963
120	FCRLM1	0.019254506	1.7570693	down	0.53679	-0.2763811
121	FGL2	6.61E-08	1.6761547	up	-0.5055029	0.23965244
122	FLJ10357	0.004686741	1.6282496	up	-0.34833077	0.3549911
123	FLJ20273	0.017583247	1.8363116	up	-0.57000357	0.30680737
124	FLJ22662	1.40E-04	1.6477797	up	-0.45327643	0.2672469
125	FLT3LG	6.61E-07	1.6907467	down	0.4784862	-0.27917433
126	FOLR3	0.024284562	2.118018	up	-0.6204037	0.46231112
127	FOS	6.16E-04	1.5530001	up	-0.3837123	0.25134563
128	FPRL1	4.32E-04	1.7971767	up	-0.58970034	0.25603193
129	G6PD	2.31E-05	1.6428543	up	-0.40454507	0.31165946
130	GALNAC4S-6ST	0.01168357	1.5734186	up	-0.39839706	0.25550553
131	GCA	2.69E-05	1.6104963	up	-0.4180822	0.26942316
132	GIMAP5	6.17E-05	1.6169097	down	0.42828014	-0.264959
133	GIMAP6	7.11E-05	1.5072603	down	0.3661282	-0.22580044
134	GLTSCR2	2.93E-06	1.5102428	down	0.35104704	-0.24373353
135	GNS	0.001315591	1.5672792	up	-0.29288337	0.3553789
136	GOLGA8B	0.019092575	1.5502025	down	0.3525721	-0.27988455
137	GPR160	0.007821544	1.5169402	up	-0.28531924	0.31584504
138	GPR18	7.06E-05	1.5627205	down	0.4083766	-0.23568314
139	GPR30	0.028008992	1.5636444	up	-0.3322477	0.3126648
140	GPR97	0.001374495	1.5649818	up	-0.3129496	0.3331963
141	GRAP	5.82E-06	1.8223768	down	0.42434028	-0.44148108
142	GRN	3.09E-04	1.664943	up	-0.44770584	0.28776696
143	GZMM	0.00463308	1.5544308	down	0.44149742	-0.19488896
144	HAL	2.61E-06	1.7816619	up	-0.6127035	0.22052002

145	HECW2	8.44E-04	1.5826768	up	-0.27963033	0.38273627
146	HIST1H4H	0.025849922	1.6558021	up	-0.3873155	0.3402147
147	HIST2H2AA3	0.001009389	1.7219576	up	-0.35815826	0.4258914
148	HIST2H2AC	0.008723038	1.5482497	up	-0.25483742	0.37580085
149	HIST2H2BE	1.52E-06	1.7038007	up	-0.43292883	0.33582774
150	HK2	0.03578569	1.811657	up	-0.37718323	0.48012656
151	HK3	0.03889349	1.5000247	up	-0.35806254	0.22692375
152	HMFN0839	7.93E-07	1.8398898	up	-0.4553827	0.42423669
153	HMGCR	1.74E-08	1.5929891	up	-0.43603715	0.23569927
154	HSDL2	1.26E-06	1.6443654	up	-0.457348	0.260183
155	HSPA1A	0.019009802	1.8337098	up	-0.49020323	0.38456213
156	IBRDC2	0.006122901	1.5297966	up	-0.3149556	0.2983843
157	ICOS	3.24E-04	1.5032278	down	0.32166818	-0.26639554
158	ID3	1.62E-07	1.8190107	down	0.46612728	-0.3970268
159	IFRD1	0.027392516	1.5545528	up	-0.4628234	0.17367627
160	IGF2R	1.66E-04	1.8231611	up	-0.39209458	0.47434753
161	IL17R	2.01E-04	1.5576218	up	-0.25372782	0.3856172
162	IL1R2	0.024156215	1.9028513	up	-0.49603036	0.43213242
163	IL2RB	0.0216144	1.5622303	down	0.28538325	-0.35822386
164	IL32	2.87E-04	1.6781938	down	0.523773	-0.22313634
165	IL32	0.003380901	1.5434707	down	0.29242566	-0.33375248
166	IL7R	6.93E-04	1.79816	down	0.39379862	-0.45272276
167	IQGAP1	2.10E-05	2.0312161	up	-0.6165189	0.4058248
168	IRAK3	5.62E-04	1.7741777	up	-0.4731234	0.35402712
169	IRS2	0.001384218	1.7316405	up	-0.33676544	0.455374
170	ITGAM	1.18E-05	1.6832777	up	-0.376963	0.37431028
171	ITK	1.57E-05	1.744029	down	0.51855147	-0.2838726
172	ITPK1	4.00E-04	1.5347396	up	-0.28709295	0.33090094
173	ITPR3	1.54E-04	1.6576486	down	0.37658942	-0.35254872
174	JMJD1C	1.55E-05	1.670232	up	-0.47736505	0.26268348
175	KCNJ15	7.00E-06	1.9459618	up	-0.4480821	0.51240134
176	KCNJ15	3.74E-04	1.5781107	up	-0.39775145	0.26044694
177	KIAA0319L	0.001731742	1.5387497	up	-0.3019117	0.3198469
178	KIAA1434	9.65E-05	1.5216612	up	-0.3586335	0.24701358
179	KIAA1600	4.60E-07	1.6111467	up	-0.4475591	0.24052879
180	KIAA1754	8.76E-06	1.6043354	up	-0.4228462	0.25912964
181	KIF1B	2.43E-09	1.8922873	up	-0.5140801	0.40605095
182	KLHL3	2.75E-06	1.5122335	down	0.31773272	-0.27894816
183	KYNU	0.003430352	1.5191779	up	-0.3092837	0.29400706
184	LAMP2	1.12E-12	1.9813491	up	-0.6557247	0.33075836
185	LAMP2	9.83E-08	1.7617275	up	-0.55963165	0.25735906
186	LAMP2	3.04E-05	1.6477886	up	-0.5334689	0.18706226
187	LEF1	0.005539591	1.6863836	down	0.3746798	-0.37925288
188	LENG4	2.56E-04	1.5949947	up	-0.38667235	0.2868792
189	LHFPL2	1.55E-06	1.6218902	up	-0.40214118	0.29553494
190	LILRB2	6.76E-05	1.5454783	up	-0.42800203	0.20005141
191	LILRB3	0.004217023	1.5993952	up	-0.3658454	0.311681
192	LIME1	3.67E-04	1.5787994	down	0.22906034	-0.42976752
193	LMNB1	0.011581204	1.5341495	up	-0.29421118	0.3232279
194	LOC90925	0.04949146	1.5222939	down	0.5025979	-0.10364902

195	LRP10	0.015376614	1.5328355	up	-0.32945156	0.28675127
196	LRRFIP1	3.33E-05	1.6990031	up	-0.46420875	0.30047974
197	LRRK2	5.82E-05	1.7916175	up	-0.42061105	0.42065164
198	LTB	4.17E-05	1.6349725	down	0.44332907	-0.2659372
199	LY9	1.70E-05	1.640391	down	0.4601098	-0.25392994
200	LY96	0.033536546	2.15995	up	-0.7933622	0.3176357
201	MAFB	0.003815692	1.5573182	up	-0.31047216	0.3285916
202	MAL	9.73E-04	1.9025011	down	0.45145655	-0.47644073
203	MAL	1.49E-05	1.816752	down	0.45890352	-0.402458
204	MANSC1	0.001718018	1.6770176	up	-0.42764106	0.31825674
205	MAP3K2	0.001394401	1.6754934	up	-0.4422439	0.3023421
206	MARCKS	1.42E-08	1.886556	up	-0.53175384	0.38400105
207	MAX	0.004743695	1.5399661	up	-0.28441906	0.33847952
208	MCEMP1	0.029402638	1.9077737	up	-0.219588	0.7123021
209	MCL1	7.70E-04	1.539178	up	-0.44949344	0.17266665
210	MFGE8	0.00110316	1.6206914	down	0.412401	-0.28420842
211	MFSD1	4.50E-07	1.5461406	up	-0.39453056	0.23414093
212	MGAM	0.001307882	1.6493242	up	-0.2990539	0.42282104
213	MGC17330	0.018769413	1.5535309	down	0.29595855	-0.3395924
214	MGC2463	2.35E-04	1.6216009	down	0.5048474	-0.1925714
215	MLSTD1	9.44E-06	1.6862478	up	-0.3698455	0.38397112
216	MME	5.07E-04	1.9353366	up	-0.47561494	0.4769696
217	MMP9	2.93E-04	2.6441047	up	-0.63609403	0.7666853
218	MOSC1	0.00227161	1.7277516	up	-0.49102435	0.2978715
219	MSL3L1	0.025648326	1.5070935	up	-0.31500092	0.2767681
220	MT1X	2.38E-04	1.5549619	down	0.41850176	-0.21837743
221	MTMR3	1.77E-06	1.6137481	up	-0.35766384	0.33275157
222	MTPN	1.08E-09	1.5344805	up	-0.4420948	0.17565541
223	MXD1	7.09E-05	1.6072506	up	-0.4908303	0.19376458
224	MYADM	0.002521299	1.6312991	up	-0.36531603	0.34070525
225	MYH9	2.17E-05	1.6730189	up	-0.4239423	0.31851146
226	NALP12	1.79E-05	1.6381502	up	-0.47598743	0.23608024
227	NCF2	0.020657025	1.614869	up	-0.4883368	0.20308037
228	NCOA1	1.51E-05	1.5352702	up	-0.31525442	0.30323818
229	NCR3	3.25E-06	1.5855395	down	0.48847985	-0.17649396
230	NDEL1	2.51E-05	1.5662156	up	-0.4093908	0.23789208
231	NDST1	0.009242461	1.5071554	up	-0.37224576	0.21958244
232	NEDD9	0.037254654	1.8163608	up	-0.72076803	0.14028278
233	NELL2	0.009759055	1.7378407	down	0.49079415	-0.30650166
234	NFIL3	1.15E-04	1.6974876	up	-0.5051343	0.25826675
235	NPL	8.49E-07	1.633246	up	-0.46844235	0.23929977
236	NT5C2	3.09E-06	1.6819997	up	-0.5887868	0.16139057
237	NT5C3	3.37E-04	1.5968759	up	-0.39640757	0.27884462
238	NUMB	1.22E-08	1.6520604	up	-0.42498946	0.299277
239	OCIAD2	0.002119827	1.5631819	down	0.29741773	-0.3470679
240	ORF1-FL49	0.019740919	1.5727817	up	-0.34972474	0.30359367
241	OSBPL8	0.011188532	1.5013292	up	-0.17578995	0.4104503
242	OSCAR	6.12E-06	1.5227277	up	-0.33202776	0.27463028
243	PACSIN2	1.08E-04	1.5298753	up	-0.3423233	0.27109072
244	PADI4	1.24E-04	2.0203402	up	-0.6873165	0.3272818

