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Hiramani Dhungana

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ABSTRACT

Ischemic stroke, characterized by sudden reduction in blood flow into one or more of the cerebral arteries, often affects the elderly population accompanied by co-morbid conditions such as atherosclerosis, infections and diabetes. Novel therapeutic compounds with neuroprotective properties in preclinical models have failed in human clinical trials. The reason for the failure may be partly explained by the use of the homogenous cohorts of young healthy animals in preclinical studies bearing little relevance to heterogenic conditions of human stroke. Especially, the interaction between aging and inflammation in patients predisposed to co-morbid conditions is poorly understood. In this study, we aimed to elucidate the impact of co-morbid conditions on ischemic inflammation in experimental settings and develop clinically more relevant rodent models that bear resemblance to human stroke.

We investigated the role of ApoE4 on aged mice fed with a high-fat diet and assessed the inflammatory and behavioral outcome following permanent middle cerebral artery occlusion (pMCAo). We show that aged transgenic mice expressing human ApoE4 isoforms fed with a high fat diet were more susceptible to sensorimotor deficits. These deficits were accompanied by increased astrogliosis, impaired neurogenesis, increased cyclooxygenase-2 immunoreactivity and increased peripheral IL-6 levels in plasma.

Next, we carried out a study where we assessed the effect of aging and predisposing peripheral infection on the outcome of cerebral ischemia in mice. Induction of chronic Th-1 polarized systemic infection by administration of gut parasite *T. muris* induced significant brain damage specifically in aged mice, and this damage correlated with pre-stroke plasma level of RANTES. In addition, significant infiltration of neutrophils in the infarct core and increased plasma levels of interleukin-17α and tumor necrosis factor-α were evident in aged infected mice when compared to their young counterparts.

Finally, we applied therapeutic intervention to inhibit proinflammatory receptor, cluster of differentiation 36 (CD36), in order to halt the brain damage following ischemia. We tested the therapeutic potential of a well-known inhibitor of CD36, sulfosuccinimidyl oleate sodium (SSO) against ischemic brain damage. Orally administered SSO reduced the infarct size of the animals that underwent surgery with the concomitant decrease in COX-2 immunoreactivity and microgliosis in the peri-ischemic area. SSO reduced the phagocytotic activity of BV2 microglial cells following LPS stimulation.

The results of this thesis show that 1) chronic peripheral infection and a high fat diet in combination with ApoE4 isoform in aged mice renders the brain more vulnerable to ischemic insults by altering the brain inflammatory status and 2) therapeutic intervention targeting the proinflammatory receptors like CD36 may be a suitable approach for designing treatment strategies. Our results show that co-morbid conditions alter the ischemia induced inflammatory status especially in aged mice pointing out the need to consider heterogeneity in preclinical modeling of stroke.

National Library of Medicine Classification: WT 104, WL 356, QZ 150, QU 55
Medical Subject Headings: Aging, Apolipoprotein E, Brain Ischemia, Cerebral Infarction, Chemokines, Co-morbidity, Cyclooxygenase 2, Cytokines, High-Fat, Infection, Inflammation, Mice, Transgenic, Neurogenesis

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TIIVISTELMÄ

Aivohalvaus on maailman toiseksi yleisin kuolinsyy. Intensiivisestä tutkimuksesta huolimatta aivohalvaukseen ei ole olemassa tehokasta hoitomuotoa. Monet uusista eläinkokeissa tehokkaiksi havaituista lääkehoidoista eivät ole osoittautuneet toimiviksi potilaskokeissa. Tätä uusien lääkeaineiden huonoa toimivuutta potilaskokeissa kutsutaan translationaalisuus ongelmaksi. Sen ajatellaan johtuvan siitä, että käytetyt prekliiniset eläinmallit eivät vastaa ihmisessä aivohalvauksen aikana tapahtuvia muutoksia. Vaikka aivohalvauspotilaat ovat iäkkäitä, usein naisia ja kärsivät erilaisista liitännäissairauksista, kuten sydän- ja verisuonisairauksista ja erilaisista infektioista, käytetään uusien lääkeaineiden testaamiseen vielä poikkeuksetta nuoria, terveitä uroshiiriä. Tämä on johtanut siihen, etteivät aivohalvaukseen suunnatut uudet lääkehoitojen kohteet eivät ole relevantteja aivohalvauspotilailla.

Tämän väitöskirjatyön tarkoituksena oli testata miten ikä ja erilaiset liitännäissairaudet vaikuttavat aivohalvauksen aiheuttamaan solutuhoon. Tutkimuksessa käytettiin kahta eri mallia, joilla pyrittiin mallintamaan ihmisillä esiintyviä, aivohalvauksen riskiin vaikuttavia tiloja. Tutkimuksen ensimmäisessä vaiheessa määritettiin, miten apolipoproteiini E4 (ApoE4) alleeli vaikuttaa aivohalvauksen aiheuttamiin motorisiin vaikeuksiin hiirillä, jotka saivat länsimaiselle väestölle tyypilliseen tapaan runsaasti kolesterolia sisältävää ruokaa. Tutkimuksessa näytettiin, että korkeakolesterolista rehua syöneiden, ihmisen ApoE4 alleelia ilmentävien hiirten aivohalvauksen jälkeiset motoriset vaikeudet olivat merkittävästi kasvaneet verrattuna ApoE3-alleelia ilmentäviin hiiriin. Nämä vaikeudet liittyivät ApoE4-alleelia ilmentävien hiirten aivojen kohonneeseen tulehdusvasteeseen, vähentyneeseen uusien hermosolujen muodostumiseen sekä lisääntyneeseen plasman IL-6 tasojen nousuun. Väitöskirjatyön toisessa vaiheessa näytettiin, että perifeerinen infektio, jota mallinnettiin Trichuris Muris parasiitilla, lisäsi aivohalvauksen aiheuttamaa solutuhoa erityisesti ikääntyneillä hiirillä. Tämä liittyi aivojen kohonneeseen tulehdussolujen infiltraatioon sekä kohonneisiin plasman tulehdusvälittäjäaineiden tasoihin. Väitöskirjatyön kolmannessa osatyössä näytettiin, että tulehdusvasteen inhiboiminen on potentiaalinen hoitomuoto aivohalvauksessa. Tähän käytettiin CD36 inhibiittoria, joka suun kautta annosteltuna pienensi aivohalvauksen aiheuttamaa solutuhoa ja vähensi tulehdusvastetta sekä aivohalvaus aivoissa että soluviljelyolosuhteissa.

Nämä tulokset osoittavat, että erilaiset aivohalvauksen liitännäissairaudet lisäävät aivohalvauksen aiheuttamaa solukuolemaa ja motorisia vaurioita. Lisäksi tulehdusvasteen säätelyn osoitettiin laskevan aivohalvauksen aiheuttamaa solutuhoa. Väitöskirjatyön tulokset osoittavat, että ikä ja erilaiset liitännäissairaudet vaikuttavat merkittävästi aivohalvauksen lopputulemaan. Tämä on erittäin tärkeää ottaa huomioon tutkimuksissa, joissa mallinnetaan tätä nimenomaan ikääntyvien ihmisten sairautta.

Yleinen Suomalainen asiasanasto: Aivohalvaus; Apolipoproteiinit, Eläinkokeet, Ikääntyminen, Infektiot, Inflammasomit, Kemokiinit; Liitännäistaudit, Mallintaminen, Riskitekijät, Sytokiinit

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Kuopio, December 2014

Hiramani Dhungana

List of the original publications

This dissertation is based on the following original publications:

I Dhungana H, Rolova T, Savchenko E, Wojciechowski S, Savolainen K, Ruotsalainen A-K, Sullivan P M, Koistinaho J and Malm T. Western-type diet modulates inflammatory responses and impairs functional outcome following permanent middle cerebral artery occlusion in aged mice expressing the human apolipoprotein E4 allele. *Journal of Neuroinflammation 10:102, 2013*

II *Dhungana H, *Malm T, Denes A, Valonen P, Wojciechowski S, Magga J, Savchenko E, Humphreys N, Grencis R, Rothwell N and Koistinaho J. Aging aggravates ischemic stroke-induced brain damage in mice with chronic peripheral infection. *Aging cell 12:842-50, 2013*

III Dhungana H, Lemarchant S, Korhonen P, Goldsteins VK, Goldsteins G, Kanninen K, Koistinaho J and Malm T. Sulfosuccinimidyl oleate sodium, a known inhibitor of CD36 protects mouse brain after permanent cerebral artery occlusion. *Manuscript*

*Equal contribution

Contents

Abbreviations

AD	Alzheimer's disease
ADP	Adenosine diphosphate
AIF	Apoptosis inducing factor
AMPA	α-amino-3-hydroxyl-5-methyl-isoxazolepropionate
AP-1	Activator protein 1
Apaf-1	Apoptotic protease activating factor 1
APOE	Apolipoprotein E (gene)
ApoE	Apolipoprotein E (protein)
ApoE3-TR	ApoE3 targeted replacement
ApoE4-TR	ApoE4 argeted replacement
APOER2	ApoE receptor 2
APP	Amyloid precursor protein
ATP	Adenosine triphosphate
Aβ	Beta amyloid
BBB	Blood brain barrier
CA1	Cornu Ammonis area 1
CAA	Cerebral amyloid angiopathy
CBA	Cytometric bead array
CBF	Cerebral blood flow
CCL	Chemokine Ligand
CCR	C-C chemokine
CD	Cluster of differentiation
CINC	Cytokines induced neutrophil chemoattractant
CNS	Central nervous system
COX-2	Cyclooxygenase-2
CXCL	Chemokine (C-X-C motif) Ligand
DCX	Doublecortin
DISC	Death inducing silencing complex
Endo G	Endonuclease G
eNOS	Endothelial Nitric oxide synthase
NOS	Nitric Oxide synthase
ER	Endoplasmic reticulum
FasR	Fas receptor
G-CSF	Granulocyte-colony stimulating factor
GFAP	Glial fibrillary acidic protein
HDL	High Density Lipoprotein
HF	High fat
HSP	Heat shock protein
Iba-1	Ionized calcium binding adaptor moleculte-1
ICAM-1	Intracellular cell adhesion molecule-1
ICH	Intracerebral hemorrhage
IFN	Interferon
IL	Interleukin
IL-1ra	IL-1 receptor antagonist
iNOS	Inducible NOS
IP3	Inositol-1, 4,5-triphosphate
JNK	C-Jun N-terminal Kinase
KC	Keratinocyte chemoattractant

LCFA	Long chain fatty acid
LDL	Low density lipoprotein
LDLR	Low density lipoprotein receptor
LPS	Lipopolysaccharides
LRP1	LDLR related protein 1
MAPK	Mitogen-associated protein Kinase
MCA	Middle cerebral artery
MCAo	MCA occlusion
MCP-1	Monocyte chemoattractant protein-1
mGluR	Metabotropic glutamate receptor
MIP	Macrophage inflammatory protein
MMP	Matrix metalloproteinase
MPTP	Mitochondrial permeability transition pores
MRI	Magnetic resonance imaging
MS	Multiple sclerosis
NADPH	Nicotinamide adenine dinucleotide phosphate
ND	Normal diet
NF-kB	Nuclear factor-Kappaβ
NMDA	N-methyl-D-aspartate
nNOS	Neuronal Nitric oxide synthase
NO	Nitric oxide
oxLDL	Oxidized low density lipoprotein
PARP	Poly ADP ribose polymerase
PD	Parkinson's disease
PI3K	Phosphoinositide 3-Kinase
RANTES	Regulated on activation, normal T cells expressed and secreted
ROS	Reactive oxygen species
SAH	Subarachnoid hemorrhage
SOD	Superoxide dismutase
SSO	Sulfosuccinimidyl Oleate Sodium
STAT-3	Signal transducer and activator of transcription 3
TBI	Traumatic Brain injury
TGF-β	Transforming growth factor-β
TLR	Toll like receptors
TNF	Tumor necrosis factor
TNFR	TNF receptor
tPA	Tissue plasminogen activator
TRADD	TNF-α receptor associated death domain
TRAF	TNF receptor associated factor
TSP	Thrombospondin
VCAM-1	Vascular Cell adhesion Molecule
VLDL	Very low density lipoprotein
VLDLR	VLDL receptor
WT	Wild-type

1 Introduction

The human brain needs a continuous supply of metabolic energy in order to maintain its cellular and molecular integrity. Since the brain cannot store enough glucose as a substrate for the production of adenosine triphosphate (ATP), there needs to be a continuous supply of oxygen and glucose in the brain. Thus, even a slight disturbance in the supply of nutrients by the blood may result in tissue damage that is characterized as stroke.

Cerebrovascular accident or stroke is the second leading cause of death and disability worldwide (Donnan et al., 2008). In the United States alone, an average of 795,000 people experiences new or recurrent stroke every year. Someone experiences stroke every 40 seconds and someone dies of stroke every 4 minutes (Roger et al., 2012). In Europe, stroke accounts for 1.1 million deaths each year (ESC, 2012). In Finland alone, the incidence of stroke is approximately 14,000 per year. Stroke is becoming an economic burden as it results in long term disability and extended hospitalization of the patients and post-stroke rehabilitation. Despite extensive research, thrombolysis is the only cure for stroke. However, the therapeutic time window is about 3 to 4.5 hours (Hacke et al., 2008, The NINDS t-PA Stroke Study Group, 1995) and only 3% to 5% of stroke patients benefit from the tissue plasminogen activator (tPA) due to timely hospitalization (Roth, 2011). There is also a risk of developing hemorrhage after thrombolysis, which contributes to mortality associated with this traditional treatment paradigm (The NINDS t-PA Stroke Study Group, 1997). Therefore, further understanding of the pathophysiology of ischemic stroke and development of suitable treatment paradigms is of utmost importance.