245	PAK2	1.63E-07	1.5011356	up	-0.30425522	0.28179914
246	PARP9	0.04576779	1.6042404	up	-0.33633992	0.34555042
247	PASK	0.001573324	1.6221251	down	0.54210055	-0.15578456
248	PBEF1	0.004247805	1.6449306	up	-0.4714088	0.24661796
249	PCNX	0.014886572	1.5285668	up	-0.3545656	0.25761405
250	PDE5A	0.018205058	1.5289818	up	-0.37917784	0.23339343
251	PDK4	2.48E-07	1.9925152	up	-0.49069417	0.5038966
252	PDLIM7	0.008335682	1.5847545	up	-0.36441982	0.2998395
253	PHC2	0.02985304	1.5579419	up	-0.32678905	0.31285232
254	PICALM	4.43E-10	1.6524075	up	-0.49607673	0.22849278
255	PJA2	2.07E-06	1.638601	up	-0.39961645	0.31284806
256	PLEKHA1	3.65E-04	1.572546	down	0.33863863	-0.31446368
257	PLXDC2	1.17E-10	1.7024015	up	-0.44182992	0.32574144
258	PLXNC1	0.003308967	1.5152813	up	-0.246924	0.35266173
259	PPP3R1	4.84E-05	1.6244959	up	-0.3795699	0.32042217
260	PPP4R1	1.27E-06	1.8080214	up	-0.56513435	0.28927743
261	PRAM1	4.55E-05	1.5166794	up	-0.3346273	0.2662889
262	PREX1	1.03E-05	1.5301241	up	-0.34114835	0.27250025
263	PRIC285	0.038515233	1.5011227	up	-0.2618407	0.32420126
264	PTGS2	0.016307665	1.5577009	up	-0.2766855	0.36273268
265	PTPRCAP	2.32E-04	1.7066637	down	0.52009594	-0.25108287
266	PYGL	2.32E-04	1.9196414	up	-0.6383079	0.30252883
267	RAB11FIP1	7.78E-04	1.5217462	up	-0.28387347	0.3218542
268	RAB27A	1.06E-07	1.5071427	up	-0.35081485	0.2410011
269	RAB31	8.00E-05	1.5792797	up	-0.4238473	0.23541942
270	RAI17	2.32E-06	1.5056869	up	-0.33112147	0.2593003
271	RASSF2	2.00E-06	1.6398529	up	-0.3305169	0.3830495
272	RASSF2	4.06E-07	1.5429187	up	-0.39883316	0.22682887
273	RBP7	2.14E-05	1.6992726	up	-0.4763657	0.28855166
274	REPS2	4.50E-04	1.8212063	up	-0.493821	0.3710734
275	RGS2	2.87E-10	1.9746453	up	-0.66856736	0.3130261
276	RNF13	1.32E-06	1.6937143	up	-0.443818	0.3163725
277	ROD1	6.08E-04	1.6012528	up	-0.5105467	0.1686544
278	RPL3	1.48E-04	1.5848476	down	0.33151624	-0.3328278
279	RPS4X	0.002112172	1.5540142	down	0.32695517	-0.3090445
280	RRAGD	7.12E-05	1.6515806	up	-0.35344505	0.37040228
281	RTN3	6.86E-06	1.6639026	up	-0.34274092	0.39183012
282	S100A12	0.010172865	2.3543987	up	-0.93866426	0.29669446
283	SAP30	0.008034376	1.6950442	up	-0.26140532	0.49991754
284	SART2	2.98E-04	1.5520507	up	-0.37208435	0.26209128
285	SDCBP	8.01E-07	1.7689717	up	-0.4568527	0.36605826
286	SERPINA1	5.01E-04	1.5274287	up	-0.22692633	0.38417876
287	SERPINA1	5.46E-05	1.6768464	up	-0.43405333	0.31169724
288	SH3GLB1	0.002829893	1.5190563	up	-0.39959073	0.20358467
289	SIRPB1	2.89E-05	1.5733961	up	-0.3352778	0.31860414
290	SIRPG	8.19E-05	1.5064514	down	0.35418966	-0.23696446
291	SKAP2	0.007368376	1.8684648	up	-0.61319184	0.28866163
292	SLA	0.001085509	1.646025	up	-0.44141772	0.2775685
293	SLAMF1	2.09E-05	1.50217	down	0.35049072	-0.23655732
294	SLC16A3	5.51E-04	1.5039946	up	-0.30064014	0.28815922

295	SLC22A4	6.54E-06	1.7355455	up	-0.5452797	0.25010952
296	SLC25A42	2.35E-06	1.5265827	down	0.41294122	-0.19736457
297	SLC2A14	0.036492433	1.5166202	up	-0.23506396	0.3657958
298	SLC2A3	8.77E-06	1.6691002	up	-0.33857092	0.40049958
299	SLC40A1	0.001077361	1.5836873	up	-0.39218852	0.27109903
300	SNRK	4.17E-05	1.535039	up	-0.38939023	0.22888504
301	SNRPF	5.71E-04	1.5414165	down	0.28002155	-0.34423518
302	SNRPN	1.98E-04	1.5017962	down	0.39556482	-0.1911243
303	SNURF	1.01E-05	1.648245	down	0.5175985	-0.20333214
304	SNX10	0.04400697	1.7789679	up	-0.5271935	0.30384696
305	SORL1	0.001287614	1.6717653	up	-0.37337554	0.36799684
306	SP100	4.03E-05	1.5320935	up	-0.32607222	0.2894322
307	SPAG9	3.72E-07	1.5381135	up	-0.36690667	0.25425524
308	SPOCK2	0.01628029	1.519338	down	0.30433238	-0.29911047
309	SRPK1	4.29E-06	1.7410624	up	-0.45548466	0.34448323
310	ST8SIA4	8.36E-10	1.7178135	up	-0.50841254	0.2721608
311	STAG2	2.95E-04	1.516143	up	-0.42515066	0.17525512
312	STK3	2.28E-09	1.5741026	up	-0.37065306	0.28387648
313	STOM	1.36E-04	1.6047417	up	-0.46994388	0.21239723
314	STX11	0.003483108	1.5377244	up	-0.3606926	0.26010433
315	STX2	3.72E-06	1.6198126	down	0.43591103	-0.25991583
316	SUSD3	2.54E-07	1.5676636	down	0.42616597	-0.22245002
317	SVIL	2.91E-09	1.8678638	up	-0.37932047	0.52206874
318	TAOK1	0.002065993	1.6937113	up	-0.27258304	0.48760498
319	TCEA3	4.77E-04	1.511375	down	0.39037618	-0.20548546
320	THOC3	9.19E-04	1.5055869	down	0.4264152	-0.16391076
321	TLE4	8.61E-08	1.5549959	up	-0.40907827	0.22783256
322	TLN1	2.48E-05	1.7057545	up	-0.29009762	0.4803124
323	TLR2	0.002389603	1.5521672	up	-0.27070537	0.36357853
324	TLR4	0.001042742	1.5305343	up	-0.28821686	0.32581848
325	TLR8	0.003152421	1.5388085	up	-0.35743284	0.26438078
326	TLR8	4.04E-05	1.6914089	up	-0.48619732	0.2720281
327	TM9SF2	1.58E-06	1.6009583	up	-0.45154515	0.22739068
328	TMEM49	4.25E-04	1.5581822	up	-0.4439707	0.19589333
329	TMEM55A	8.74E-06	1.6568463	up	-0.48433432	0.24410544
330	TMEM91	0.006200825	1.5575422	up	-0.43001485	0.20925644
331	TNFRSF25	1.10E-04	1.7684107	down	0.48251605	-0.33993736
332	TNFRSF7	1.09E-04	1.5374962	down	0.37414774	-0.24643512
333	TNFSF10	2.31E-07	1.8427252	up	-0.50972724	0.37211373
334	TNFSF13B	5.04E-08	1.7849591	up	-0.6033955	0.2324955
335	TRAF5	4.21E-04	1.5331247	down	0.3040305	-0.31244445
336	TRIB1	0.001714893	1.5124131	up	-0.33436236	0.26248994
337	TRIB2	7.96E-04	1.5173746	down	0.3352317	-0.26634556
338	TSC22D3	7.15E-05	1.6924903	up	-0.41409767	0.34505
339	TSC22D3	2.15E-09	1.8774674	up	-0.5252797	0.38350818
340	TSEN34	1.60E-06	1.6337733	up	-0.4572495	0.25095838
341	TXNDC13	0.005317458	1.5814332	up	-0.41053784	0.25069478
342	VASP	2.68E-05	1.7724652	up	-0.4458139	0.37994343
343	VCL	6.95E-05	1.5214889	up	-0.36566982	0.23981395
344	VEGFB	4.56E-06	1.6234121	down	0.40394688	-0.29508245

345	VMD2	0.001219483	1.5103511	up	-0.30331692	0.29156697
346	VNN2	1.45E-08	1.7898074	up	-0.55726606	0.28253838
347	VNN2	0.04293769	1.5314873	up	-0.5372459	0.07768749
348	VNN3	2.23E-06	1.6555603	up	-0.44342068	0.28389886
349	VNN3	1.29E-06	1.9406601	up	-0.5721719	0.38437554
350	VPREB3	0.004627587	1.9301076	down	0.6043759	-0.34430534
351	WBSCR18	3.12E-05	1.6152911	down	0.3094127	-0.38238144
352	WSB1	3.73E-05	1.622479	up	-0.4006877	0.29751208
353	ZAP70	6.08E-05	1.5332873	down	0.36498556	-0.25164247
354	ZCCHC6	6.02E-05	1.5224382	up	-0.2387861	0.36759755
355	ZNF337	6.54E-07	1.5926328	down	0.40538433	-0.2660293

APPENDIX R

GENE SPRING 2 FOLD, P<0.05, GENE LIST 16 GENES

	Symbol	Corrected p-value	Fold change	Regulation	Control	Stroke
1	ACSL1	4.03E-04	2.1238022	up	-0.7834313	0.30321807
2	AKAP7	0.001062506	2.1777713	up	-0.4427783	0.68007416
3	APOBEC3A	0.03293885	2.2190735	up	-0.5995044	0.55045307
4	ARG1	2.84E-07	3.1748993	up	-0.6286387	1.0380721
5	CA4	2.00E-04	2.1216044	up	-0.5995044	0.5403645
6	CCR7	4.37E-05	2.0941548	down	0.40388396	-0.6624842
7	CRISPLD2	4.74E-06	2.0627022	up	-0.562452	0.48208353
8	CSPG2	3.45E-05	2.0874317	up	-0.5293819	0.53234696
9	FCGR3B	0.024003983	2.235583	up	-0.6427389	0.5179123
10	FOLR3	9.23E-04	2.118018	up	-0.6204037	0.46231112
11	IQGAP1	7.97E-07	2.0312161	up	-0.6165189	0.4058248
12	LY96	0.001274389	2.15995	up	-0.7933622	0.3176357
13	MMP9	1.11E-05	2.6441047	up	-0.636094	0.7666853
14	ORM1	0.00609218	2.2461765	up	-0.5504059	0.6170654
15	PADI4	4.70E-06	2.0203402	up	-0.6873165	0.3272818
16	S100A12	3.87E-04	2.3543987	up	-0.9386643	0.29669446

APPENDIX S

GENES REGULATED BY AGE IN STROKE GROUP

	Gene	Fold change	Regulation	Corrected p-value	Stroke <60y	Stroke >60y
1	ACSL1	2.484374	up	0.0446085	-0.74035513	0.5725272
2	ARFGEF1	2.213192	up	0.008257919	-0.7595796	0.38654903
3	ARPP-19	2.1646469	up	0.01195357	-0.90462375	0.209508
4	ATP11B	2.3637316	up	0.03603059	-0.8622775	0.37878868
5	BIN2	2.1958306	up	0.031200677	-0.7576958	0.377071
6	CAST	2.252003	up	0.029694267	-0.98087955	0.19032918
7	DHRS8	2.2953622	up	0.009798116	-0.93625236	0.2624695
8	F11R	3.1309345	up	0.007976374	-1.25972	0.38687336
9	FAM49B	3.2131665	up	0.040724937	-1.1172744	0.5667214
10	FBXO38	2.5393336	up	0.025979264	-0.960459	0.38399088
11	FLJ11151	2.7070642	up	0.014563354	-0.7309369	0.7057922
12	GNA13	2.5798671	up	0.043621372	-0.88985026	0.4774464
13	GPBP1	2.0558481	up	0.04165832	-0.7248635	0.3148702
14	HEBP2	2.1112642	up	0.002986089	-0.65289176	0.4252153
15	HECA	2.5280104	up	0.004940345	-1.0853611	0.25264132
16	HMGB2	3.197031	up	0.008333158	-1.0408751	0.63585764
17	HNRPC	2.4634032	up	0.020800604	-1.0007415	0.2999113
18	MCTS1	2.4712584	up	0.030100342	-1.0774565	0.22778945
19	METTL7A	2.5580256	up	0.04897627	-0.713773	0.64125764
20	MYADM	2.6774745	up	0.04692652	-0.7664151	0.6544577
21	NEDD9	2.1855106	up	0.033739414	-0.75630903	0.3716613
22	PAPSS1	2.4646783	up	0.048576247	-0.8755778	0.4258215
23	PIK3AP1	2.8321755	up	0.035703324	-0.81097174	0.6909389
24	RTN3	2.834121	up	0.004845684	-0.89699674	0.60590464
25	RTN4	2.2975173	up	0.020294828	-0.7311121	0.46896365
26	SF3B14	2.135227	up	0.03467603	-0.7967069	0.29768255
27	SLC2A11	2.752874	up	0.041122623	-0.6690398	0.7918988
28	SULF2	2.012786	up	0.021191685	-0.51547337	0.49372038
29	TUBA1	2.221746	up	0.011949345	-0.79954374	0.3521501
30	USP8	2.414108	up	0.016071992	-0.8737992	0.39769098
31	VHL	2.0118108	up	0.045644812	-0.72845244	0.28004214

APPENDIX T

LOGISTIC REGRESSION FOR GENES ASSOCIATED WITH HARM

Model One: *HARM categories*-None, mild, moderate, or severe with the following covariates: signal intensities for the *16 genes specific for stroke, age, gender, rtPA treatment, hypertension, diabetes, hyperlipidemia, and smoking*

Parameter	Wald χ^2	*p*-value	Odds Ratio	95% CI
LY96	7.78	0.005	0.244	0.091-0.658
ORM1	4.05	0.044	2.064	1.019-4.181
Age	6.58	0.01	0.924	0.870-0.982
rtPA treatment	6.12	0.013	0.099	0.016-0.618

Percent Concordant 84.1	Somers' D 0.685
Percent Discordant 15.6	Gamma 0.687
Percent Tied 0.3	Tau-a 0.520
Pairs 308	c 0.843

Model Two: *HARM categories*-None, mild, moderate, or severe with the following covariates: signal intensities for the *16 genes specific for stroke, age, gender, rtPA treatment,*

Parameter	Wald χ^2	*p*-value	Odds Ratio	95% CI
LY96	5.54	0.019	0.381	0.17-0.851
Age	5.59	0.018	0.936	0.887-0.989
rtPA treatment	6.14	0.013	0.109	0.019-0.629

Percent Concordant 81.5	Somers' D 0.629
Percent Discordant 18.5	Gamma 0.629
Percent Tied 0.0	Tau-a 0.476
Pairs 329	c 0.815

Model Three: *Severe Harm* (yes or no) with the following covariates: signal intensities for the *16 genes specific for stroke, age, gender, rtPA treatment, hypertension, diabetes, hyperlipidemia, and smoking*

Parameter	Wald χ^2	*p*-value	Odds Ratio	95% CI
AKAP7	4.12	0.042	0.41	0.174-0.969

Percent Concordant 75.6	Somers' D 0.528
Percent Discordant 22.8	Gamma 0.537
Percent Tied 1.7	Tau-a 0.234
Pairs 180	c 0.764

Model Four: Severe Harm (yes or no) with the following covariates: signal intensities for the *16 genes specific for stroke, age, gender, rtPA treatment,*

Parameter	Wald χ^2	*p*-value	Odds Ratio	95% CI
AKAP7	4.48	0.034	0.399	0.170-0.934

Percent Concordant 76.7	Somers' D 0.550
Percent Discordant 21.7	Gamma 0.559
Percent Tied 1.6	Tau-a 0.239
Pairs 189	c 0.775

BIBLIOGRAPHY

Healthy People 2010 2nd ed. With Understanding and Improving Health and Objectives for Improving Health 2 vols. U.S. Department of Health and Human Services, Washington, DC: U.S. Government Printing Office, November 2000.

(1995).Thrombolysis in stroke--results of the ECASS study (European Cooperative Acute Stroke Study)." Nervenarzt 66(8 Suppl): 1-8.

(1995). Tissue plasminogen activator for acute ischemic stroke. The National Institute of Neurological Disorders and Stroke rt-PA Stroke Study Group. N Engl J Med 333(24): 1581-7.