Stroke can be either ischemic or hemorrhagic. Ischemic stroke caused by blockage of arteries by either thrombus or embolus accounts for 85% of all stroke cases. Hemorrhagic stroke results from the rupture of the artery supplying blood and it accounts for the remaining 15 percent (Roger et al., 2012). Blockage of blood flow to the brain results in irreversible brain damage at the core area. The infarct can further evolve to the surrounding area known as the penumbra that can be salvaged by therapeutic means. Ischemic damage to the neuronal tissue initiates a variety of inflammatory responses as defense mechanisms to maintain tissue homeostasis through clearance of cellular debris as well as promoting neurogenesis and tissue repair (Iadecola and Anrather, 2011). However, in the acute phase of stroke, inflammatory reactions appear to be detrimental and also contribute to the development of delayed brain damage over time. Inflammation in the ischemic area is mediated by astrocytes, microglia and infiltrating peripheral leukocytes, primarily neutrophils and monocytes into the brain parenchyma. These inflammatory cells then produce a variety of cytotoxic molecules, including pro-inflammatory cytokines and chemokines, and reactive oxygen (ROS) and –nitrogen species. Thus, anti-inflammatory strategies have been suggested to be beneficial for stroke patients. A number of therapeutic compounds that have been reported to be beneficial in pre-clinical trials have undergone clinical trials, but none of them has been effective in humans. One of the reasons for the translational failure is suggested to be the heterogeneity of human stroke when compared to homogenous cohorts of young animals used in pre-clinical trials (Endres et al., 2008). In clinical condition, stroke typically affects elderly individuals with significant co-morbidity risk factors, such as hypertension, diabetes, atherosclerosis, Alzheimer's disease (AD), and infections, etc. So, inclusion of these factors in the preclinical setting should provide the next level of understanding and provide insights into effective therapeutic strategies in treating the disease.

Several lines of evidence suggest the role of aging, atherosclerosis, infections and genetic risk factors to the outcome of cerebral ischemia and hence to the pathophysiology of ischemic brain damage that is often accompanied by neurological deficits. However, the impact of

confounding risk factors such as infections, different apolipoprotein E (ApoE) isoforms and atherogenic diet in aging are not well studied. In this thesis, we studied the impact of high fat (HF) diet on sensorimotor functions and inflammation in aged mice expressing E3 and E4 isoforms of human APOE in C57Bl/6j background. Second, we investigated the impact of age and peripheral infection on the outcome of ischemic stroke. Finally, we studied the therapeutic efficacy SSO, a known inhibitor of CD36, against ischemic damage.

2 Review of the Literature

2.1 STROKE

2.1.1 Epidemiology

2.1.1.1 Mortality and prevalence

Stroke is characterized by diminished blood flow and hence compromised delivery of oxygen and nutrients into the discrete area of the brain. In addition to life-threatening properties, stroke often leaves the patients with long term disability and long term institutionalization and therefore adds to the economic burden of the society. Stroke is a disease affecting mostly older people. Annually, about 16 million people suffer from new stroke which claim the lives of nearly 5.7 million worldwide (Strong et al., 2007). About 85%-87% of the deaths are accounted on low and middle income countries. In the United States alone, approximately 610 000 of new stroke cases are reported every year. In addition, 185 000 people are susceptible to recurrent stroke. Between the ages of 55-75, the lifetime risk of stroke in women is about 20% and in men is 14%-17% (Roger et al., 2012). The situation is worse in Europe where stroke accounts for almost 1.1 million deaths each year. In the European Union alone, stroke is the second most common cause of death after cardiovascular disease and accounts for over 460 000 death each year. In particular, women have higher mortality and poorer outcome compared to men (ESC, 2012). Mortality from stroke is higher in Eastern and Central Europe (Truelsen et al., 2006). In Finland, approximately 14 000 people suffer from stroke every year according to the data from 2007 (Ruuskanen et al., 2010). Stroke prevalence in Finland in 2009 was estimated around 82,000 which is approximately 1.5% of the total population (Meretoja et al., 2010).

2.1.1.2 Economic burden

Given the second largest cause of disease worldwide, it is no wonder that the economic global burden caused by cerebral infarction is huge. Stroke accounts for approximately 3% of the total health care expenditure in the industrialized nations. The direct and indirect cost of stroke in US in 2008 was around $34.3 billion (Roger et al., 2012). In EU, Stroke cost around €19 billion of the health care system due to direct health care costs. An additional cost of productivity losses and informal care is estimated to cost €19 billion more. In Finland, annual nationwide cost for stroke is around €1.1 billion which is around 7% of the national health care expenses (Meretoja et al., 2011).

2.1.2 Types of stroke

2.1.2.1 Ischemic stroke

Ischemic stroke can be classified into thrombotic, embolic, lacunar stroke and stroke of other known and unknown etiologies. Cerebral thrombosis or thrombotic stroke accounts for 50% of all ischemic strokes and results from thrombus formation at the clogged part of the vessels. Embolic stroke constitutes about 20% of all cases and results from the clot formed at a distinct site that often dislodges and enters into the bloodstream and travels to block one of the brain arteries. Lacunar stroke or small vessel disease is caused by blockage of deep penetrating branches of large vessels and accounts for 20% of stroke cases (Warlow et al., 2003). Occlusion of the blood vessels by either thrombus or embolus limits the supply of nutrients and oxygen to the brain tissue. This event activates ischemic cascade that ultimately damages the core area of

the brain and often leads to sensorimotor deficits and cognitive impairments on the affected individuals.

2.1.2.2 Hemorrhagic stroke

Hemorrhagic stroke is caused by the rupture of the weaker vessels inside the skull, either into the brain or into the fluid that surrounds the brain. Intracerebral hemorrhage (ICH) occurs when the blood vessels inside the brain rupture and leak into the surrounding area of the brain tissue. It accounts for 10% of all stroke cases (Roger et al., 2012). ICH is usually caused by hypertension, brain tumors, aneurysms, amyloid angiopathy, arteriovenous malfunctions, etc. (Caplan, 2006). Primary ICH caused by rupture of arteries due to hypertension and amyloid angiopathy accounts for 78-88 % of all cases. Secondary ICH is caused by vascular abnormalities and accounts for the remaining cases (Qureshi et al., 2001). ICH mainly occurs in basal ganglia, cerebral lobes, thalamus, cerebellum and brain stem and is usually associated with hematoma formation that may expand over time into the surrounding area due to continuous bleeding from the primary source (Brott et al., 1997).

Subarachnoid hemorrhage (SAH) occurs due to rupture of the arteries between the pia mater and arachnoid space and hence the extravasation of blood into the subarachnoid space (Suarez et al., 2006). It is most commonly due to aneurysm, ballooning of the weakened region of the blood vessels and accounts for 3% of all stroke cases (Roger et al., 2012). The mortality from this type of hemorrhage is high, and 40% of the patients die within 30 days following the accident (Kissela et al., 2002). Smoking, hypertension and alcohol abuse are the most common cause of SAH. Other types of hemorrhage, epidural and subdural hemorrhage, are most commonly caused by traumatic brain injury (TBI).

2.1.3 Cerebral metabolism and ischemic stroke

The brain's metabolic requirement accounts for 20% of the total oxygen consumed by our body. The brain cannot store enough glucose substrate necessary to produce ATP for the maintenance of membrane potentials, ionic transport and transport of neurotransmitter. Thus, the brain needs a constant supply of oxygen and glucose in order to maintain functional and structural integrity.

In a normal adult brain, Cerebral Blood Flow (CBF) through the whole brain is approximately 46ml/100g/min (Lund Peter et al., 1993). CBF threshold for gray and white matter are about 75ml/100g/min and 45ml/100g/min respectively. CBF remains relatively constant despite moderate hypoperfusion through a mechanism of cerebral autoregulation. In the case of ischemia or hypoperfusion, the reduction in blood flow is compensated by the vasodilation of blood vessels, cumulative extraction of oxygen and glucose from the circulation. With the further fall in CBF below 15-20ml/100g/min, neuronal electrical activity cease, ATP declines, lactate level rises and pH declines. With further decline in CFB, potassium floods out of the cell, the intracellular level of calcium and sodium increases and the cell viability is lost (Hossmann, 1994, Markus, 2004). The amount of permanent damage depends on the degree and the duration of ischemia. The reduction in CBF is most severe in the central territory known as the core. However, the peripheral area is less severely affected due to additional flow provided by collateral circulation (Symon et al., 1976). Cells that are in the central area or the core are destined for death while those in the periphery or penumbra area are potentially salvageable by intervention (Astrup et al., 1981). In rodent models, when the blood flow is reduced to about 5-10%, cells in the core area die within a matter of minutes due to homeostatic failure, neuronal and glial depolarization and loss of synaptic transmission. Energy failure further leads to the accumulation of glutamate in the extracellular space. Elevated glutamate in synaptic terminals binds to NMDA and AMPA receptors in post synaptic neurons and results in excessive calcium influx into the postsynaptic neurons ultimately initiating ischemic cascades contributing to cell death (Dirnagl et al., 1999).

Cells in the penumbra area are functionally impaired but might recover if the biochemical processes leading to cell death are interrupted by therapeutic means. In the penumbra, CBF is reduced to about 10-20ml/100g/min (Ginsberg, 2003). In this state, electrical neuronal function does not exist but the levels of ATP are maintained to preserve cellular energy stores. Cells in the penumbra region can thus be rescued if the CBF is restored.

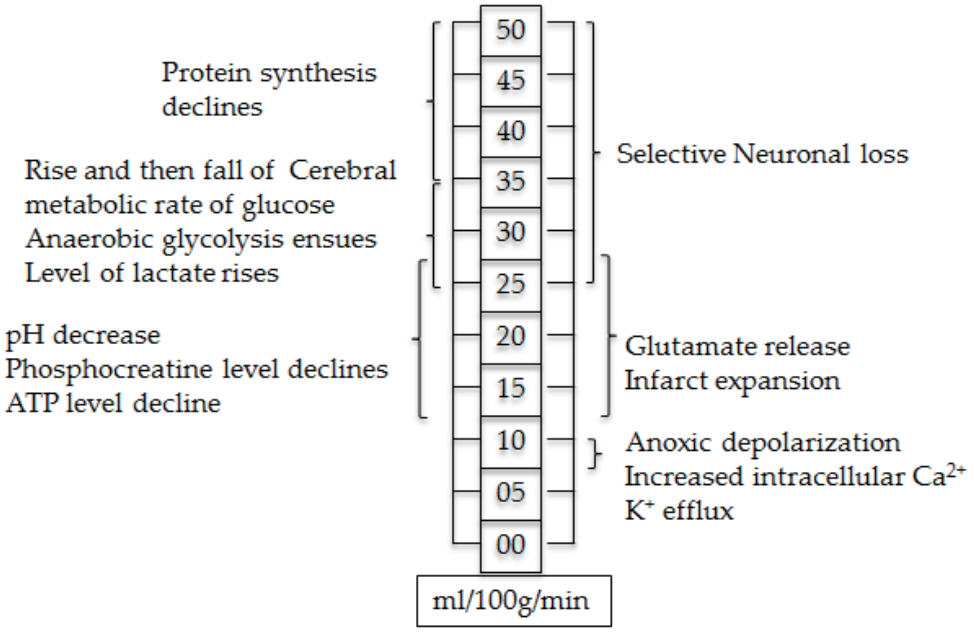

Figure 1: Ischemia thresholds for various metabolic and functional disturbance and formation of ischemic lesion. The values of CBF might vary between the animals. (Figure modified from Markus, 2004)

2.2 PATHOPHYSIOLOGY OF ISCHEMIC STROKE

This blockage of blood flow during ischemic stroke results in destruction and damage of the brain cells and is often accompanied by sensorimotor deficits and cognitive impairment in both clinical and preclinical subjects. Ischemia can be either of short duration with subsequent restoration of blood flow and hence minimal damage to the brain tissues or of long duration without restoration of blood flow and significant damage to brain tissues. Ischemic stroke is a cascade of complex and coordinated events and leads to cell death through either necrosis or apoptosis. Ischemic insult for a few minutes results in a dramatic reduction of blood flow and subsequent energy failures that are accompanied by ionic imbalance, acidosis, excitotoxicity, oxidative stress, cytotoxicity, blood brain barrier (BBB) disruption, glial cell activation and leukocyte infiltration. These series of events lead to acute, irreversible necrotic cell death at the core area which is characterized by cellular swelling, disruption and disintegration of cellular components and extrusion of cellular content into the extracellular space (Woodruff et al., 2011). In addition, an excitotoxic mechanism during the early phase of ischemia triggers a number of cellular and molecular events that contribute to delayed cell death through inflammation and apoptosis (Dirnagl et al., 1999).

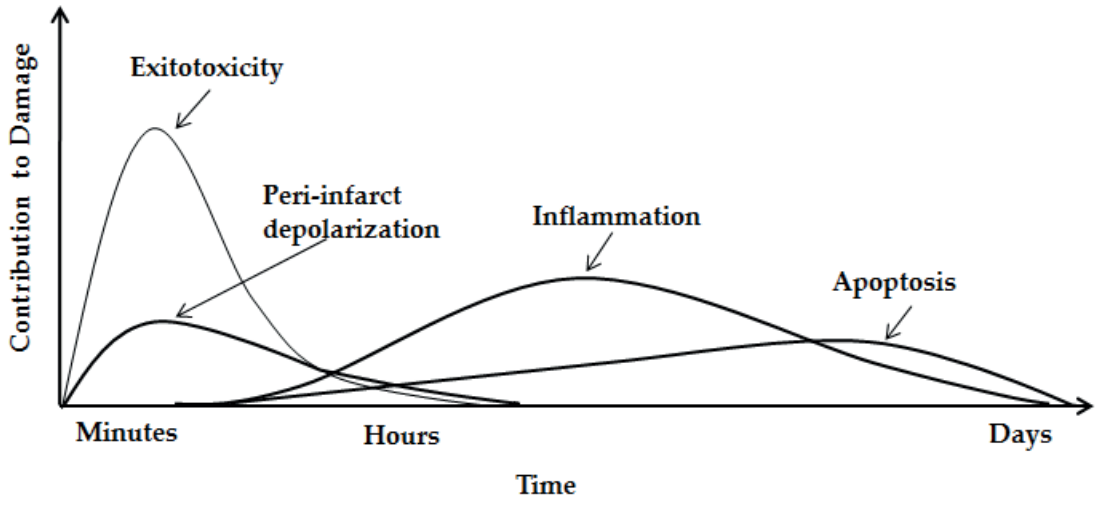

Figure 2: Acute and delayed damage following focal cerebral ischemia. Excitotoxic mechanisms can damage neurons and glial cells very early following the ischemic insults. Excitotoxicity can give rise to peri-infarct depolarization, inflammation and apoptosis that can lead to subsequent brain damage over the course of days. The X and Y axis represent the evolution of the cascade over time and the impact of individual elements of the cascade respectively. (Figure modified from Dirnagl *et al.* 1999).