(1999). "Recommendations for standards regarding preclinical neuroprotective and restorative drug development." Stroke 30(12): 2752-8.

(2001). "Enlimomab acute stroke trial investigators.

Use of anti-ICAM-1 therapy in ischemic stroke: results of the Enlimomab acute stroke trial." Neurology(57(8):1428-34.).

(2001). "Recommendations for clinical trial evaluation of acute stroke therapies." Stroke 32(7): 1598-606.

(2006). "Application Note: Amplification efficiency of TaqMan Gene Expression Assays." Applied Biosystems.

(2006). "Microarray Platform Comparisons- Commentary on Barnes et al. (2005)." from http://www.affymetrix.com/support/technical/whitepapers.affx.

(2006). "White Paper: TaqMan gene expression assays for validating hits from fluorescent microarrays." Applied Biosystems.

(2007). Technical Note: Expression profiling of whole blood specimens on Illumina BeadChips, Expression Analysis, http://www.expressionanalysis.com/technical_notes.html.

Adam-Vizi, V. (2005). "Production of reactive oxygen species in brain mitochondria: contribution by electron transport chain and non-electron transport chain sources." Antioxid Redox Signal 7(9-10): 1140-9.

Albers, G. W., P. Amarenco, et al. (2008). "Antithrombotic and thrombolytic therapy for ischemic stroke: American College of Chest Physicians Evidence-Based Clinical Practice Guidelines (8th Edition)." Chest 133(6 Suppl): 630S-669S.

Albers, G. W., V. N. Thijs, et al. (2006). "Magnetic resonance imaging profiles predict clinical response to early reperfusion: the diffusion and perfusion imaging evaluation for understanding stroke evolution (DEFUSE) study." Ann Neurol 60(5): 508-17.

Alberts, M. J., G. Hademenos, et al. (2000). "Recommendations for the establishment of primary stroke centers. Brain Attack Coalition." JAMA 283(23): 3102-9.

Alpert, J. S., K. Thygesen, et al. (2000). "Myocardial infarction redefined- a consensus document of the Joint European Society of Cardiology/American College of Cardiology Committee for the redifination of myocardial infarction." J Am Coll Cardiol 36(3):959-69.

Amantea, D., R. Russo, et al. (2007). "Early upregulation of matrix metalloproteinases following reperfusion triggers neuroinflammatory mediators in brain ischemia in rat." Int Rev Neurobiol 82: 149-69.

Andersson, A., C. Ritz, et al. (2007). "Microarray-based classification of a consecutive series of 121 childhood acute leukemias: prediction of leukeminc and genetic subtype as well as of minimal residual disease status." Leukemia. 21: 1198-1203.

Aronowski, J., R. Strong, et al. (1997). "Reperfusion injury: demonstration of brain damage produced by reperfusion after transient focal ischemia in rats." J Cereb Blood Flow Metab 17(10): 1048-56.

Badimon, L. and G. Vilahur (2007). "Platelets, arterial thrombosis and cerebral ischemia." Cerebrovasc Dis 24 Suppl 1: 30-9.

Baird, A. E. (2006). "Blood genomic profiling: novel diagnostic and therapeutic strategies for stroke?" Biochem Soc Trans 34(Pt 6): 1313-7.

Baird, A. E. (2006). "The blood option: transcriptional profiling in clinical trials." Pharmacogenomics 7(2): 141-4.

Baird, A. E. (2007). "Blood genomics in human stroke." Stroke 38(2 Suppl): 694-8.

Bak, S., D. Gaist, et al. (2002). "Genetic liability in stroke: a long-term follow-up study of Danish twins." Stroke 33(3): 769-74.

Barnes, M., J. Freudenberg, et al. (2005). "Experimental comparison and cross-validation of the Affymetrix and Illumina gene expression analysis platforms." Nucleic Acids Res 33(18): 5914-23.

Barr, T. L. and Y. P. Conley (2007). "Poly(ADP-ribose) polymerase-1 and its clinical applications in brain injury." J Neurosci Nurs 39(5): 278-84.

Basso, G., C. Case, et al. (2007). "Diagnosis and genetic subtypes of leukemia combining gene expression and flow cytometry." Blood Cells, Molecules, and Diseases 39: 164-168.

Becker, K. J. (2002). "Anti-leukocyte antibodies: LeukArrest (Hu23F2G) and Enlimomab (R6.5) in acute stroke." Curr Med Res Opin(Suppl2:s18-22.).

Becker, K. J. and T. G. Brott (2005). "Approval of the MERCI clot retriever: a critical view." Stroke 36(2): 400-3.

Bengrine, A., J. C. Li, et al. (2007). "The A-kinase anchoring protein 15 regulates feedback inhibition of the eipthelial Na+channel." FASEB J(21(4):1198-201.).

Berlis, A., H. Lutsep, et al. (2004). "Mechanical thrombolysis in acute ischemic stroke with endovascular photoacoustic recanalization." Stroke 35(5): 1112-6.

Bianchi, M. (2007). "DAMPs, PAMPs and alarmins: all we need to know about danger." J Leukocyte Biology 81:1-5.

Bonzano, L., L. Roccatagliata, et al. (2008). "In vitro investigation of poor cerebrospinal fluid suppression on fluid-attenuated inversion recovery images in the presence of a gadolinium-based contrast agent." Magn Reson Med 60(1): 220-3.

Brass, L. M., J. L. Isaacsohn, et al. (1992). "A study of twins and stroke." Stroke 23(2): 221-3.

Brott, T. and J. Bogousslavsky (2000). "Treatment of acute ischemic stroke." N Engl J Med 343(10): 710-22.

Buttner, F., C. Cordes, et al. (2009). "Genomic response of the rat brain to global ischemia and reperfusion." Brain Res 1252: 1-14.

Cantrell, A. R., V. C. Tibbs, et al. (2002). "Molecular mechanims of convergent regulation of brain Na(+) channels by protein kinase C and protein kinase A anchored to AKAP-15." Mol Cell Neurosci.(21(1):63-80.).

Caplan, L., Ed. (2000). Caplan's Stroke: A clinical approach. Woburn, MA, Butterworth: Heinemann.

Cardoso, F., L. Van't-Verr, et al. (2008). "Clinical application of the 70-gene profile: The MINDACT trial." Journal of Clinical Oncology 26(5): 729-735.

Carmichael, S. T., I. Archibeque, et al. (2005). "Growth-associated gene expression after stroke: Evidence for a growth-promoting region in peri-infarct cortex." Exp Neurol 193:291-311.

Caso, J. R., J. M. Pradillo, et al. (2008). "Toll-like receptor 4 is involved in subactue stress-induced neuroinflammation and in the worsening of experimental stroke." Stroke(39(4):1314-20.).

Caso, J. R., J. M. Pradillo, et al. (2007.). "Toll-like receptor 4 is involved in brain damage and inflammation after experimental stroke." Circulation.(115(12):1599-608.).

Castellanos, M., R. Leira, et al. (2003). "Plasma metalloproteinase-9 concentration predicts hemorrhagic transformation in acute ischemic stroke." Stroke 34(1): 40-6.

Castellanos, M., T. Sobrino, et al. (2007). "Serum cellular fibronectin and matrix metalloproteinase-9 as screening biomarkers for the prediction of parenchymal hematoma after thrombolytic therapy in acute ischemic stroke: a multicenter confirmatory study." Stroke 38(6): 1855-9.

Chacon, M. R., M. B. Jensen, et al. (2008). "Neuroprotection in cerebral ischemia: emphasis on the SAINT trial." Curr Cardiol Rep 10(1): 37-42.

Chalela, J. A., C. S. Kidwell, et al. (2007). "Magnetic resonance imaging and computed tomography in emergency assessment of patients with suspected acute stroke: a prospective comparison." Lancet 369(9558): 293-8.

Chang, J. H., J. C. Pratt, et al. (1998). "The small GTP-binding protein Rho potentiates AP-1 transcription in T cells." Mol Cell Biol(18(9):4986-93).

Chrissobolis, S. and F. M. Faraci (2008). "The role of oxidative stress and NADPH oxidase in cerebrovascular disease." Trends Mol Med 14(11): 495-502.

Cipolla, M. J. and A. B. Curry (2002). "Middle cerebral artery function after stroke: the threshold duration of reperfusion for myogenic activity." Stroke 33(8): 2094-9.

Clark, W. M. and K. P. Madden (2009). "Keep the three hour TPA window: the lost study of Atlantis." J Stroke Cerebrovasc Dis 18(1): 78-9.

Clark, W. M., S. Wissman, et al. (1999). "Recombinant tissue-type plasminogen activator (Alteplase) for ischemic stroke 3 to 5 hours after symptom onset. The ATLANTIS Study: a randomized controlled trial. Alteplase Thrombolysis for Acute Noninterventional Therapy in Ischemic Stroke." JAMA 282(21): 2019-26.

Clynes, R., B. Moser, et al. (2007). "Receptor for AGE (RAGE): weaving tangled webs within the inflammatory response." Curr Mol Med 7(8):743-51.

Coghlan, M. P., M. M. Chou, et al. (2000). "Atypical protein kinase Clambda and -zeta associate with the GTP-binding protein Cdc42 and mediate stress fiber loss." Mol Cell Biol(20(8):2880-9).