2.2.1 Lactic acidosis

The brain needs a constant supply of ATP to maintain the ionic gradient across the cell and synaptic integrity. However, following ischemia, brain cells cannot produce ATP and the metabolism shifts towards anaerobic respiration that contributes to the rise in the level of lactic acid and hence a fall in pH (Combs et al., 1990). The increased amount of H^+ ions attracts water molecules inside the cells and causes swelling and edema. Even though lactic acidosis is not a sole contributor to injury following cerebral ischemia, it does contribute to the pathophysiology of ischemia and impairs post-ischemic outcome especially in hyperglycemic cases (Rehncrona et al., 1981, Siesjö et al., 1990). The levels of lactate are more in the penumbra region than the core due to residual blood flow. In addition, pre-ischemic hyperglycemia has been shown to further increase the lactate production resulting in deleterious effects in experimental animals (Folbergrova et al., 1992, Young et al., 1992).

2.2.2 Necrosis

Necrosis results in premature death of cells and is morphologically characterized by swelling of the cell and organelles, disruption of plasma membrane and loss of intracellular contents. (Kroemer et al., 2009). Necrosis, also known as mortification of tissues, results from extrinsic insults like hypoxia, ischemia, hypoglycemia, TBI, etc. Disruption of membrane structure and functional integrity, influx of water and Ca^{2+} , impairment of oxidative phosphorylation, depletion of high energy phosphate and subsequent dissolution of the cells are hallmarks of necrotic cell death (Martin et al., 1998). Necrotic cell death can be classified into edematous and homogenizing cell death. Edematous cell death is characterized by irregular clumping of chromatin and fragmentation of the endoplasmic reticulum (ER), Golgi apparatus and polysome. Ribosomes often accumulate around the nucleus. In this type of cell death, microtubules and other filamentous structure are absent, and cytoplasm is often clear (Lipton, 1999). Homogenizing cell change is accompanied by pronounced darkening and shrinkage of

the nucleus and cytoplasm with irregular chromatin clumping (pyknosis). The cells often assume triangular shape with strong acidophilic cytoplasm (Lipton, 1999).

Necrotic cell death has been widely described in both focal ischemia and global ischemia. Cornu Ammonis area 1 (CA1) neurons of the hippocampus in an animal model of global ischemia has been shown to exhibit morphological features of necrotic cell death (Colbourne et al., 1999, De Souza Pagnussat et al., 2007). In focal ischemia, necrosis accounts for acute cell death within the core area in contrast to apoptosis that primarily occurs in the penumbra area (Kametsu et al., 2003, Li et al., 1995). Metabolic changes leading to cell death by necrosis in focal ischemia involves excitotoxicity triggered by overactivation of glutamate receptors, intracellular calcium overload, and downstream activation of catabolic enzymes and increased production of free radicals (Snider et al., 1999). Oxidative stress is the key mediator of cell death in ischemia reperfusion injury. During this process, mitochondrial permeability transition pore (MPTP) opens in response to Ca^{2+} overload. Opening of the MPTP pore allows water, ions and large molecules to enter freely into the mitochondrial matrix and hence impairs the mitochondrial respiratory chain. The impairment in mitochondrial respiratory chain is followed by release of a large amount of ROS and subsequent necrotic cell death (Gouriou et al., 2011). Inflammation often accompanies necrosis due to cytoplasmic contents spilling into the extracellular space triggering the activation of inflammatory mediator cells (Festjens et al., 2006). In the case of cerebral ischemia, necrosis and apoptosis overlap and elements of both processes can occur within the same cell. Programmed necrosis or necroptosis has been shown to be initiated by death domain receptors including TNF receptor (TNFR)-1, Fas receptor (FasR) and TRAIL-Receptor (Kroemer et al., 2009). In addition, Toll like receptors (TLR)-3 and TLR4 also participate in necrosis (Kroemer et al., 2009).

2.2.3 Apoptosis

Apoptosis or programmed cell death is essential for embryonic development, normal cell turnover, and development and functioning of the immune system (Elmore, 2007). However, apoptosis also contributes to neuronal death in various neurodegenerative diseases including ischemic stroke, AD and Parkinson's disease (PD) (Elmore, 2007). During apoptosis, there is rounding-up of cells, chromatin condensation, nuclear fragmentation, reduction in cellular volume, plasma membrane blebbing and finally engulfment by phagocytic cells (Kroemer et al., 2009). Cell death by apoptosis occurs via several receptors or factors and involves two different mechanisms; caspase dependent pathways and caspase independent mechanism

Caspases are the family of structurally related cysteine protease enzymes consisting of a pentapeptide motif Gln-Ala-Cys-X-Gly where X is Arg, Gln or Gly (Earnshaw et al., 1999). These proteases cleave target proteins after an aspartate residue and irreversibly commit the cells to die (Earnshaw et al., 1999). Caspases are synthesized as inactive zymogens. These inactive zymogens contain a prodomain which when cleaved in presence of several apoptotic upstream signals results in two separate subunits of 10kD and 20kD that dimerize to form the active enzyme (Rotonda et al., 1996). Caspases can be classified into two different subfamilies, pro-apoptotic and non- apoptotic. In human, caspases 2, 3, 6, 7, 8, 9 and 10 are pro-apoptotic candidates and are involved in mediating cell death signaling. Non-apoptotic caspases include caspase 1, 4, 5 and 14 and are involved in cytokine maturation during inflammation (Pop and Salvesen, 2009). Alternatively, caspases can be classified as "initiator caspases" that are activated by oligomerization induced autoprocessing and "effector caspases" that are activated by initiator caspases and cleave a diverse array of cellular target. Initiator caspases include caspases 1, 2, 4, 5, 8, 9 and 10; and effector caspases includes caspases 3, 6 and 7 (Li and Yuan, 2008, Pop and Salvesen, 2009).

Depending on the origin of death stimuli, the caspase cascade can be activated by either intrinsic pathway involving mitochondria or extrinsic pathway involving death receptors (Danial and Korsmeyer, 2004). The intrinsic pathway mediated by mitochondria involves

release of several proteins into the cytoplasm from the intermembrane space of the mitochondria in response to apoptotic stimuli. Of the various proteins released into the cytoplasm, proapoptotic protein cytochrome c binds to ATP and activates apoptotic protease activating factor 1 (Apaf-1). Apaf-1 oligomerizes and recruits procaspase 9 to form apoptosome. Activated caspase 9 further cleaves procaspase 3 to generate active caspase 3 to induce apoptosis by cleavage of downstream mediators (Zou et al., 1997). The extrinsic or death receptor pathway involves binding of the death ligand like FasL to its receptor FasR and forming a death-inducing signaling complex (DISC) (Muzio et al., 1996). The DISC complex catalyzes the proteolytic cleavage of procaspase 8 to form caspase 8. The activated caspase 8 is then released from the DISC complex to initiate downstream cleavage of caspase 3 (Kischkel et al., 1995).

Another form of caspase independent apoptotic cell death has been documented in a number of studies in which proapoptotic protein like apoptosis inducing factor (AIF) and endonuclease G (Endo G) induces neuronal death due to increase intracellular Ca^{2+} concentration. Endo G and AIF are released from mitochondria in response to cellular stress and translocate to the nucleus where they promote DNA fragmentation and chromatin condensation (Joza et al., 2001, Li et al., 2001a).

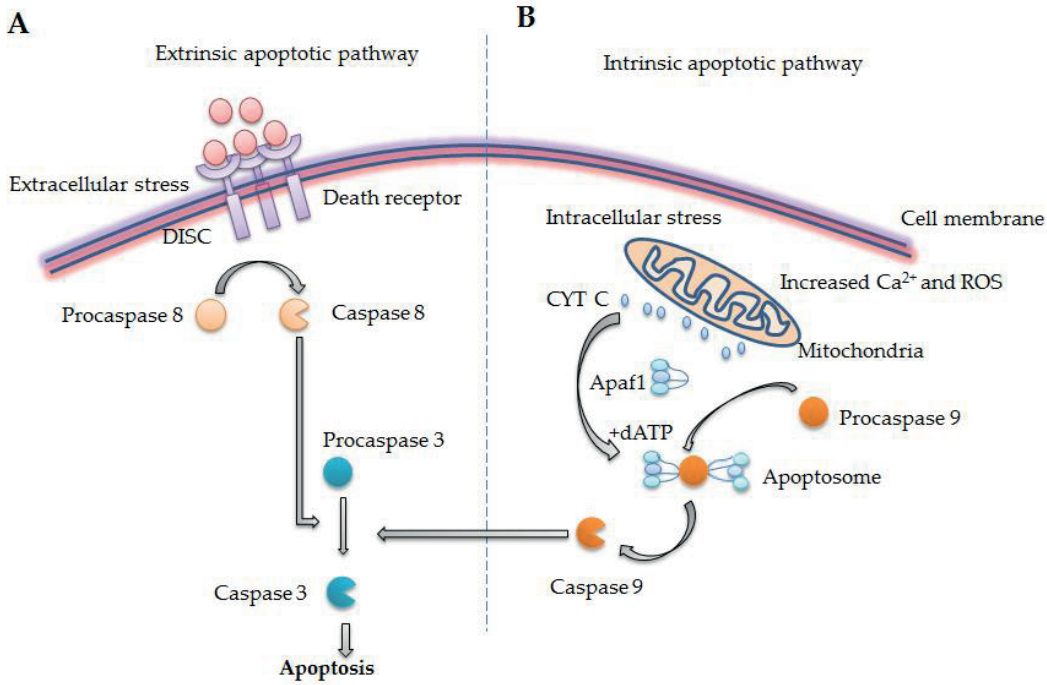

Figure 3: Activation of the caspase cascade by a) extrinsic pathway and b) intrinsic or mitochondrial pathways. The extrinsic or death receptor pathway is initiated at the plasma membrane whereas the intrinsic or mitochondrial pathway is triggered by intracellular stimuli. In both pathways, initiator caspases catalyze the activation of executioner caspase like caspase 3 ultimately leading to cell death by apoptosis. Figure modified from (Galluzzi *et al.* 2009).

In an animal model of focal ischemia, rapid depletion of energy leads to cytotoxic accumulation of intracellular Ca^{2+} that in turn initiates a series of events in both cytoplasm and nucleus ultimately leading to cell death. Focal ischemia promotes delayed neuronal death in the penumbra through FasR activation, mitochondrial release of cytochrome c, AIF and calpain in response to elevated intracellular Ca^{2+} and ROS thus leading to activation of caspase 3. Caspase

3 activation results in DNA fragmentation and cell death (Cho and Toledo-Pereyra, 2008, D'Orsi et al., 2012, Fujimura et al., 1998, Martin-Villalba et al., 1999, Namura et al., 1998).

2.2.4 Glutamate and excitotoxicity

Neurotransmitters such as glutamate and aspartate are vital for neuronal plasticity and are essential for normal neuronal function. Following ischemia, glutamate is released in excessive amounts into the extracellular space as a result of ionic pump failure as well as failure of reuptake mechanism which is an active energy dependent process (Choi and Rothman, 1990). This subsequent accumulation of glutamate leads to the prolonged activation of glutamate receptors in surrounding neurons enhancing the influx of Ca^{2+}, Na^{+} and water into the postsynaptic neurons. Excessive accumulation of Ca^{2+} in the postsynaptic neurons or calcium overload triggers various downstream processes that have a detrimental effects on cellular integrity through activation of proteases, lipases and nucleases (Ankarcrona et al., 1995, Lo et al., 2003). In addition, production of nitric oxide (NO), arachidonic acid and superoxides by enzymatic reaction of neuronal nitric oxide synthase (nNOS), phospholipase A2 and other calcium dependent enzyme further enhances the cell death (Moskowitz et al., 2010).

Glutamate receptors can be subdivided into two main groups, ionotropic and metabotropic glutamate receptors (mGluRs). Ionotropic glutamate receptors are directly coupled to ion channels. Unlike ionotropic glutamate receptors, mGluRs are coupled G protein and phospholipase C to produces inositol-1, 4, 5-triphosphate (IP3) and diacylglycerol both of which act as intracellular second messenger (Traynelis et al., 2010, Niswender and Conn, 2010). The directly coupled ionotropic receptors are of three different types: N-methyl-D-aspartate (NMDA) receptors, α-amino-3-hydroxy-5-methyl-isoxazolepropionate (AMPA) receptors and kainate receptors (Traynelis et al., 2010). The mGluRs are divided into 3 families (group I, II and III) consisting of eight different receptors (Niswender and Conn, 2010).

The NMDA receptors are glutamate gated ion channels that have an essential role in many neurological processes ranging from regulation of synaptic function, memory, cognition and learning (Carroll and Zukin, 2002, Mori and Mishina, 1995). In the brain, functional NMDA receptors are expressed in microglia, astrocytes and oligodendrocytes in addition to neurons. The NMDA receptors are composed of an NR1 subunit that combines with one or more NR2 subunits (NR2A-NR2D) and in some cases an NR3 subunit (Chen et al., 2008). Thus, the identity of the NR2 subunit determines biophysical and pharmacological properties of NMDA receptors that ultimately influence the downstream signaling.

AMPA receptors are also widespread in the CNS like NMDA receptors with fast synaptic transmission at excitatory synapses. AMPA receptors are composed of four subunits GluR1-GluR4 and are encoded by separate genes. These receptors are crucial for normal neuronal development, synaptic plasticity, and structural remodeling (Liu and Zukin, 2007). AMPA receptors devoid of GluR2 subunit typically present in spiny neurons are permeable to Ca^{2+} and can contribute to neuronal injury following ischemia (Szydlowska and Tymianski, 2010). In addition, ischemic events promote internalization AMPA receptors containing GluR2 subunit from the synaptic terminals and mobilize AMPA receptors lacking GluR2 to the synaptic site thus contributing to bigger damage (Liu et al., 2006).

Kainate receptors, also known as non-NMDA receptors, are difficult to distinguish from NMDA receptors. Kainate receptors are involved in synaptic plasticity (Lerma, 2003). They are localized in presynaptic and postsynaptic terminals of excitatory neurons. Subunits of kainate receptors bear resemblance to NMDA receptors in transmembrane topology and stoichiometry. Kainate receptors can be divided into two groups based on the affinity to kainic acids. High affinity KA1 and KA2 and low-affinity GluR5-7 are found to be involved in excitotoxic cell death (Collingridge et al., 2004).

Metabotropic glutamate receptors, mGluRs, are also important for synaptic plasticity in addition to contributing to excitotoxicity (Doyle et al., 2008). There are 8 subtypes of receptors

that are divided into three groups. Group I receptors (mGlu1 and mGlu5) are abundantly expressed in the postsynaptic membrane and modulate NMDA and AMPA receptors activity thus increasing neuronal excitability. Group II (mGlu2 and mGlu3) and group III (mGlu4, mGlu6, mGlu7, mGlu8) are found in presynaptic terminals of neurons as well as in astrocytes and are involved in inhibition of glutamate release at synaptic cleft (Bruno et al., 2001).

Both ionotropic and mGluR have been implicated in the pathophysiology of stroke. NMDA receptors are calcium permeable channels and further opening of these channels by glutamate leads to greater Ca^{2+} load contributing the excitotoxic cell death (Doyle et al., 2008). Likewise, Ca^{2+} impermeable AMPA receptors containing GluR2 is reduced with a concomitant increase of Ca^{2+} permeable AMPA receptor contributing to delayed cell death in hippocampal cell in response to global ischemia (Liu et al., 2006, Pellegrini-Giampietro et al., 1992). MGluR like mGluR1/5 acts via phospholipase C and IP3 and triggers the release of Ca^{2+} from intracellular stores like ER thereby triggering ER stress and apoptosis. The potential culprit, Ca^{2+}, in addition to activating various phospholipase, protease and nucleases can also trigger mitochondrial outer membrane depolarization and contribute to excitotoxic cell death (Green and Kroemer, 2004).

Given the plethora of effect of calcium toxicity in the preclinical settings, it has attracted numerous attentions to developing a therapeutic antagonist or drugs against glutamate receptors to treat stroke patients. Indeed, a number of drug targeting AMPA and NMDA receptors have undergone clinical trials but with no positive outcome (Ginsberg, 2008). This might be attributed to the fact that the NMDA-AMPA pathway is just part of a bigger picture given that glutamate independent calcium influx also ensues following ischemia. In addition, subunit composition of NMDA receptors also differentially regulates neuronal survival and death. Moreover, the drugs might have targeted almost all the receptors so an alternative approach should be chosen to target only the excessive channel opening. Thus, more in-depth knowledge about the diverse response following the receptor interaction and response of secondary messenger during ischemic stroke would provide a clue for successfully targeting excitotoxicity for the treatment of ischemic stroke (Moskowitz et al., 2010).

2.2.5 Oxidative and nitrosative stress

Cells can detoxify oxidants and free radicals that are produced during normal aerobic metabolism. However, during ischemia and other neurodegenerative events, ROS such as hydrogen peroxide (H_2O_2), superoxide anion (O_2^-) and hydroxyl radicals ($^{\cdot}OH$) are produced in overwhelming amounts through various mechanisms leading to oxidative stress. Mitochondria, for example, are the main producers of oxygen free radicals following ischemic injury due to high levels of calcium, sodium and adenosine diphosphate (ADP). The formation of MPTP following ischemia in response to Ca^{2+} overload leads to an oxygen-free radical burst. In addition, inflammatory processes involving synthesis of prostanoid and hypoxanthine degradation also produces oxygen radicals (Lo et al., 2003). Likewise, Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase also produces superoxide anions during NMDA receptor activation following ischemia devoid of mitochondrial origin (Girouard et al., 2009).

Brain have limited amount of antioxidant enzymes (superoxide dismutase (SOD), catalase and glutathione peroxidase) and antioxidant molecules (α-tocopherol, ascorbic acids and glutathione). Following neuronal injury, endogenous scavenging mechanism cannot detoxify excessive generation of free radicals. These excessive free radical thus damages lipids, proteins and nucleic acids (Chan, 2001). Free radicals inactivate and damage cellular proteins like ionic Ca^{2+} and Na^+ pumps, creatine kinase and mitochondrial dehydrogenases through oxidation of side chains and disulphide bonds. Nucleic acid damage by free radicals occurs through the breakage of single- and double-stranded DNA and chemical modification that link the protein to DNA (Mohr et al., 2011). In mitochondria, oxygen radicals and oxidative stress participate in the release of apoptosis-related protein like cytochrome c through the formation of MPTP and

thus triggers apoptosis (Kroemer and Reed, 2000). Beside cellular damage, free radicals also promote BBB damage through the activation of matrix metalloproteinase (MMP) and endothelial dysfunction (Brouns and De Deyn, 2009).

NO and related oxidation products are also key players in metabolic stress. NO, synthesized by nNOS in neurons, is an important mediator for NMDA mediated downstream signaling and synaptic plasticity. In addition, NO is involved in NMDA mediated excitotoxicity, inhibition of key mitochondrial enzymes, DNA damage and Poly ADP ribose polymerase (PARP) activation, etc. (Moskowitz et al., 2010). NO can combine to superoxide anions thereby forming perioxynitrite. Following ischemia, peroxynitrite can oxidize key mitochondrial enzymes like cytochrome c oxidase promoting the formation of a mitochondrial transition pore thus releasing AIF and causing the apoptosis of the cells. Peroxynitrite can produce hydroxyl radicals, the most reactive oxygen free radicals, and superoxide anions that are efficient in damaging the tissues in a very short time (Brouns and De Deyn, 2009, Kunz et al., 2007b).

The importance of oxidative and nitrosative stress in cerebral ischemia have been highlighted in a number of studies. In experimental stroke models, transgenic mice overexpressing SOD have been demonstrated to have smaller brain infarcts while those with reduced expression (SOD deficient mouse) have been found to have bigger ischemic damage (Fujimura et al., 1999, Kinouchi et al., 1991, Sheng et al., 1999). Likewise, nNOS and inducible nitric oxide synthase (iNOS) deficient mice are also found to exhibit reduced ischemic damage compared to their wild-type counterparts (Huang et al., 1994, Iadecola et al., 1997). Neuroprotection in cerebral ischemia have also been shown in a number of rodent studies that used agents with antioxidant properties like vitamin E, ebselen, tirilizad, etc. (McColl, 2004). Owing to the neuroprotective effect of antioxidants, a novel way of suppressing deleterious radicals without interfering their endogenous functions is critical for designing and testing the efficacy of antioxidant therapies (Moskowitz et al., 2010).

2.2.6 Inflammation

Inflammation is a biological response or defense mechanism of the immune system to harmful stimuli, such as pathogens, irritants or damaged cells in order to eliminate them and repair the surrounding tissues. Classically, inflammation is characterized by redness, swelling, heat and pain as well as loss of function in some cases. Inflammation can be divided into two types. Acute inflammation is the transient and early response of tissue to injury and is characterized by movement of leukocytes and plasma to the injured tissues (Medzhitov, 2008). Chronic inflammation is an inflammatory response of prolonged duration lasting for months to years due to the persistence of the causative stimulus like persistent infections, immune-mediated inflammatory disease and prolonged exposure to harmful toxins (Kumar et al., 2012, Medzhitov, 2008). Inflammation is mediated by complex sets of mechanisms that involve both the cellular and molecular components.

In cerebrovascular disease, acute inflammatory reaction occurs in the affected area for days to weeks post injury and contributes to the progression of the delayed brain damage with worsened neurological outcome. Following ischemic stroke, depletion of energy and increase in ROS induce the synthesis of several transcription factors, including nuclear factor-kappaβ (NF-kB), hypoxia inducible factors and Stat3, ultimately leading to the production of inflammatory cytokines and chemokines (Dirnagl et al., 1999). Likewise, expression and activation of adhesion molecules on the endothelial surface leads the early accumulation of inflammatory cells from the periphery into the ischemic brain. These blood borne inflammatory cells along with cells in the brain like microglia and astrocytes constitute the cellular components of inflammation. They together produce a variety of cytotoxic cytokines and chemokines which function as molecular mediators of inflammation contributing to the tissue damage (Emsley and Tyrrell, 2002, Wang et al., 2007)

2.2.6.1 Cellular components of inflammation

Following ischemia, inflammatory response is characterized by a rapid activation of the resident phagocytic microglial cells. This activation is followed by the subsequent infiltration of circulating inflammatory leukocytic cells including neutrophils, monocytes/macrophage and T cells (Jin et al., 2010). Recently, the role of astrocytes in mediating post-ischemic inflammation has also been addressed in many studies.

Leukocytes

Leukocytes have been demonstrated to be involved in a number of preclinical and clinical stroke studies (Kochanek and Hallenbeck, 1992). Leukocytes are produced and derived from multipotent hematopoietic stem cells and can be divided into granulocytes (polymorphonuclear leukocytes) and agranulocytes (mononuclear leukocytes). Granulocytes include neutrophils, basophils and eosinophils while agranulocytes include lymphocytes, monocytes and macrophages. Acute phase of ischemia is characterized by the production of cytokines like IL-1β, IL-6 and TNF-α, and chemokines including monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory proteins (MIP)-1α. These mediators induce the expression of the adhesion molecules such as intracellular cell adhesion molecule-1 (ICAM-1), E-selectin, P-selectin and integrins on the endothelial cells and leukocytes and promote the transmigration of circulating leukocytes (Amantea et al., 2009, Yilmaz and Granger, 2008). Infiltrating leukocytes have the capacity to release a wide array of cytokines and chemokines further activating resident cells as well as promoting even more infiltration of leukocytes thus leading to BBB damage, neuronal death and edema (Amantea et al., 2009, Jin et al., 2010).

Neutrophils are the first leukocytes to infiltrate into the brain in response to ischemia and may cause injury by secreting inflammatory mediators (Hallenbeck, 1996). They appear as early as 30 minutes to a few hours after the onset of ischemia with the peak at 24-72 hours and then diminish rapidly within 7 days in ischemic brain parenchyma of rodent models (Garcia et al., 1994, Jin et al., 2010, Kriz, 2006). However, in disagreement with current views, one study proposed the infiltration of other inflammatory cells, including macrophages, lymphocytes and dendritic cells prior to neutrophils (Gelderblom et al., 2009). The importance of neutrophil infiltration into the brain parenchyma have been demonstrated in a number of studies and inhibition of neutrophil infiltration has been linked with the significant reduction in brain damage (Wang et al., 2007).

Monocytes/macrophages are also the abundant immune cells that enter the brain after ischemia. Circulating monocytes are detected in capillaries of the ischemic area 4-6 hours after the onset (Garcia et al., 1994). Concomitantly, macrophages infiltrate to the core within 24 hours and persist for up to 14 days (Schroeter et al., 1994). Blood derived macrophages are found to be abundantly recruited into the ischemic area at day 3-7 post-stroke in contrast to resident microglial cells which are already activated at day 1 after ischemic onset (Jin et al., 2010).

Lymphocytes, especially T lymphocytes, recruit into the brain at the later stage of brain injury. T cells were found to infiltrate the border zone 3 days after an ischemic event and their infiltration further increased between days 3 and 7 (Jander et al., 1995, Stevens et al., 2002). However, other studies have shown that T cells accumulate in ischemic brain within 24 hours following ischemia/reperfusion injury (Jander et al., 1995, Schroeter et al., 1994). Like neutrophils, lymphocytes are also associated with an increase ischemic brain damage and attenuation of lymphocytes was found to reduce stroke induced cell death (Becker et al., 2001). Clinical studies have shown lymphocytes to be implicated in stroke recurrence and death (Nadareishvili et al., 2004).

Microglia

Microglial cells are the resident immunocompetent and phagocytic cells in the CNS that renew throughout adult lifetime. Microglia have a plethora of physiologically important functions in

the brain. They control the synapse number, remodel the developing brain and remove cellular debris (Patel et al., 2013). Microglia exist in several different morphological phenotypes and exhibit various stages of activation. However, three major morphological phenotypes are described in the literature: amoeboid (activated phagocytic state), ramified (resting) and intermediate forms (activated state) (Kettenmann et al., 2011). Resting ramified microglia comprise about 5-20% of the neuroglia cell population in the CNS and act together with astrocytes and neurons to maintain normal brain homeostasis (Hansson and Rönnbäck, 2003, Kim and de Vellis, 2005). Resting microglia are capable of extensive movement through their thin ramified processes and provide surveillance system in mature and uninjured adult brain (Hanisch and Kettenmann, 2007, Nimmerjahn et al., 2005). This population of cells is less common in white matter compared to gray matter and can alter their morphology according to the brain microenvironment (Lawson et al., 1993). In response to various insults, resting or surveying microglia undergo morphologic transformation to the activated amoeboid phagocytes and increase their expression of cell surface markers that make them virtually indistinguishable from peripheral macrophage (Yenari et al., 2010).