Columba-Cabezas, S., B. Serafini, et al. (2003). "Lymphoid chemokines CCL19 and CCL21 are expressed in the central nervous system during experimental autoimmune encephalomyelitis: implications for the maintenance of chronic neuroinflammation." Brain Pathol. 13(1):38-51.

Davis, S. M., G. A. Donnan, et al. (2008). "Effects of alteplase beyond 3 h after stroke in the Echoplanar Imaging Thrombolytic Evaluation Trial (EPITHET): a placebo-controlled randomised trial." Lancet Neurol 7(4): 299-309.

Debey, S., U. Schoenbeck, et al. (2004). "Comparison of different isolation techniques prior gene expression profiling of blood derived cells: impact on physiological responses, on overall expression and the role of different cell types." Pharmacogenomics J 4(3): 193-207.

Diener, H. C., K. R. Lees, et al. (2008). "NXY-059 for the treatment of acute stroke: pooled analysis of the SAINT I and II Trials." Stroke 39(6): 1751-8.

Dinapoli, V. A., J. D. Huber, et al. (2007). "Early disruptions of the blood-brain barrier may contribute to exacerbated neuronal damage and prolonged functional recovery following stroke in aged rats." Neurobiol Aging.

DiNapoli, V. A., J. D. Huber, et al. (2008). "Early disruptions of the blood-brain barrier may contribute to exacerbated neuronal damage and prolonged functional recovery following stroke in aged rats." Neurobiol Aging 29(5): 753-64.

Dirnagl, U., C. Iadecola, et al. (1999). "Pathobiology of ischaemic stroke: an integrated view." Trends Neurosci 22(9): 391-7.

Dohmen, C., B. Bosche, et al. (2007). "Identification and clinical impact of impaired cerebrovascular autoregulation in patients with malignant middle cerebral artery infarction." Stroke 38(1): 56-61.

Drake, C. T. and C. Iadecola (2007). "The role of neuronal signaling in controlling cerebral blood flow." Brain Lang 102(2): 141-52.

Du, X., Y. Tang, et al. (2006). "Genomic profiles for human peripheral blood T cells, B cells, natural killer cells, monocytes, and polymorphonuclear cells: comparisons to ischemic stroke, migraine, and Tourette syndrome." Genomics 87(6): 693-703.

Durante, W., F. K. Johnson, et al. (2007). "Arginase: A critical regulator of nitric oxide synthesis and vascular function." Clin Exp Pharm Physiol 34:906-911.

Ebert, S., T. Schoeberl, et al. (2008). "Chondroitin sulfate disacchardie stimulates microglia to adopt a novel regulatory phenotype." J Leukocyte Biology 84:736-740.

Edwards, A. S. and J. D. Scott (2000). "A-kinase anchoring proteins: protein kinase A and beyond." Curr Opin Cell Biol(12(2):217-21.).

Emsley, H. C., C. J. Smith, et al. (2003). "An early and sustained peripheral inflammatory response in acute ischaemic stroke: relationships with infection and atherosclerosis." J Neuroimmunol 139(1-2): 93-101.

Etienne-Manneville, S., N. Chaverot, et al. (1999). "ICAM-1 coupled signaling pathways in astrocytes converge to cyclic AMP response element-binding protein phosphorylation and TNF-alpha secertion." J Immunol(163(2):668-74.).

Feuerstein, G. Z., M. M. Zaleska, et al. (2008). "Missing steps in the STAIR case: a Translational Medicine perspective on the development of NXY-059 for treatment of acute ischemic stroke." J Cereb Blood Flow Metab 28(1): 217-9.

Few, W. P., T. Scheuer, et al. (2007). "Dopamine modulation of neuronal Na(+) channels requires binding of A kinase-anchoring protein 15 and PKA by a modified leucine zipper motif." Proc. Natl. Acad. Sci(104(12):5187-92.).

Fisher, M. (2003). "Recommendations for advancing development of acute stroke therapies: Stroke Therapy Academic Industry Roundtable 3." Stroke 34(6): 1539-46.

Fisher, M., G. W. Albers, et al. (2005). "Enhancing the development and approval of acute stroke therapies: Stroke Therapy Academic Industry roundtable." Stroke 36(8): 1808-13.

Foell, D., H. Wittkowski, et al. (2007). "S100 proteins expressed in phagocytes: a novel group of damage-associated molecular pattern molecules." J Leukocyte Biology 81:28-37.
Fraser, I. D., S. J. Tavalin, et al. (1998). "A novel lipid-anchored A-kinase anchoring protein facilitates cAMP responsive membrane events." EMBO J 17(8):2261-72.
Fukata, M., M. Nakagawa, et al. (2003). "Roles of Rho-family GTPases in cell polarisation and directional migration." Curr Opin Cell Biol 15:590-597.
Furlan, A., R. Higashida, et al. (1999). "Intra-arterial prourokinase for acute ischemic stroke. The PROACT II study: a randomized controlled trial. Prolyse in Acute Cerebral Thromboembolism." Jama 282(21): 2003-11.
Ganter, B., N. Zidek, et al. (2008). "Pathway analysis tools and toxicogenomics reference databases for risk assessment." Pharmacogenomics 9(1): 35-54.
Gartshore, G., J. Patterson, et al. (1997). "Influence of ischemia and reperfusion on the course of brain tissue swelling and blood-brain barrier permeability in a rodent model of transient focal cerebral ischemia." Exp Neurol 147(2): 353-60.
Gasche, Y., J. C. Copin, et al. (2001). "Matrix metalloproteinase inhibition prevents oxidative stress-associated blood-brain barrier disruption after transient focal cerebral ischemia." J Cereb Blood Flow Metab 21(12): 1393-400.
Gasche, Y., M. Fujimura, et al. (1999). "Early appearance of activated matrix metalloproteinase-9 after focal cerebral ischemia in mice: a possible role in blood-brain barrier dysfunction." J Cereb Blood Flow Metab 19(9): 1020-8.
Ghandour, M. S., O. K. Langley, et al. (1992). "Carbonic anhydrase IV on brain capillary endothelial cells: A marker associated with the blood-brain barrier." Proc. Natl. Acad. Sci 89:6823-6827.
Gidday, J. M., Y. G. Gasche, et al. (2005). "Leukocyte-derived matrix metalloproteinase-9 mediates blood-brain barrier breakdown and is proinflammatory after transient focal cerebral ischemia." Am J Physiol Heart Circ Physiol 289(2): H558-68.
Ginis, I., R. Jaiswal, et al. (2002). "TNFalpha induced tolerance to ischemic injury involves differential control of NF-kB transactivation: The role of NF-kB assocation with p300 adaptor." J Cereb Blood Flow Metab(22:142-152.).
Ginsberg, M. D., M. D. Hill, et al. (2006). "The ALIAS Pilot Trial: a dose-escalation and safety study of albumin therapy for acute ischemic stroke--I: Physiological responses and safety results." Stroke 37(8): 2100-6.
Gladstone, D. J., S. E. Black, et al. (2002). "Toward wisdom from failure: lessons from neuroprotective stroke trials and new therapeutic directions." Stroke 33(8): 2123-36.
Grammas, P., P. Moore, et al. (1998). "Anoxic injury of endothelial cells causes divergent changes in protein kinase C and protein kinase A signaling pathways." Mol Chem Neuropathol(33(2):113-21.).
Grond-Ginsbach, C., M. Hummel, et al. (2008). "Gene expression in human peripheral blood mononuclear cells upon acute ischemic stroke." J Neurol 255(5): 723-31.
Guo, L., E. K. Lobenhofer, et al. (2006). "Rat toxicogenomic study reveals analytical consistency across microarray platforms." Nat Biotechnol 24(9): 1162-9.
Gur, A. Y., D. Gucuyener, et al. (2007). "Cerebral vasomotor reactivity of patients with acute ischemic stroke: Cortical versus subcortical infarcts: an Israeli-Turkish collaborative study." J Neurol Sci 257(1-2): 121-5.
Guttmacher, A. E. and F. S. Collins (2002). "Genomic medicine--a primer." N Engl J Med 347(19): 1512-20.

Hacke, W., G. Albers, et al. (2005). "The Desmoteplase in Acute Ischemic Stroke Trial (DIAS): a phase II MRI-based 9-hour window acute stroke thrombolysis trial with intravenous desmoteplase." Stroke 36(1): 66-73.
Hacke, W., M. Kaste, et al. (2008). "Thrombolysis with alteplase 3 to 4.5 hours after acute ischemic stroke." N Engl J Med 359(13): 1317-29.
Hallenbeck, J. M. (1996). "Inflammatory reactions at the blood-endothelial interface in acute stroke." Adv Neurol 71: 281-97; discussion 297-300.
Hara, H., R. M. Friedlander, et al. (1997). "Inhibition of interleukin 1beta converting enzyme family proteases reduces ischemic and excitotoxic neuronal damage." Proc Natl Acad Sci U S A 94(5): 2007-12.
Henning, E. C., L. L. Latour, et al. (2008). "Verification of enhancement of the CSF space, not parenchyma, in acute stroke patients with early blood-brain barrier disruption." J Cereb Blood Flow Metab 28(5): 882-6.
Hochepied, T., F. Berger, et al. (2003). "Alpha1-acid glycoprotein: an acute phase protein with inflammatory and immunomodulating properties." Cytokine and Growth Factor Reviews 14:25-34.
Hossmann, K. A. (1994). "Viability thresholds and the penumbra of focal ischemia." Ann Neurol 36(4): 557-65.
Huang, Z. G., D. Xue, et al. (1999). "Biphasic opening of the blood-brain barrier following transient focal ischemia: effects of hypothermia." Can J Neurol Sci 26(4): 298-304.
Jaffe, A. S. (2008). "The clinical impact of the universal diagnosis of myocardiatl infarction." Clin Chem Lab Med 46(11):1485-1488.
Janoff, A. (1964.). "Alterations in lysosomes (intracellular enzymes) during shock; effects of preconditioning (tolerance) and protective drugs." Int Anesthesiol Clin 2:251-259.
Jerne, N. K. (1985). "The generative grammar of the immune system." Embo J 4(4): 847-52.
Jordan, J., T. Sequra, et al. (2008). "Inflammation as the therapeutic objective in stroke." Curr Pharm Des(14(33):3549-64.).
Joutel, A., C. Corpechot, et al. (1996). "Notch3 mutations in CADASIL, a hereditary adult-onset condition causing stroke and dementia." Nature 383(6602): 707-10.
Kahles, T., C. Foerch, et al. (2005). "Tissue plasminogen activator mediated blood-brain barrier damage in transient focal cerebral ischemia in rats: relevance of interactions between thrombotic material and thrombolytic agent." Vascul Pharmacol 43(4): 254-9.
Kamada, H., F. Yu, et al. (2007). "Influence of hyperglycemia on oxidative stress and matrix metalloproteinase-9 activation after focal cerebral ischemia/reperfusion in rats: relation to blood-brain barrier dysfunction." Stroke 38(3): 1044-9.
Kariko, K., D. Weissman, et al. (2004). "Inhibition of toll-like receptor and cytokine signalling-a unifying then in ischemic tolerance." J Cereb Blood Flow Metab(24(11):1288-304.).
Kelly, P. J., J. D. Morrow, et al. (2008). "Oxidative stress and matrix metalloproteinase-9 in acute ischemic stroke: the Biomarker Evaluation for Antioxidant Therapies in Stroke (BEAT-Stroke) study." Stroke 39(1): 100-4.
Kidwell, C. S., L. Latour, et al. (2008). "Thrombolytic Toxicity: Blood Brain Barrier Disruption in Human Ischemic Stroke." Cerebrovasc Dis 25(4): 338-343.
Kidwell, C. S., D. S. Liebeskind, et al. (2001). "Trends in acute ischemic stroke trials through the 20th century." Stroke 32(6): 1349-59.
Kidwell, C. S. and S. Warach (2003). "Acute Ischemic Cerebrovascular Syndrome. Diagnostic Criteria." Stroke.

Kidwell, C. S. and S. Warach (2003). "Acute ischemic cerebrovascular syndrome: diagnostic criteria." Stroke 34(12): 2995-8.
Kilic, U., E. Kilic, et al. (2008). "TLR-4 deficiency protects against focal cerebral ischemia and axotomy-induced neurodegeneration." Neurobiol Dis.(31(1):33-40.
Kim, D. and D. S. Liebeskind (2005). "Neuroimaging advances and the transformation of acute stroke care." Semin Neurol 25(4): 345-61.
Kim, J. B., C. S. Piao, et al. (2004). "Delayed genomic responses to transient middle cerebral artery occlusion in the rat." J Neurochem 89(5): 1271-82.
Kochanek, P. M. and J. M. Hallenbeck (1992). "Polymorphonuclear leukocytes and monocytes/macrophages in the pathogenesis of cerebral ischemia and stroke." Stroke 23(9): 1367-79.
Kolev, K., J. Skopal, et al. (2003). "Matrix metalloproteinase-9 expression in post-hypoxic human brain capillary endothelial cells: H2O2 as a trigger and NF-kappaB as a signal transducer." Thromb Haemost 90(3): 528-37.
Krizbai, I. A. and M. A. Deli (2003). "Singaling pathways regulating the tight junction permeability in the blood brain barrier." Cell Mol Biol(49(1):23-31.).
Kuge, Y., C. Yokota, et al. (2001). "Serial changes in cerebral blood flow and flow-metabolism uncoupling in primates with acute thromboembolic stroke." J Cereb Blood Flow Metab 21(3): 202-10.
Kunz, A., L. Park, et al. (2007). "Neurovascular protection by ischemic tolerance: role of nitric oxide and reactive oxygen species." J Neurosci 27(27): 7083-93.
Kuroiwa, T., P. Ting, et al. (1985). "The biphasic opening of the blood-brain barrier to proteins following temporary middle cerebral artery occlusion." Acta Neuropathol 68(2): 122-9.
Lampl, Y., M. Boaz, et al. (2007). "Minocycline treatment in acute stroke: an open-label, evaluator-blinded study." Neurology(69(14):1404-10.).
Laskowitz, D. T., S. E. Kasner, et al. (2009). "Clinical usefulness of a biomarker-based diagnostic test for acute stroke: the Biomarker Rapid Assessment in Ischemic Injury (BRAIN) study." Stroke 40(1): 77-85.
Latour, L. L., D. W. Kang, et al. (2004). "Early blood-brain barrier disruption in human focal brain ischemia." Ann Neurol 56(4): 468-77.
Lattimore, S. U., J. Chalela, et al. (2003). "Impact of establishing a primary stroke center at a community hospital on the use of thrombolytic therapy: the NINDS Suburban Hospital Stroke Center experience." Stroke 34(6): e55-7.
Lee, S. R., S. Z. Guo, et al. (2007). "Induction of matrix metalloproteinase, cytokines and chemokines in rat cortical astrocytes exposed to plasminogen activators." Neurosci Lett 417(1): 1-5.
Lees, K. R., K. Asplund, et al. (2000). "Glycine antagonist (gavestinel) in neuroprotection (GAIN International) in patients with acute stroke: a randomised controlled trial. GAIN International Investigators." Lancet 355(9219): 1949-54.
Lees, K. R., J. A. Zivin, et al. (2006). "NXY-059 for acute ischemic stroke." N Engl J Med 354(6): 588-600.
Li, F., Z. Z. Chong, et al. (2005). "Vital elements of the Wnt-frizzled signalling pathway in the nervous system." Curr Neurovasc Res 2(4):331-40.
Liao, D., R. Myers, et al. (1997). "Familial history of stroke and stroke risk. The Family Heart Study." Stroke 28(10): 1908-12.

Lipshutz, R. J., S. P. Fodor, et al. (1999). "High density synthetic oligonucleotide arrays." Nat Genet 21(1 Suppl): 20-4.
Lo, E. H. (2008). "A new penumbra: transitioning from injury into repair after stroke." Nat Med 14(5): 497-500.
Lu, A., Y. Tang, et al. (2003). "Genomics of the periinfarction cortex after focal cerebral ischemia." J Cereb Blood Flow Metab 23(7): 786-810.
Luo, B. Y., Z. Chen, et al. (2003). "Study of apolipoprotein E genetic polymorphism in patients with atherosclerotic cerebral infarction." J Zhejiang Univ Sci 4(6): 749-52.
Maas, A. I., A. Marmarou, et al. (2007). "Prognosis and clinical trial design in traumatic brain injury: the IMPACT study." J Neurotrauma 24(2): 232-8.
Maier, C. M., L. Hsieh, et al. (2006). "A new approach for the investigation of reperfusion-related brain injury." Biochem Soc Trans 34(Pt 6): 1366-9.
Maouche, S., O. Poirier, et al. (2008). "Performance comparison of two microarray platforms to assess differential gene expression in human monocyte and macrophage cells." BMC Genomics 9: 302.
Marciano, P. G., J. H. Eberwine, et al. (2002). "Expression profiling following traumatic brain injury: a review." Neurochem Res 27(10): 1147-55.
Markus, H. S., J. Barley, et al. (1995). "Angiotensin-converting enzyme gene deletion polymorphism. A new risk factor for lacunar stroke but not carotid atheroma." Stroke 26(8): 1329-33.
Marler, J. R., B. C. Tilley, et al. (2000). "Early stroke treatment associated with better outcome: the NINDS rt-PA stroke study." Neurology 55(11): 1649-55.
Marsh, B. J., S. L. Stevens, et al. (2009). "Inflammation and the emerging role of the toll-like receptor system in acute brain ischemia." Stroke(40(3 Suppl):S34-7.).
Matarin, M., W. M. Brown, et al. (2007). "A genome-wide genotyping study in patients with ischaemic stroke: initial analysis and data release." Lancet Neurol 6(5): 414-20.
Mathews, V. P., K. S. Caldemeyer, et al. (1999). "Brain: gadolinium-enhanced fast fluid-attenuated inversion-recovery MR imaging." Radiology 211(1): 257-63.
McKay, J., G. A. Mensah, et al. (2005). The atlas of heart disease and stroke. Geneva, World Health Organization.
Millan, M. and J. Arenillas (2006). "Gene expression in cerebral ischemia: a new approach for neuroprotection." Cerebrovasc Dis 21 Suppl 2: 30-7.
Mines, M., Y. Ding, et al. (2007). "The many roles of chemokine receptors in neurodegenerative disorders: Emerging new therapeutical strategies." Curr Med Chem 14:2456-2470.
Mitsios, N., M. Saka, et al. (2007). "A microarray study of gene and protein regulation in human and rat brain following middle cerebral artery occlusion." BMC Neurosci 8: 93.
Miyake, K. (2003). "Innate recognition of lipopolysaccharide by CD14 and toll-like receptor 4-MD-2: unique roles for MD-2." International Immunopharmacology 3:119-128.
Moeller, J. J., J. Kurniawan, et al. (2008). "Diagnostic accuracy of neurological problems in the emergency department." Can J Neurol Sci 35(3): 335-41.
Moncayo, J., G. R. de Freitas, et al. (2000.). "Do transient ischemic attacks have a neuroprotective effect?" Neurology(54:2089-2094.).
Montaner, J., P. Chacon, et al. (2008). "Simvastatin in the acute phase of ischemic stroke: a safety and efficacy pilot trial." Eur J Neurol 15(1): 82-90.