In response to the changing environment, activated microglia can undergo either classical activation (M1) or alternate activation (M2) and can release a wide variety of both cytotoxic and cytoprotective molecules. M1 is a proinflammatory state and is associated with upregulation of proinflammatory mediators, production of ROS and NO and secretion of proteolytic enzymes ultimately leading to destructive neuronal cell death (Kreutzberg, 1996, Patel et al., 2013, Yenari et al., 2010). Proinflammatory mediators released through M1 activation include interferon (IFN)-γ, IL-1β, TNFα and IL-6 (Hanisch, 2002, Patel et al., 2013). Likewise, proteases such as MMP9 and MMP3 can lead to BBB damage by damaging extracellular matrix.

In contrast, M2 state is characterized by resolution of inflammation and maintenance of homeostasis through release of anti-inflammatory mediators like IL-10, transforming growth factors-β (TGF-β), IL-4, IL-13, Insulin-like growth factor 1, etc. TGF-β with a special role in tissue development and immune response acts to reduce the proinflammatory mediators thus alleviating the injury (Hanisch, 2002). The shift in the activation of microglia from resting stage to the activated stage involves various stages including the replacement of the ramified branches with a new type of mortile protusions (Stence et al., 2001).

Upon ischemia, microglia are rapidly activated within hours and the activation persists for weeks. Highly ramified resting microglia predominate the penumbra area whereas amoeboid microglia are abundant in the ischemic core (Perego et al., 2011). Amoeboid microglia and their shortened retracted processes are clearly seen in the ischemic core area 24 hours after reperfusion following 60 minutes of transient middle cerebral artery (MCA) occlusion (MCAo) (Mabuchi et al., 2000, Morrison and Filosa, 2013). Microglial activation after ischemic stroke involves activation of a variety of receptors in microglia. Upon ischemia, purinergic receptors mainly $P2X_7$ and $P2X_{12}$ on microglia are upregulated in the ischemic area. Upregulation of $P2X_7$ have been shown to promote microglial proliferation in addition to production of superoxide and release of proinflammatory IL-1β and TNFα (Yenari et al., 2010). In contrast, $P2X_{12}$ receptor interact with integrin-β1 and is involved in microglial process extension and microglial migration towards the injury sites (Ohsawa et al., 2010). Microglia are also activated by stimulation of TLR, namely TLR-2 and TLR-4 leading to upregulation of several proinflammatory genes via NF-kB signaling. Endogenous ligands like purines, peroxyredoxin, heat shock proteins (HSP), high-mobility group box-1, etc. can act on TLRs and produce proinflammatory cytokines like IL-17, IL-33, IL-1β, TNFα and IL-6 (Patel et al., 2013, Yenari et al., 2010). Many experimental studies have demonstrated an adverse role of TLR-4 upon ischemia and a TLR-4 deficient mouse has been shown to have a robust neurological outcome following stroke (Becker et al., 2001, Nadareishvili et al., 2004, Perego et al., 2011). In addition, microglia expresses a wide variety of other receptors including cytokine receptors,

prostaglandin receptors and glutamates receptors. The activation of these receptors may ultimately influence the extent of brain damage following cerebral ischemia (Yenari et al., 2010).

Astrocytes

Astrocytes are the most abundant glial cells in the CNS and account for up to 50% of the human brain volume (Abbott et al., 2006). These cells contain 8-10 nm wide microfilaments composed of polymerized strands called glial fibrillary acidic protein (GFAP), a biomarker for astrocytes that can be visualized by immunohistochemistry. Astrocytes, mainly protoplasmic astrocytes, are endowed by extremely elaborated fine processes and are localized in gray matter. The processes of the protoplasmic astrocytes are in contact with the blood vessels to form perivascular endfeet in addition to forming multiple contacts with neurons. In contrast, fibrous astrocytes have less elaborate but long processes and are found in the white matter (Sofroniew and Vinters, 2010).

Astrocytes serve a variety of functions that are vital for brain development and physiology. They guide the migration of developing axons and are involved in the regulation of synaptogenesis (Barres, 2008, Powell and Geller, 1999). In addition, astrocytes provide structural support and form functional architecture for neurons in the CNS through scaffold formation. Astrocytes in particular form a glial-vascular interface and are involved in the regulation of cerebral microcirculation across the BBB. These astrocytes provide metabolic support to neurons by providing energy substrates without which the neurons will essentially die (Sofroniew and Vinters, 2010). Astrocytes also control the CNS microenvironment through regulation of extracellular ion concentrations, extracellular pH, removal of neurotransmitters from extracellular space and maintenance of brain water homeostasis (Verkhratsky and Butt, 2007). A growing number of evidence also support the notion that astrocytes are also involved in the synaptic signaling via intracellular calcium wave (Volterra and Meldolesi, 2005).

Astrocytes are affected by ischemic injury very rapidly. Failure of energy driven pumps on astrocytes impair their ability to uptake glutamate that can subsequently lead to excitotoxic cell death and infarct expansion. In addition, reduced potassium spatial buffering also initiates a wave of peri-infarct depolarization further increasing the ischemic injury (Anderson and Swanson, 2000, Nedergaard and Hansen, 1993). Following neuroinjury, astrocytes can produce and respond to a broad array of cytokines including IL-1, -4, -6, - 10 and -12, IFN-α,-β and $-\gamma$, colony stimulating factors like granulocyte macrophage-colony stimulating factor, macrophage-colony stimulating factor and granulocyte-colony stimulating factor (G-CSF), TNF-α, TGF-β. In addition, they also produce chemokines such as regulated on activation, normal T cells expressed and secreted (RANTES), IL-8, MCP-1, and IFN-Υ induced protein 10 (IP-10) (Benveniste, 1998). Following ischemia, astrocytes undergo proliferation, change in morphology and increase their GFAP expression, collectively known as astrogliosis (Hatten et al., 1991). Astrogliosis can affect the injured tissue in both positive and negative ways. It can promote neuronal survival through the secretion of growth factors and neurotrophins. Astrocytes can also protect the compromised tissue from further damage by taking up excess glutamate, rebuilding the blood brain barrier and providing essential metabolic support (Barreto et al., 2011, del Zoppo, 2009, Sofroniew and Vinters, 2010). In contrast, astrocytes can inhibit neuronal survival and function through formation of a physical barrier known as an astroglial scar in the affected area. Following ischemia, the astrocytic gap junctions can open leading to the influx of proapoptotic factors through syncytium ultimately expanding the infarct size (Lin et al., 1998). Production of high levels of proinflammatory cytokines can be detrimental to ischemic recovery through apoptosis of neurons and inhibition of neurogenesis (Barreto et al., 2011).

Astrocytes are almost dead in the ischemic core area whereas in the penumbra or peri-infarct area viable astrocytes undergo reactive astrogliosis. Astroglial activation can be seen within hours after ischemic insults rapidly peaking within 24 hours (Chen et al., 1993, Raivich et al.,

1999). Taken together, these data suggest an important role of astrocytes in the pathophysiology of ischemic stroke and other neurodegenerative diseases.

2.2.6.2 Molecular components of inflammation

During the course of ischemic stroke and other neurodegenerative diseases, a wide array of inflammatory molecules is secreted by various signaling that can ultimately affect the outcome after the injury. Several cytokines, chemokines and inflammatory proteins are produced during the time course of an ischemic cascade and these are described hereunder.

2.2.6.2.1 Cytokines

Cytokines are a group of low molecular weight glycoproteins that are produced in response to an antigen and thus regulate both innate and adaptive immune responses. Cytokines are essential for the regulation of normal cellular functions during development in addition to maintaining homeostasis during infection, inflammation and traumatic and ischemic injury (Holloway et al., 2002). In the periphery, cytokines are secreted by activated macrophages, monocytes, endothelial cells, platelets and other cell types. However, in the CNS, cytokines are also expressed by resident neurons and glial cells in addition to being expressed by the cells of the immune system (Barone and Feuerstein, 1999). Peripheral immune cells like T lymphocytes, natural killer (NK) cells and polymorphonuclear leukocytes produce a variety of cytokines that can also contribute to the inflammation of the CNS thus affecting the clinical outcome (Ferrarese et al., 1999). Following cerebral ischemia, proinflammatory cytokines like IL-1β, TNF-α and IL-6 aggravate the inflammatory response. In contrast, anti-inflammatory cytokines like TGF-β, IL-10 and IL-1 receptor antagonist (IL-1ra) inhibit the expression of proinflammatory cytokines and reduce inflammation (Allan et al., 2005, Lakhan et al., 2009, Wang et al., 2007). Other cytokines are also involved in tissue damage and repair; however, the effects of these cytokines are less pronounced.

Interleukin 1 (IL-1)

The IL-1 family comprises of a group of 11 cytokines and is closely linked to innate immune responses. All IL-1 proteins except IL-1ra are synthesized as a precursor protein that is enzymatically cleaved to the mature forms by specific cellular proteases. Of these cytokines, IL-1α, IL-1β and IL-1ra are extensively studied in experimental stroke (Dinarello, 2009).

IL-1α is a dual-function cytokine as it takes part in extracellular receptor mediated effect characteristics of classical cytokines as well as its role in the nucleus (Cohen et al., 2010). IL-1α precursor protein is proteolytically cleaved by to its mature form by calcium activated cysteine protease, calpain. Biologically active precursor IL-1α are constitutively expressed in cell membrane of different cell types and can contributes to inflammatory properties of necrotic cells in response to insults (Werman et al., 2004).

IL-1β is metabolically inactive and is converted to mature active IL-1β by an enzyme called IL-1β converting enzyme (Cerretti et al., 1992). IL-1β is expressed at very low levels in the brain. However, in response to insult or injury, IL-1β is dramatically upregulated for several days (Allan and Rothwell, 2001). Following ischemia, elevation of IL-1β mRNA is observed within 15-30 minutes followed by increased levels of IL-1β protein in the next few hours (Buttini et al., 1994, Davies et al., 1999). Biphasic expression of IL-1β is also observed at 1 hours and 6-24 hours after reperfusion in transient global cerebral ischemia in the rat (Haqqani et al., 2005). Activated microglia, astrocytes, neurons and endothelial cells account for the early production of IL-1β while the late production is accompanied by the influx of inflammatory cells into the CNS (Ceulemans et al., 2010, Legos et al., 2000, Rothwell and Luheshi, 2000). The toxic effect of IL-1β on brain damage was shown after the intraventricular administration of recombinant IL-1β after MCAo in rat (Yamasaki et al., 1995b). Even though IL-1β has been found to have a deleterious effect on cerebral ischemia; studies using knockout and transgenic

animals show how the situation is far more complex. For example, mice deficient in both IL-1α and IL-1β exhibited significantly attenuated ischemic damage after 30 minutes of transient occlusion whereas deficiency in either IL-1α or IL-1β had no effect on infarct size. This might be attributed to the compensatory mechanism of the IL-1 system (Boutin et al., 2001). IL-1β contributes to the neuronal loss through production and expression of other proinflammatory mediators in addition to stimulating its own production. In addition, it also contributes to the activation and proliferation of glial cells, stimulation of calcium influx into neurons and priming of endothelial cells for leukocyte adherence. Moreover, induction of IL-1β will also lead to increase level of IL-1ra that serves as an antagonist to IL-1β suggesting there is a balance between the level of IL-1β and IL-1ra following ischemia (Ceulemans et al., 2010). Likewise, IL-1β also elevates the levels of IL-6 that is involved in worse neurological outcome and aggravated brain damage (Acalovschi et al., 2003, Vila et al., 2000).

IL-1ra is an endogenous inhibitor for both IL-1α and IL-1β without any known agonist activity in the periphery and brain (Rothwell, 1999). IL-1ra increases right after brain damage in rodents. Generally, expression of IL-1ra peaks after 30-60 minutes following IL-1β expression mainly in neurons (Loddick et al., 1997, Toulmond and Rothwell, 1995). In animal models of permanent and transient MCAo, administration of IL-1ra has been shown to reduce the infarct size, edema, glial activation and neuronal loss and improve neurological outcome (Banwell et al., 2009, Rothwell, 1999). In contrast, a neutralizing antibody to IL-1ra has been shown to increase brain damage (Deb et al., 2010). Delayed administration of IL-1ra after 3 hours following 60 min occlusion of MCA was still significantly reducing the damage volume in rat (Mulcahy et al., 2003). Even though IL-1α and IL-1β are upregulated after the ischemic insults, the primary targets for IL-1ra might be IL-1β since of these two IL-1β is predominantly upregulated early after brain ischemia (Rothwell, 1999). IL-1ra has garnered a lot of attention since methionylated form of human IL-1ra was proven to be safe and effective in improving the neurological outcomes in patients with cortical infarct (Emsley et al., 2005).

Tumor Necrosis factor-α (TNF-α)

Tumor necrosis factor is a pleiotropic inflammatory cytokine with both neurotoxic and neuroprotective effects and is rapidly upregulated in response to CNS injury and inflammation (Pan and Kastin, 2007). TNF-α exerts its effects by binding to two different high affinity receptors, TNFR1 and TNFR2. TNF-α activates a number of other secondary proteins including transcription factors like NF-kB, activator protein 1 (AP-1), protein kinases, phospholipases, mitochondrial proteins and caspases to elicit the inflammatory process (Idriss and Naismith, 2000). For example, TNF-α upon binding to TNFR can recruit TNF-α receptor associated death domain (TRADD) that serves as a platform for additional protein binding. Binding of Fas associated death domain to TRADD can activate caspases leading to cell death. In addition, TRADD can recruit TNF-α receptor associated factors (TRAFs) like TRAF-1 and TRAF-2 that can lead to activation of NF-kB. Especially, TRAF-2 is involved in transcriptional regulation of NF-kB. NF-kB can in turn regulate the expression of several pro and anti-inflammatory cytokines. Likewise, Mitogen-associated protein kinases (MAPKs) like JNKs and p38 kinases are also activated. These kinases lead to the activation of transcription factor AP-1 that is involved in several inflammatory processes (Chen and Goeddel, 2002, Gaur and Aggarwal, 2003, Wajant et al., 2003).

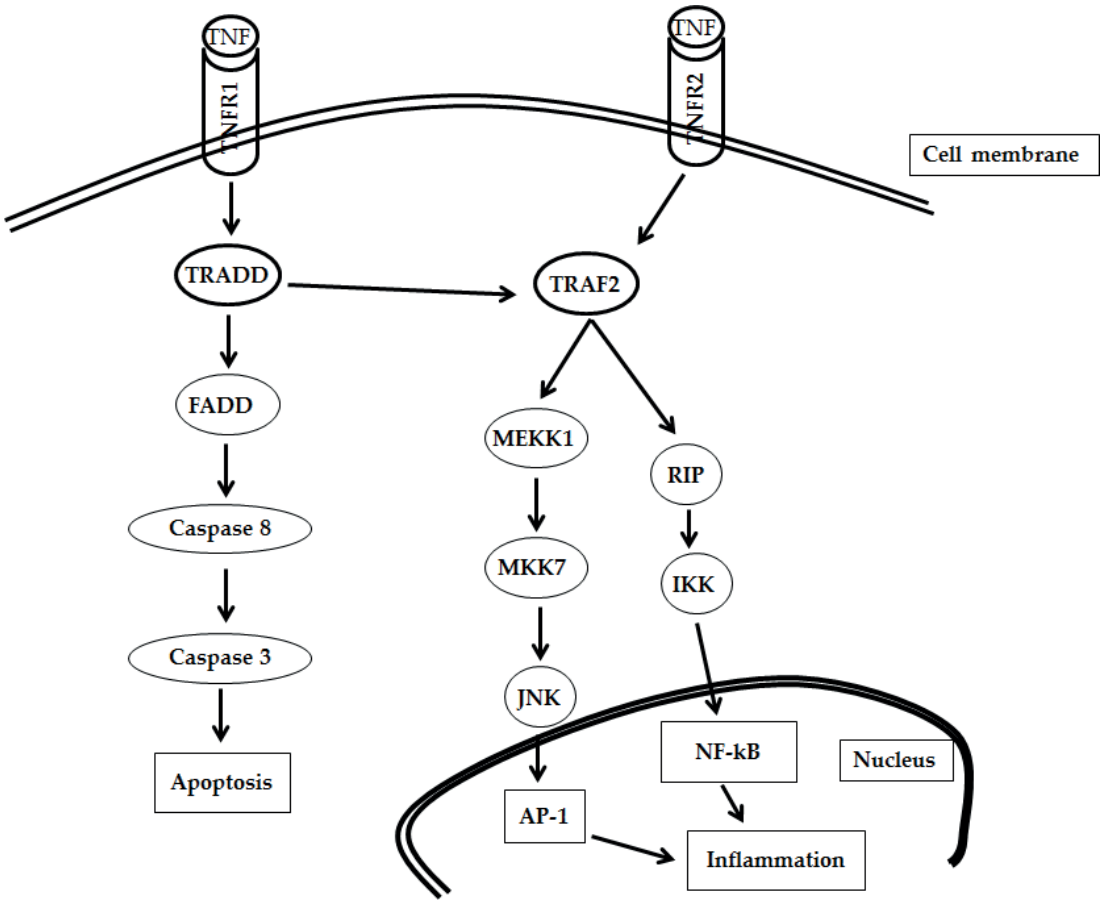

Figure 4: TNF signaling pathways. TNFR1 recruits and forms a complex with TRADD. More adapter molecules are then assembled on this platform where they initiate downstream events ultimately leading to inflammation and apoptosis. Figure modified from (Aggarwal, 2003).

In addition to IL-1β, TNF-α also has a biphasic release pattern following ischemia, first peak at 1-3 hours and a second peak at 24 hours (Wang et al., 2007). TNF-α is expressed by both neurons and glia (Liu et al., 1994, Uno et al., 1997). The expression of TNF-α in neurons occurs earlier than in glial cells. A detrimental role of TNF-α has been documented in many studies where it contributes to neuroinflammation and neurodegeneration following brain injury. Inhibition of TNF-α has been shown to reduce brain injury while administration of recombinant TNF-α has been shown to exacerbate brain injury (Barone et al., 1997, Yang et al., 1998a). TNF-α promotes activation and proliferation of glial cells thus upregulating its own production and production of other neurotoxic mediators (Ceulemans et al., 2010). However, other studies have reported the protective effects of TNF-α and deletion of TNFR appears to have larger infarcts (Bruce et al., 1996). This inconsistency can be attributed due to different signaling pathways induced by binding of TNF-α to its receptors. Binding of the soluble form of TNF-α to TNFR1 can activate both pro and anti-inflammatory signaling via death receptors and can be a turning point for cell death or cell survival (Wang et al., 2007). Alternatively, membrane bound TNF-α that binds to TNFR-2 might be responsible for neuroprotection following injury (Ceulemans et al., 2010). Thus, neuroprotective and neurotoxic effects depend on the extent of glial cell activation, soluble and membrane forms of TNF-α signaling as well as the receptors that are activated in question.

Interleukin 6 (IL-6)
IL-6 is another proinflammatory cytokine that is upregulated upon ischemia. IL-6 is detected as early as 4 hours following stroke and remains elevated in neurons and microglia in ischemic penumbra up to 14 days post-stroke (Block et al., 2000, Ceulemans et al., 2010, Suzuki et al., 1999). Even though IL-6 contributes to inflammation, IL-6 deficient mice don't show exacerbated lesions and neurological deficits compared to their wild type (WT) counterparts (Clark et al., 2000). This suggests that IL-6 has no influence in acute ischemic injury. However, there are other studies reporting either the beneficial or deleterious role of IL-6 (Clark et al., 2000, Herrmann et al., 2003, Loddick et al., 1998, Matsuda et al., 1996). Following acute ischemic injury, levels of IL-6 are also upregulated in peripheral circulation and several studies have reported the association between peripheral levels of IL-6 and infarct volume (Acalovschi et al., 2003, Fassbender et al., 1994, Smith et al., 2004, Vila et al., 2000). So, IL6 is often suggested as a good peripheral marker in patients with acute ischemic stroke (Smith et al., 2004).

Transforming growth factor-β (TGF-β)
TGF-β is an anti-inflammatory cytokine that is involved in a variety of cellular processes ranging from apoptosis, cell proliferation and differentiation to initiation and resolution of inflammation (Buckwalter and Wyss-Coray, 2004). In the CNS, three isoforms of TGF-β (TGF-β1, TGF-β2 and TGF-β3) are widely expressed in neurons and glial cells. Specifically, TGF-β1 is primarily produced by activated microglia with some production occurring in neurons as well. In contrast, TGF-β2 is primarily secreted by astrocytes and neurons (Ceulemans et al., 2010). Following ischemia, TGF-β can have both neuroprotective and neurotoxic effects. It can protect neurons by reducing glial activation and hence expression of cytokines, suppressing oxidative and nitrosative stress, promoting angiogenesis and reducing neutrophil adherence to endothelial cells (Pantoni et al., 1998). On the other hand, TGF-β can also promote glial scarring and fibrosis (Buckwalter and Wyss-Coray, 2004).

Following stroke, increased expression of TGF-β1 mRNA and its protein have been observed in both human and rodent subjects (Krupinski et al., 1996, Yamashita et al., 1999). Several studies have suggested a link between increased levels of TGF-β1 and neuroprotection after cerebral ischemia (Ma et al., 2008, Pang et al., 2001, Wang et al., 1995b) while inhibition of TGF-β1 leads to exacerbated brain damage (Ruocco et al., 1999). Since TGF-β expression mostly occurs in the recovery phase of some CNS diseases, it is suggested to have a protective function, and targeted overexpression of TGF-β1 might be a candidate for neuroprotection in stroke.

Interleukin 10 (IL-10)
IL-10 is another constitutively expressed anti-inflammatory cytokine that is produced mainly by microglia and astrocytes. IL-10 can inhibit cytokines like IL-1 and TNF-α and their receptors thus providing neuroprotection after stroke. IL-10 can also promote cell survival by inhibiting both ligand and mitochondrial induced apoptotic pathways (Strle et al., 2001). Administrations, as well as gene transfer of IL-10, have been shown to reduce brain damage following brain ischemia (Ooboshi et al., 2005, Spera et al., 1998).

2.2.6.2.2 Chemokines
Chemokines also known as chemotactic cytokines are small inducible molecules that are structurally and functionally related to cytokines. Chemokines are 8-10 KDa molecules and are classified into four main subfamilies: CXC, CC, CX3C and XC (Mélik-Parsadaniantz and Rostène, 2008) and include more than 40 different members (Mennicken et al., 1999). Even though the majority of cytokines promote inflammation and adhesion between leukocytes and endothelial cells, they are poor attractants for guiding leukocytes and monocytes to the brain parenchyma (Pantoni et al., 1998). Thus, chemokines come into play to attract these inflammatory cells and guide their migration into the brain parenchyma. In addition to their

chemotactic properties, chemokines also participate in cytotoxicity, tumor cell growth, degranulation, T-cell activation, cell migration, apoptosis and cellular adhesion (Mélik-Parsadaniantz and Rostène, 2008, Mennicken et al., 1999). Chemokines and their receptors are constitutively expressed in astrocytes, microglia and neurons and are upregulated in response to various neurodegenerative diseases and disorders (Luster, 1998). Chemokines bind to seven specific transmembrane domains and exert their effects through activation of G-protein and subsequently intracellular kinases (Baggiolini et al., 1997).

Cytokines like IL-1α, IL-1β and TNF-α stimulate the production and secretion of chemokines following injury mediated inflammation. Chemokines, including cytokines induced neutrophil chemoattractant (CINC), monocyte chemoattractant protein (MCP), MIP, IL-8 and RANTES are expressed following cerebral ischemia and are thought to have deleterious effects by increasing leukocyte infiltration (Lakhan et al., 2009, Mennicken et al., 1999).

Monocyte chemoattractant protein-1 (MCP-1)

MCP-1, also known as chemokine ligand (CCL)2, is a member of the CC chemokine subfamily and is involved in the recruitment of monocytes to the site of injury and inflammation (Fuentes et al., 1995). MCP-1 is rapidly induced in neurons and glial cells following ischemia and is thought to exacerbate brain injury (Mélik-Parsadaniantz and Rostène, 2008). MCP-1 mRNA expression was shown to increase in the ischemic cortex at 6 hours followed by a peak at day 2 after transient MCAo in models (Wang et al., 1995a, Yamagami et al., 1999). Blocking the activity of MCP-1 with antibodies or using MCP-1 deficient mice in transient and permanent MCAo are associated with smaller infarct size demonstrating that MCP-1 is involved in cerebral injury (Hughes et al., 2002, Kumai et al., 2004, Ono et al., 1999). In contrast, upregulation of MCP-1 was shown to have larger infarcts and increased infiltration of monocytes and macrophages to the ischemic core (Chen et al., 2003). MCP-1 also contributes to the migration of bone marrow stromal cells into the ischemic brain (Wang et al., 2002). In addition, MCP-1 promotes the migration of neuroblast from ventricular region to the injury area suggesting its role in neurogenesis (Yan et al., 2007). Thus, MCP-1 might be deleterious in the early stage after stroke while beneficial at later stages.

Macrophage inflammatory protein (MIP-1α and MIP-1β)

MIP-1α, also known as CCL3, is involved in microglial and monocyte infiltration into the injured brain parenchyma. Increased expression of MIP-1α mRNA was observed at 6 hours and persisted for up to 48 hours following MCAo in rats (Kim et al., 1995). Microglia/macrophages were found to be the main sources of MIP-1α (Takami et al., 1997). MIP-1α can also mediate neutrophil infiltration into the injured brain through the release of other inflammatory mediators like leukotrienes (Reichel et al., 2009). MIP-1β or CCL4 binds to C-C chemokine receptor (CCR)5 receptors and induces the recruitment of monocytes to inflammatory sites (Mirabelli-Badenier et al., 2011).

Regulated on activation, normal T-cell expressed and secreted (RANTES)

RANTES, also known as CCL5, binds to three different transmembrane chemokine receptors (CCR1, CCR3 and CCR5) and plays an active role in recruiting leukocytes into the inflammatory sites (Mirabelli-Badenier et al., 2011). RANTES is produced by a variety of cells including T lymphocytes, endothelial cells and glial cells that can ultimately contribute to the pathogenesis of stroke (Terao et al., 2008). Mice deficient in RANTES have significantly smaller infarcts and decreased BBB permeability in focal cerebral ischemia highlighting the deleterious role of blood cell derived RANTES in cerebral ischemia and reperfusion (Terao et al., 2008). In contrast, another recent study showed that mice deficient in CCR5, the major receptor of RANTES, have

larger ischemic brain damage when compared to wild-type counterparts suggesting the neuroprotective role of CCR5 and other CCR5 ligands.