Montaner, J., I. Fernandez-Cadenas, et al. (2003). "Safety profile of tissue plasminogen activator treatment among stroke patients carrying a common polymorphism (C-1562T) in the promoter region of the matrix metalloproteinase-9 gene." Stroke 34(12): 2851-5.

Montaner, J., C. A. Molina, et al. (2003). "Matrix metalloproteinase-9 pretreatment level predicts intracranial hemorrhagic complications after thrombolysis in human stroke." Circulation 107(4): 598-603.

Montaner, J., M. Perea-Gainza, et al. (2008). "Etiologic diagnosis of ischemic stroke subtypes with plasma biomarkers." Stroke 39(8): 2280-7.

Mooradian, A. D. (1988). "Effect of aging on the blood-brain barrier." Neurobiol Aging 9(1): 31-9.

Mooradian, A. D. (1994). "Potential mechanisms of the age-related changes in the blood-brain barrier." Neurobiol Aging 15(6): 751-5; discussion 761-2, 767.

Mooradian, A. D., M. J. Haas, et al. (2003). "Age-related changes in rat cerebral occludin and zonula occludens-1 (ZO-1)." Mech Ageing Dev 124(2): 143-6.

Moore, D. F., H. Li, et al. (2005). "Using peripheral blood mononuclear cells to determine a gene expression profile of acute ischemic stroke: a pilot investigation." Circulation 111(2): 212-21.

Murkin, J. M. (2002). "Hemodynamic changes during cardiac manipulation in off-CPB surgery: relevance in brain perfusion." Heart Surg Forum 5(3): 221-4.

Nakajima, T., H. Ohtani, et al. (2008). "Natural selection in the TLR-realted genes in the course of primate evolution." Immunogenetics 60:727-735.

Neuberger, M. S. (2008). "Antibody diversification by somatic mutation: from Burnet onwards." Immunol Cell Biol 86(2): 124-32.

Pardridge, W., Ed. (1998). Introduction to the Blood-Brain Barrier: Methodology, Biology, and Pathology. Cambridge, Cambridge University Press.

Pardridge, W. M. (2007). "Blood-brain barrier delivery." Drug Discov Today 12(1-2): 54-61.

Parham, P. (2003). "Innate immunity: The unsung heroes." Nature(423(6935):20.

Patterson, T. A., E. K. Lobenhofer, et al. (2006). "Performance comparison of one-color and two-color platforms within the MicroArray Quality Control (MAQC) project." Nat Biotechnol 24(9): 1140-50.

Persidsky, Y., S. H. Ramirez, et al. (2006). "Blood-brain barrier: structural components and function under physiologic and pathologic conditions." J Neuroimmune Pharmacol 1(3): 223-36.

Pfefferkorn, T. and G. A. Rosenberg (2003). "Closure of the blood-brain barrier by matrix metalloproteinase inhibition reduces rtPA-mediated mortality in cerebral ischemia with delayed reperfusion." Stroke 34(8): 2025-30.

Popovic, P. J., H. J. Zeh, et al. (2007). "Arginine and Immunity" J Nutr 137:1681S-1686S.

Qi, X., K. Inagaki, et al. (2008). "Sustained pharmacological inhibition of deltaPKC protects against hypertensive encephalopathy through prevention of blood brain barrier breakdown in rats." J Clin Invest(118(1):173-82.).

Rainen, L., U. Oelmueller, et al. (2002). "Stabilization of mRNA expression in whole blood samples." Clin Chem 48(11): 1883-90.

Reeves, M. J., S. Arora, et al. (2005). "Acute stroke care in the US: results from 4 pilot prototypes of the Paul Coverdell National Acute Stroke Registry." Stroke 36(6): 1232-40.

Relton, J. K. and N. J. Rothwell (1992). "Interleukin-1 receptor antagonist inhibits ischaemic and excitotoxic neuronal damage in the rat." Brain Res Bull 29(2): 243-6.

Rodriguez-Yanez, M. and J. Castillo (2008). "Role of inflammatory markers in brain ischemia." Curr Opin Neurol 21(3): 353-7.
Rosamond, W., K. Flegal, et al. (2008). "Heart disease and stroke statistics--2008 update: a report from the American Heart Association Statistics Committee and Stroke Statistics Subcommittee." Circulation 117(4): e25-146.
Rosenberg, G. A. (1999). "Ischemic brain edema." Prog Cardiovasc Dis 42(3): 209-16.
Rosenberg, G. A. (2002). "Matrix metalloproteinases in neuroinflammation." Glia 39(3): 279-91.
Rosenberg, G. A. (2009). "Matrix metalloproteinases and their multiple roles in neurodegenerative diseases." Lancet Neurol 8(2): 205-16.
Rosenberg, G. A., E. Y. Estrada, et al. (1998). "Matrix metalloproteinases and TIMPs are associated with blood-brain barrier opening after reperfusion in rat brain." Stroke 29(10): 2189-95.
Rosenberg, G. A. and Y. Yang (2007). "Vasogenic edema due to tight junction disruption by matrix metalloproteinases in cerebral ischemia." Neurosurg Focus 22(5): E4.
Rubartelli, A. and M. Lotze (2007.). "Inside, outside, upside down: damage-associated molecular-pattern molecules (DAMPs) and redox." Trends in Immunology.(28(10):429-436.).
Rupalla, K., P. R. Allegrini, et al. (1998). "Time course of microglia activation and apoptosis in various brain regions after permanent focal cerebral ischemia in mice." Acta Neuropathol 96(2): 172-8.
Russo, R., E. Siviglia, et al. (2007). "Evidence implicating matrix metalloproteinases in the mechanism underlying accumulation of IL-1beta and neuronal apoptosis in the neocortex of HIV/gp120-exposed rats." Int Rev Neurobiol 82: 407-21.
Savitz, S. I. and W. R. Schabitz (2008). "A Critique of SAINT II: wishful thinking, dashed hopes, and the future of neuroprotection for acute stroke." Stroke 39(4): 1389-91.
Schabitz, W. R., A. Schneider, et al. (2008). "Minocycline treatment in acute stroke: an open-label, evaluator-blinded study." Neurology(71(18):1461;author reply 1461.).
Schmidt, A. M. and D. M. Stern (2001). "Receptor for age (RAGE) is a gene within the major histocompatibility class III region: implications for host response mechanisms in homeostasis and chronic disease." Front Biosci 6:D1151-60.
Schmitz, H. P., A. Lorberg, et al. (2002). "Regulation of yeast protein kinase C activity by interaction with the small GTPase Rho1p through its amino-terminal HR1 domain." Mol Microbiol(44(3):829-40.).
Schoch, C., M. Dugas, et al. (2004). ""Deep insight" into microarray technology. ." Atlas Genet Cytogenet Oncol Haematol.
Segura, T., S. Calleja, et al. (2008). "Recommendations and treatment strategies for the management of acute ischemic stroke." Expert Opin Pharmacother 9(7): 1071-85.
Sharief, M. K., M. A. Noori, et al. (1993). "Increased levels of circulating ICAM-1 in serum and cerebrospinal fluid of patients with active multiple sclerosis: Correlation with TNF-alpha and blood brain barrier damage." J Neuroimmunol(43(1-2):15-21.).
Sharp, F. R., H. Xu, et al. (2006). "The future of genomic profiling of neurological diseases using blood." Arch Neurol 63(11): 1529-36.
Sharp, F. R., H. Xu, et al. (2007). "Genomic profiles of stroke in blood." Stroke 38(2 Suppl): 691-3.

Shi, L., L. H. Reid, et al. (2006). "The MicroArray Quality Control (MAQC) project shows inter- and intraplatform reproducibility of gene expression measurements." Nat Biotechnol 24(9): 1151-61.

Shuaib, A., K. R. Lees, et al. (2007). "NXY-059 for the treatment of acute ischemic stroke." N Engl J Med 357(6): 562-71.

Sitzer, M., C. Foerch, et al. (2004). "Transient ischaemic attack preceding anterior circulation infarction is independently associated with favourable outcome." J Neurol Neurosurg Psychiatry.(75:659-660.).

Slater, S. J., J. L. Seiz, et al. (2001). "Interaction of protein kinase C isozymes with Rho GTPases." Biochemistry(40(14):4437-45.).

Smith, N. L., J. C. Bis, et al. (2008). "Variation in 24 hemostatic genes and associations with non-fatal myocardial infarction and ischemic stroke." J Thromb Haemost 6(1): 45-53.

Smith, W. S. (2006). "Safety of mechanical thrombectomy and intravenous tissue plasminogen activator in acute ischemic stroke. Results of the multi Mechanical Embolus Removal in Cerebral Ischemia (MERCI) trial, part I." AJNR Am J Neuroradiol 27(6): 1177-82.

Smith, W. S., G. Sung, et al. (2008). "Mechanical thrombectomy for acute ischemic stroke: final results of the Multi MERCI trial." Stroke 39(4): 1205-12.

Smith, W. S., G. Sung, et al. (2005). "Safety and efficacy of mechanical embolectomy in acute ischemic stroke: results of the MERCI trial." Stroke 36(7): 1432-8.

Stamatovic, S. M., O. B. Dimitrijevic, et al. (2006). "Protein kinase Calpha-RhoA cross-talk in CCL2-induced alterations in brain endothelial permeability." J Biol Chem(281(13):8397-88).

Stamatovic, S. M., R. F. Keep, et al. (2003). "Potential role of MCP-1 in endothelial cell tight junction "opening": signaling via Rho and Rho kinase." J Cell Sci(116(Pt2):4615-28).

Steiner, T., E. Bluhmki, et al. (1998). "The ECASS 3-hour cohort. Secondary analysis of ECASS data by time stratification. ECASS Study Group. European Cooperative Acute Stroke Study." Cerebrovasc Dis 8(4): 198-203.

Sturgeon, J. D., A. R. Folsom, et al. (2005). "Apolipoprotein E genotype and incident ischemic stroke: the Atherosclerosis Risk in Communities Study." Stroke 36(11): 2484-6.

Sumii, T. and E. H. Lo (2002). "Involvement of matrix metalloproteinase in thrombolysis-associated hemorrhagic transformation after embolic focal ischemia in rats." Stroke 33(3): 831-6.

Svedin, P., H. Hagberg, et al. (2007). "Matrix metalloproteinase-9 gene knock-out protects the immature brain after cerebral hypoxia-ischemia." J Neurosci 27(7): 1511-8.

Tang, Y., A. Lu, et al. (2001). "Blood genomic responses differ after stroke, seizures, hypoglycemia, and hypoxia: blood genomic fingerprints of disease." Ann Neurol 50(6): 699-707.

Tang, Y., A. Lu, et al. (2002). "Genomic responses of the brain to ischemic stroke, intracerebral haemorrhage, kainate seizures, hypoglycemia, and hypoxia." Eur J Neurosci 15(12): 1937-52.

Tang, Y., A. C. Nee, et al. (2003). "Blood genomic expression profile for neuronal injury." J Cereb Blood Flow Metab 23(3): 310-9.

Tang, Y., H. Xu, et al. (2006). "Gene expression in blood changes rapidly in neutrophils and monocytes after ischemic stroke in humans: a microarray study." J Cereb Blood Flow Metab 26(8): 1089-102.

Thom, T., N. Haase, et al. (2006). "Heart disease and stroke statistics--2006 update: a report from the American Heart Association Statistics Committee and Stroke Statistics Subcommittee." Circulation 113(6): e85-151.

Tibbs, V. C., P. C. Gray, et al. (1998). "AKAP15 anchors cAMP-dependent protein kinase to brain sodium channels." J Biol Chem(273(40):25783-8.).

Tournier-Lasserve, E., A. Joutel, et al. (1993). "Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy maps to chromosome 19q12." Nat Genet 3(3): 256-9.

Trebst, C., S. M. Staugaitis, et al. (2003). "CC chemokine receptor 8 in the central nervous system is associated with phagocytic macrophages." Am J. Pathol 162(2):427-38.

Trotter, K. W., I. D. Fraser, et al. (1999). "Alternative splicing regulates the subcellular localization of A-kinase anchoring protein 18 isoforms." J Cell Biol(147(7):1481-92.).

Tsuei, B. J., A. C. Bernard, et al. (2001). "Surgery induces human mononuclear cell arginase i expressions." J Trauma 51:497-502.

Tuttolomondo, A., D. Di Raimondo, et al. (2008). "Inflammatory cytokines in acute ischemic stroke." Curr Pharm Des 14(33): 3574-89.

Tyagi, N., W. Gillespie, et al. (2009). "Activation of GABA-A receptor ameliorates homocysteine-induced MMP9 activation by ERK pathway." J Cell Physiol(Epub ahead of print).

Ueda, S., C. J. Weir, et al. (1995). "Lack of association between angiotensin converting enzyme gene insertion/deletion polymorphism and stroke." J Hypertens 13(12 Pt 2): 1597-601.

Urra, X., A. Cervera, et al. (2009). "Monocytes are major players in the prognosis and risk of infection after acute stroke." Stroke(40:epub ahead of print.).

Vernino, S., R. D. Brown, Jr., et al. (2003). "Cause-specific mortality after first cerebral infarction: a population-based study." Stroke 34(8): 1828-32.

von Gertten, C., A. Flores Morales, et al. (2005). "Genomic responses in rat cerebral cortex after traumatic brain injury." BMC Neurosci 6: 69.

Wahlgren, N., N. Ahmed, et al. (2007). "Thrombolysis with alteplase for acute ischaemic stroke in the Safe Implementation of Thrombolysis in Stroke-Monitoring Study (SITS-MOST): an observational study." Lancet 369(9558): 275-82.

Wang, S., S. R. Lee, et al. (2006). "Reduction of tissue plasminogen activator-induced matrix metalloproteinase-9 by simvastatin in astrocytes." Stroke 37(7): 1910-2.

Wang, X., T. L. Yue, et al. (1994). "Concomitant cortical expression of TNF-alpha and IL-1 beta mRNAs follows early response gene expression in transient focal ischemia." Mol Chem Neuropathol 23(2-3): 103-14.

Warach, S., D. Kaufman, et al. (2006). "Effect of the Glycine Antagonist Gavestinel on cerebral infarcts in acute stroke patients, a randomized placebo-controlled trial: The GAIN MRI Substudy." Cerebrovasc Dis 21(1-2): 106-11.

Warach, S. and L. L. Latour (2004). "Evidence of reperfusion injury, exacerbated by thrombolytic therapy, in human focal brain ischemia using a novel imaging marker of early blood-brain barrier disruption." Stroke 35(11 Suppl 1): 2659-61.

Warach, S., L. L. Latour, et al. (2003). HARM: A potential marker of reperfusion injury in human stroke following intra-arterial thrombolysis. J. Cereb Blood Flow Metab, Philadelphia, Lippincott Williams & Wilkins, Inc.

Wei, L. H., A. T. Jacobs, et al. (2000). "IL-4 and Il-13 upregulate arginase I expression by cAMP and JAK/STAT6 pathways in vascular smooth muscle cells." Am J. Physiol Cell Physiol 279:C248-56.

Weih, M., K. Kallenberg, et al. (1999.). "Attenuated stroke severity after prodromal TIA: a role for ischemic tolerance in the brain?" Stroke(30:1851-1854.).

Weir, N. U. (2008). "An update on cardioembolic stroke." Postgrad Med J 84(989): 133-42; quiz 139-40.

Whiteley, W., M. C. Tseng, et al. (2008). "Blood Biomarkers in the Diagnosis of Ischemic Stroke. A Systematic Review." Stroke.

Wojciak-Stothard, B. and A. Ridley (2003). "Rho GTPases and the regulation of endothelial permeability." Vascular Pharmacology 39(4-5):187-199.

Wojner-Alexander, A. W., Z. Garami, et al. (2005). "Heads down: flat positioning improves blood flow velocity in acute ischemic stroke." Neurology 64(8): 1354-7.

Wong, C. H. and P. J. Crack (2008). "Modulation of neuro-inflammation and vascular response by oxidative stress following cerebral ischemia-reperfusion injury." Curr Med Chem 15(1): 1-14.

Woo, Y., J. Affourtit, et al. (2004). "A comparison of cDNA, oligonucleotide, and Affymetrix GeneChip gene expression microarray platforms." J Biomol Tech 15(4): 276-84.

Xu, H., Y. Tang, et al. (2008). "Gene expression in peripheral blood differs after cardioembolic compared with large-vessel atherosclerotic stroke: biomarkers for the etiology of ischemic stroke." J Cereb Blood Flow Metab 28(7): 1320-8.

Yang, Q. W., J. C. Li, et al. (2008). "Upregulated expression of toll-like receptor 4 in monocytes correlates with severity of acute cerebral infarction." J Cereb Blood Flow Metab(28(9):1588-96.

Zee, R. Y., P. M. Ridker, et al. (1999). "Prospective evaluation of the angiotensin-converting enzyme insertion/deletion polymorphism and the risk of stroke." Circulation 99(3): 340-3.

Zhao, B. Q., Y. Ikeda, et al. (2004). "Essential role of endogenous tissue plasminogen activator through matrix metalloproteinase 9 induction and expression on heparin-produced cerebral hemorrhage after cerebral ischemia in mice." Blood 103(7): 2610-6.

Zhao, B. Q., S. Wang, et al. (2006). "Role of matrix metalloproteinases in delayed cortical responses after stroke." Nat Med 12(4): 441-5.

Ziegler, G., D. Harhausen, et al. (2007). "TLR2 has a detrimental role in mouse transient focal cerebral ischemia." Biochem Biophys Res Commun(359(3):574-9.).

Zlokovic, B. V. (2008). "The blood-brain barrier in health and chronic neurodegenerative disorders." Neuron 57(2): 178-201.