Interleukin 8 (IL-8)
IL-8, also known as Chemokine (C-X-C motif) Ligand (CXCL)-8, is reported to be present in humans but not in rodents. Rodents have a similar CINC, which is equivalent to human IL-8. In the rabbit, IL-8 was shown to be produced in the brain, and the anti-IL-8 antibody was associated with reduced infarct size and brain edema (Matsumoto et al., 1997). The levels of CNIC in brain and blood was found to precede and correlate with brain edema and cerebral neutrophil infiltration during early reperfusion in the murine model (Yamasaki et al., 1995a). Concurrently, inhibition of CINC with neutralizing antibodies reduces ischemic brain damage in transient MCAo models when administered 24 hours before and immediately after reperfusion (Yamasaki et al., 1997)

2.2.6.2.3 Adhesion molecules
The recruitment of leukocytes and platelets in the cerebral microvasculature following cerebral ischemia and other inflammatory processes involves different adhesion molecules that are expressed on vascular endothelial cells and circulating cells. Selectins, integrins and cellular adhesion molecules (CAMs) are the adhesion molecules that participate in the transmigration of inflammatory cells into the brain parenchyma from the blood vessels (Ceulemans et al., 2010). During this process, the leukocytes roll on the endothelial surface in a process mediated by E- and P selectins. This rolling is followed by adhesion which is mediated by interaction of β-integrins on leukocytes with ICAM-1 on endothelial cells (Yilmaz and Granger, 2008). Following ischemic stroke, upregulation in the levels of P-selectin occur as early as 15 min while E-selection upregulation is induced within 2 hours after the onset of ischemia (Zhang et al., 1998). Vascular upregulation of P- and E-selectins has been demonstrated to promote an inflammatory response and exacerbate brain injury following ischemic stroke (Wang et al., 2007). Consequently, overexpression of P-selectin exacerbates brain infarcts while inhibition of P- and E-selectins leads to improved neurological outcome (Huang et al., 2000, Mocco et al., 2002).

Among the cellular adhesion molecules, vascular cell adhesion molecule (VCAM-1) and ICAM-1 are widely reported in ischemic stroke. ICAM-1 expression is increased within hours after stroke in both primates and human subjects upon stimulation by cytokines such as IL-1β and TNF-α (Lindsberg et al., 1996, Okada et al., 1994). ICAM-1 also contributes to a proinflammatory status following cerebral ischemia and blockage or inhibition of ICAM-1 was associated with improved outcome following stroke (Wang et al., 2007). Likewise, ICAM-1 deficient mice were shown to have a smaller infarct compared to WT mice (Connolly et al., 1996, Kitagawa et al., 1998). Integrins are transmembrane cell surface proteins found in leukocytes and are activated by various chemokines and cytokines. Binding of leukocytes to the endothelium is mediated by CD18 or β2 integrins after stroke and inhibition of β2 integrins that include three subclasses is neuroprotective in stroke (Wang et al., 2007).

2.2.6.3 Inflammatory and other enzymes
Cerebral ischemia is involved in the upregulation of various pro- and anti-inflammatory mediators and proteins that ultimately influence the outcome after stroke. In addition, upregulation of various enzymes including cyclooxygenase (COX)-2, nitric oxide synthase (NOS) and MMPs are also observed during the course of ischemia and contribute to ischemic brain damage.

2.2.6.3.1 Cyclooxygenase

Cyclooxygenase is an integral glycoprotein involved in normal pathological processes such as vascular function, wound healing and renal maintenance (Nurmi, 2004). It is the rate-limiting enzyme for the synthesis of prostanoids like prostaglandins and thromboxanes from arachidonic acid substrates. Two major isozymes known to catalyze the conversion of arachidonic acid to its end products are COX-1 and COX-2. COX-1 is constitutively expressed in virtually all types of cells and produces physiological levels of prostanoids (Vane et al., 1998). COX-2 is the product of immediate early response gene and is rapidly induced upon various stimuli, such as growth factors, hypoxia and inflammatory mediators (Andreasson, 2010, Seibert et al., 1995).

In the brain, controversial data exist for the role of COX-1 in cerebral ischemia. One study showed that mice deficient in COX-1 were more vulnerable to ischemic brain damage supporting a protective role for COX-1 in MCAo (Iadecola et al., 2001). On the other hand, in transient global ischemia, COX-1 was found to be involved in the delayed neuronal damage of hippocampal CA1 neurons (Candelario-Jalil et al., 2003).

The role of COX-2 and its participation in the progression of cerebral ischemic damage have been demonstrated in a number of studies (Iadecola et al., 2001, Koistinaho et al., 1999, Nagayama et al., 1999, Nogawa et al., 1997). In rodent models of cerebral ischemia, COX-2 mRNA and proteins are upregulated in neurons, microglia and vascular cells 12-24 hours after injury (Miettinen et al., 1997, Nogawa et al., 1997). In addition, COX-2 activation has been demonstrated in the human brain after ischemic stroke (Iadecola et al., 1999, Sairanen et al., 1998). Inhibition of COX-2 has been shown to improve neurological outcome in several studies whereas its overexpression is associated with exacerbated brain damage (Doré et al., 2003, Nogawa et al., 1997, Sugimoto and Iadecola, 2003). It is suggested that the deleterious effects of COX-2 are associated with the production of superoxides and prostanoids (Candelario-Jalil and Fiebich, 2008). However, other reports have concluded that prostanoids and not ROS contribute to the COX-2 dependent neurotoxicity (Kawano et al., 2006, Kunz et al., 2007a, Manabe et al., 2004). Specifically, the interaction of prostaglandin E2 with its receptor prostaglandin E1 was found to contribute to neurotoxicity through Ca^{2+} dysregulation and suppression of protein kinase B (Akt) (Kawano et al., 2006, Zhou et al., 2008).

2.2.6.3.2 Matrix Metalloproteinases

MMPs are the proteases that are responsible for degradation as well as remodeling of the extracellular matrix. MMPs are tightly regulated and secreted as inactive enzymes and are cleaved by other proteases and free radicals to yield their activated state (Rosenberg, 2002). MMPs are nearly undetectable in the brain under normal physiologic conditions. However, following brain injury, MMPs are expressed in astrocytes, microglia, neurons and endothelial cells (Lakhan et al., 2009, Montaner et al., 2001). Inhibition of MMPs has been shown to reduce BBB damage and thus ischemia induced infarct size, brain edema and hemorrhage transformation (Pfefferkorn and Rosenberg, 2003, Ramos-Fernandez et al., 2011). Even though both MMP-2 (gelatinase A) and MMP-9 (gelatinase B) are implicated in cerebral ischemia, mice deficient in MMP-9 but not MMP-2 have been shown to develop smaller infarcts compared to wild-type mice. Thus, MMP-9 plays a more detrimental and prominent role in cerebral ischemia (Asahi et al., 2000, Asahi et al., 2001). Rather than brain cell-derived MMP-9, peripheral inflammatory leukocyte-derived MMP-9 was shown to contribute to ischemic brain damage following transient focal cerebral ischemia (Gidday et al., 2005). MMP-9 can disrupt the BBB by degrading tight junction proteins and basal membrane proteins like fibronectin, laminin and collagen (Yamashita and Abe, 2011). However, in later phases of ischemia, MMPs are involved in plasticity, recovery and repair through their association with vascular endothelial growth factor, a known growth factor for regulating angiogenesis (Zhao et al., 2006). In addition, delayed appearance of MMPs facilitates the migration of macrophages into the ischemic area in

order to clear the cellular debris (Romanic et al., 1998). MMP-9 in plasma have been suggested to predict the volume of infarcted tissue, stroke severity and functional outcome following acute cerebral ischemia (Ramos-Fernandez et al., 2011)

2.2.6.3.3 Nitric oxide synthase

NO is a potent dilator of cerebral vessel and is produced during the enzymatic conversion of L-arginine to L-citrulline by NOS. There are three subtypes of NOS: nNOS, iNOS and endothelial NOS (eNOS) (Moncada et al., 1991). Neuronal NOS and eNOS are constitutively expressed in blood vessels under normal conditions. However, iNOS is upregulated upon pathological conditions such as ischemic stroke and contributes to increased amounts of NO (Faraci and Heistad, 1998).

As described earlier, nNOS synthesized by neurons has potential harmful effects on ischemic stroke through NMDA mediated toxicity, DNA damage, PARP activation and formation of peroxynitrites (Brouns and De Deyn, 2009, Kunz et al., 2007b, Moskowitz et al., 2010). Following ischemia, nNOS also strongly interacts with postsynaptic density protein-95 thus negatively affecting neurogenesis and dendritic remodeling. Disruption in the nNOS-PSD95 interaction has been shown to rescue ischemic damage in rodent models of transient middle MCAo (Luo et al., 2014, Zhou et al., 2010). Similarly, iNOS is also involved in cytotoxicity by inducing inflammation and is thought to be present in cells involved in the inflammatory response mediated by leukocytes, microglia and astrocytes (Murphy and Gibson, 2007, Wang et al., 2007). In the brain following transient or permanent ischemia, iNOS mRNA and protein levels are upregulated and associated with increased iNOS enzymatic activity and NO production as evident by accumulation of peroxynitrites (Grandati et al., 1997, Hirabayashi et al., 2000, Iadecola et al., 1995). There are a number of studies showing the neurotoxic effect of iNOS in ischemic brain damage. Administration of an iNOS inhibitor or using iNOS deficient mice has been associated with smaller brain infarct and better neurological outcome compared to wild-type controls (Iadecola et al., 1996, Iadecola et al., 1995, Zhao et al., 2000). Likewise, suppression of iNOS expression by hypothermia, estrogen and progesterone appeared to have neuroprotective effects (Wang et al., 2007).

2.2.7 Endogenous protective mechanism

Depending on the time course of ischemic brain damage, cell death and cell survival pathways are activated at several points. Research has mainly focused on the cytotoxic mechanisms since the impact of endogenous protective mechanisms induced upon injury are overwhelmed by destructive pathways. However, boosting the endogenous protection following stroke has been suggested to be a potential therapeutic strategy to combat ischemia induced brain damage.

Following ischemic brain injury, various protective mechanisms are activated as a defense mechanism to combat necrotic and apoptotic cell death. One such protein that is rapidly activated is heat shock protein (HSP) 70 and its induction in animal models was shown to attenuate infarct volume (Yasuda et al., 2005). HSP is known to protect the cells from both necrosis and apoptosis. Specifically, HSP 70 is suggested to interfere proapoptotic molecules while also increasing the levels of anti-apoptotic Bcl-2 proteins (Giffard and Yenari, 2004). Bcl-2 inhibits not only apoptosis but also promotes cell survival in response to necrotic cell death (Li and Yuan, 1999, Tamatani et al., 1999). HSP-70 also can interacts with Apaf-1 and hinders caspase 9 activation (Saleh et al., 2000). In addition, HSP-70 interacts with AIF and JNK to prevent apoptosis.

Neurotrophins are the tropic growth factors required for neuronal survival and maintenance. Following ischemia, neurotrophins promote cell survival by binding to tyrosine kinase receptors and activating phosphoinositide 3-kinase (PI3K) and MAPK pathway. Activated PI3K phosphorylates Akt which in turn phosphorylates Bad and procaspase 9 thus inhibiting apoptosis. MAPK pathway also phosphorylates Bad in addition to cAMP response element

binding and interferes with cell death (Mohr et al., 2011). Neurotrophin-3 has been shown to be altered during ischemia and treatment with a low-dose of Neuroptophin-3 was associated with protection against neuronal loss in neonatal hypoxic/ischemic brain injury (Galvin and Oorschot, 2003, Yang et al., 1998b).

G-CSF is a hematopoietic growth factor produced by many types of cells like neurons, monocytes and endothelial cells (Schneider et al., 2005b). The neuroprotective effect of G-CSF in stroke has been discussed in a number of studies (Lee et al., 2005, Ohmori et al., 2011, Schäbitz et al., 2003b). In addition, G-CSF has been shown to protect human cerebral neurons following oxygen glucose deprivation *in vitro* using cerebral-neuroblastoma hybrid cell lines (Jung et al., 2006). G-CSF is involved in neuroprotection through the activation of various cell survival pathways including the Akt/PKB pathway, reduction of infarct volume and promotion of plasticity and angiogenesis (Lee et al., 2005, Schneider et al., 2005b). Even though G-CSF has been shown to be beneficial in preclinical stroke, it failed to improve neurological outcome in acute ischemic stroke patients in clinical trials (Ringelstein et al., 2013).

Post-stroke neurogenesis where acute brain injury signals the stem cell population to divide and migrate towards the injured area is also one mechanism of endogenous neuroprotection after stroke. Neurogenesis occurs in the subventricular zone and subgranular layer of the dentate gyrus of the hippocampus in rodent models of both transient and permanent ischemia (Jin et al., 2001, Komitova et al., 2002, Zhang et al., 2001). In a mouse model of transient ischemia, neurogenesis has been shown to be induced bilaterally in both brain sides of the subventricular zone and dentate gyrus at around 2 days following ischemia and peaking at 7-14 days post-stroke (Jin et al., 2001, Takasawa et al., 2002, Zhang et al., 2001). Neurogenesis then returns to basal levels by 3-4 weeks after stroke. Most of the neural progenitor cells that survive migrate from the subgranular layer to the granular cell layer and from the subventricular zone to the striatum and neocortex where they mature into functional neurons (Tobin et al., 2014). Also, the amount of newly born neurons is affected by the type and the severity of cerebral ischemia. For example, MCA occlusion for 2 hours was shown to induce more proliferation than an occlusion of a 30 min (Arvidsson et al., 2001). In permanent focal ischemia, cellular proliferation in the SVZ was also dependent on the size of the infarct (Moraga et al., 2014). Neurogenesis and angiogenesis are a coupled process and endothelial cells when activated by ischemia promote the release of stromal derived factor 1α that attracts neuroblasts expressing its receptor CXCR4 and thus promotes proliferation and migration (Imitola et al., 2004, Robin et al., 2006). In addition, angiogenesis after stroke promotes the formation of new vessels, and these vessels allow the migration of the newly formed neuroblast to migrate out of subventricular zone (Thored et al., 2007).

In addition to growth factors and chaperones, anti-inflammatory cytokines like IL-10, IL-1ra, and TGF-β are also involved in the neuroprotective mechanism following brain injury as already discussed above.

2.3 CO-MORBIDITIES AND RISK FACTOR FOR STROKE

Stroke is a heterogeneous disease that develops due to the predisposition of multiple underlying pathological risk factors in individual during their lifetime. The risk factors can be classified as either modifiable or non-modifiable. Major modifiable risk factors include hypertension, hyperlipidemia, smoking, diet and physical inactivity accounting for more than 80% of the risk of all stroke (Goldstein et al., 2006, O'Donnell et al., 2010). Non-modifiable risk factors include age, race, sex, and genetics.

2.3.1 Hypertension

Hypertension is one of the most important modifiable risk factor for both ischemic and hemorrhagic stroke. Framingham Heart Study has shown that hypertension was associated with about 3 times the risk for stroke in both men and women (Burt et al., 1995, Kannel et al., 1970) when compared to non-hypertensive subjects. Hypertension increases the risk of stroke with both increasing systolic and diastolic blood pressure. People with systolic blood pressure of 160 mmHg or higher and diastolic blood pressure of 95 mmHg have a higher risk of stroke when compared to normotensive people (Sacco et al., 1997). Systolic increase in blood pressure alone has been shown to increase the risk of stroke (Kannel et al., 1981).

In ischemic stroke, hypertension causes sheer stress in endothelium thus affecting atheroma formation and subsequent atherosclerosis (Dubow and Fink, 2011). In addition, elevation of blood pressure is associated with endothelial damage and enhances leukocyte adhesion and local thrombus formation subsequently leading to thrombotic stroke (Johansson, 1999). Moreover, hypertension can cause lipohyalinosis of small penetrating arteries and cause lacunar stroke as well as deep hemispheric hemorrhage (Fisher, 1971). Chronic hypertension can cause pathological changes in smooth muscle cells and endothelium and contribute to ICH (Johansson, 1999).

2.3.2 Atherosclerosis

Atherosclerosis is one of the major risk factors for stroke and about half of the entire stroke is caused by atherosclerosis either by thrombus or by embolus. Atherosclerosis is a disease characterized by thickening of the artery due to the inflammatory response of macrophage and lymphocytes to pathogenic lipoproteins into the arterial walls (Libby et al., 2002). Atherosclerosis is a slow and progressive disease that advances with age. Various risk factors for atherosclerosis like smoking, hypertension, diabetes, hyperlipidemia, etc. cause the damage to the endothelial cells on the inner lining of the arteries. The damage to the endothelial lining upregulate adhesion molecules on the endothelial cell surface and leukocytes and platelets adhere to the endothelium (Weber et al., 2008). Monocytes migrate into the arterial wall where they differentiate into macrophages. The differentiated macrophages then take up the lipids and oxidized low density lipoprotein (oxLDL), leading to foam cell formation and development of fatty streak lesions. As the lesion progresses, foam cells macrophages die and contribute to the formation of necrotic core through release of cytoplasmic content that further induce inflammation (Weber et al., 2008). During this process, vascular smooth muscle cells migrate from the tunica media to tunica intima, proliferate and form fibrous caps which ultimately lead to narrowing of the artery and plaque formation and eventually rupture (Figure 5). The rupture of the plaque and narrowing of the arteries lead to clinical events such as stroke and cardiovascular disease (Weber et al., 2008).

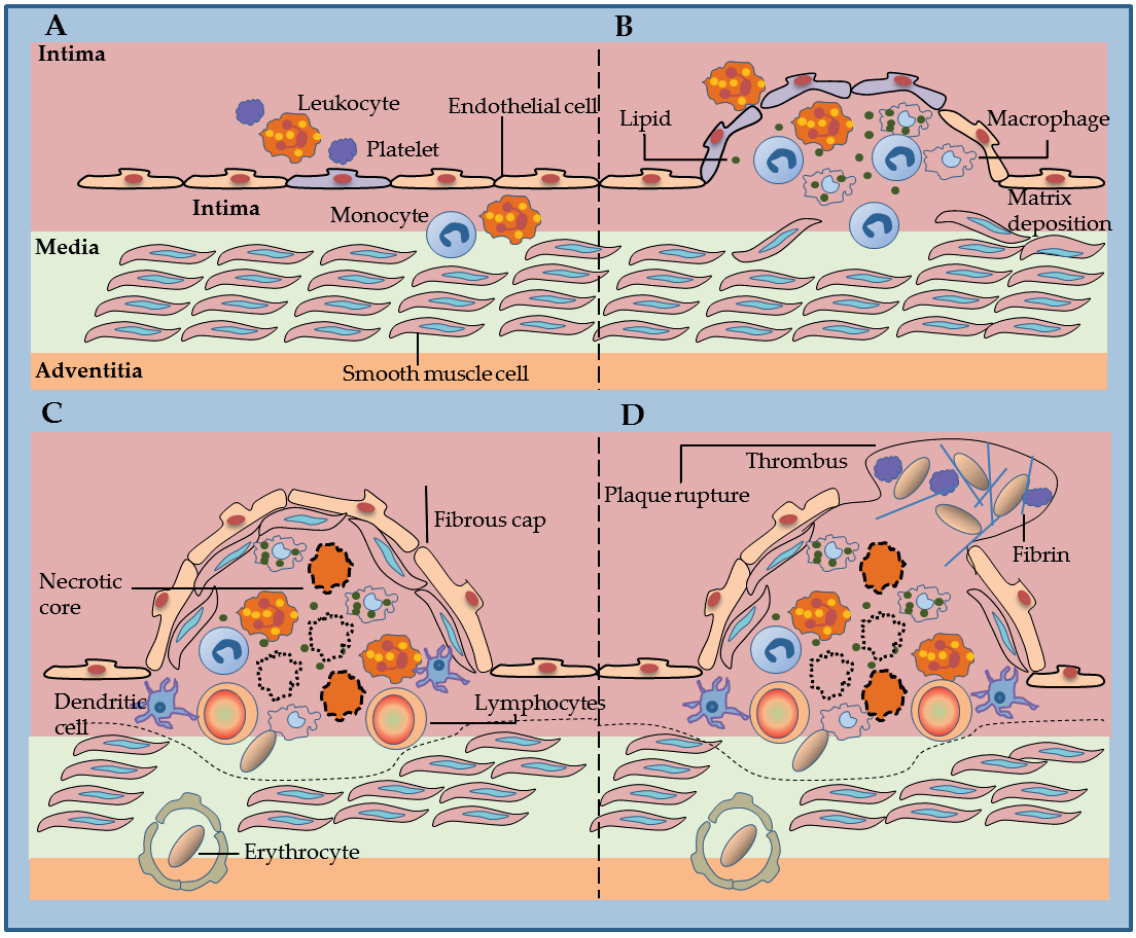

Figure 5: Progression of an atherosclerotic lesion. a) Endothelial dysfunction and leukocyte and platelet adhesion on the endothelial cell. b) Recruitment and differentiation of monocytes into macrophages that take up lipids to form foam cells and build up fatty streaks and subsequent plaques. c) Formation of necrotic core and fibrous caps due to the apoptosis of macrophage and other plaque cells. d) Thinning and erosion of fibrous caps into unstable plaques due to matrix degradation by protease and ultimate rupture of the plaque. This further leads to arterial occlusion which is the main cause of myocardial infarction and stroke. (Figure adopted from Weber *et al*, 2008)

Large artery atherosclerosis causes stroke in three different ways; (1) hypoperfusion due to severe narrowing of the arterial lumen that transport blood to the brain, (2) hypoperfusion contributed by thrombi that block the main branch of the occluded arteries and, (3) emboli causing the disturbances and blockage of the distal artery (Higashida et al., 2005)

2.3.3 Hyperlipidemia

Hyperlipidemia refers to elevated amounts of lipids and lipoproteins in the blood. Increased serum cholesterol has been shown to increase the incidence of coronary heart disease. However, such types of associations are less well documented in ischemic stroke and are rather conflicting. Hypercholesterolemia has been shown to increase the plasma levels of inflammatory sensitive proteins and contributes to increased incidence of stroke (Engström et al., 2002). Secondary to that, hypercholesterolemia in combination with inflammation might

accelerate the progression of atherosclerosis. Several studies have shown that the total serum cholesterol, high levels of low density lipoproteins (LDL) and triglycerides increase the risk of stroke where as high density lipoproteins (HDL) are associated with decreased risk of stroke. In contrast, low levels of serum cholesterol have been found to increase the incidence of ICH (Yano et al., 1989). Indeed, ischemic stroke and ICH are differently related to cholesterol levels. Hyperlipidemia conditions modeled by feeding the high cholesterol diet in ApoE deficient mice was found to attenuate angiogenesis and pericyte coverage of endothelial cells thus impairing cerebral blood flow (Zechariah et al., 2013). Upregulation of Rho-associated kinase activity has been proposed as one of the mechanisms of vascular dysfunction in hyperlipidemic condition in ischemic stroke and that its inhibition is associated with improved stroke outcome (Shin et al., 2008, Shin et al., 2014). In addition, CD36 has been shown to exacerbate brain injury in hyperlipidemic conditions through MCP1/CCR2 axis (Kim et al., 2008).

2.3.4 Diabetes

Diabetes mellitus is another highly ranked independent risk factor for stroke, and people with diabetes have 2-3 fold risk of having stroke compared to people without diabetes (Air and Kissela, 2007, The Emerging Risk Factors, 2010). The effect of diabetes mellitus has been demonstrated by Honolulu Heart Program, where subjects with diabetes and asymptomatic hyperglycemia were prone to increased ischemic stroke independent of age and other risk factors (Burchfiel et al., 1994). People with both type I and type II diabetes have increased susceptibility for atherosclerosis and small artery occlusive disease and, therefore, develop thromboembolic, larger arteries and lacunar stroke (Jackson and Sudlow, 2005, Mankovsky et al., 1996, Ohira et al., 2006). Diabetes and hyperglycemia contribute to progression of atherosclerosis through endothelial dysfunction which in turn promotes proinflammatory, vasoconstrictive and prothrombotic processes leading to plaque formation and rupture (Castilla-Guerra and Fernandez-Moreno, 2007). Diabetes also promotes hypertension and dyslipidemia which are the major risk factors of stroke. Moreover, diabetes increases the level of fibrinogen that subsequently leads to thrombosis and stroke (Lukovits et al., 1999).

Increased blood glucose or hyperglycemia through intraperitoneal injection of dextrose was found to increase the infarction volume in both permanent and transient MCAo (Liu et al., 2007). Hyperglycemia has been reported to increase the oxidative stress and MMP-9 activation and these events are linked to promote BBB dysfunction (Kawai et al., 1998, Kamada et al., 2007). In addition, the mechanism of ischemic damage by hyperglycemia was attributed to exacerbated leukocyte-endothelial cell adhesion, increased IL-1 and ICAM expression (Howells et al., 2010). However, gender specific response to ischemic insult where females were more resistant to damage than males was observed in genetically engineered diabetic db/db mice (Vannucci et al., 2001). Even though the mechanism of brain damage in diabetic condition are less than clear, recent study points towards decreased levels of MCP-1, IL-6 and CCR-2 gene expression in brains of mice fed with a diabetic diet (Kim et al., 2014).

2.3.5 Infections

Pre- and post-ischemic infections have been known to be associated with stroke with pre-ischemic infections increasing the risk of stroke and post-ischemic infections aggravating the damage and neurological functions (Emsley and Tyrrell, 2002, Grau et al., 2010). Both acute and chronic infections are involved in the aggravation of brain damage. Inflammation is a mechanism to combat the infection, however, aggravated inflammation caused by infections can subsequently increase the deleterious effect of stroke (Emsley and Hopkins, 2008). The incidence of stroke is more pronounced during cold season (Hindfelt and Nilsson, 1977). A number of infectious diseases of bacterial, viral and fungal origin have been found to increase the risk of ischemic stroke. Infections, especially of bacterial origin, affecting the respiratory and urinary tracts have been shown to increase the risk of stroke 3 days after the preceding infection

(Clayton et al., 2008, Smeeth et al., 2004). Bacterial infections have been shown to increase the prevalence of brain infarction in young and middle aged patients (Syrjänen et al., 1988). Viral infections like influenza, also predict the outcome after stroke (Grau et al., 2005, Lavallée et al., 2002). Chronic infections by several pathogens like *Chlamydia pneumonie, Helicobacter pylori and porphyromonas gingivalis* have also been suggested to contribute to stroke outcome. However, the results are either inconclusive or conflicting.

Both acute and chronic infections induce brain infarctions by several mechanisms. Infections induce procoagulant pathways via cytokines, interleukin (IL)-1 and tumor necrosis factor (TNF). These cytokines released by monocytes and other inflammatory cells can transform the endothelium from anticoagulant to the procoagulant cell layer by lowering endogenous tPA and thrombomodulin and increasing tissue factor and plasminogen-activator inhibitor 1 expression (Grau et al., 2010). In addition, these cytokines along with C-reactive protein also induce the expression of tissue factors by circulating monocytes and macrophages (Cermak et al., 1993). Acute infection has also been shown to decrease the level of circulating antithrombotic activated protein C and increase plasma concentration of C4b-binding protein which is an inhibitor of anticoagulant protein (Macko et al., 1996). Likewise, increased fibrin D-dimer, cardiolipin immunoreactivity and fibrinogen concentration was also observed in stroke patients with preceding infections (Ameriso et al., 1991). Gingivalis infections have been shown to induce platelet aggregation (Lourbakos et al., 2001). Recently, *Streptococcus pneumonia* was also shown to exacerbate inflammation in the brain via the activation of IL-1 and platelet glycoprotein 1bα (Dénes et al., 2014). Specifically, the production of microglial IL-1α was augmented. Thus, infections can further enhance the coagulation cascade to form thrombus and stroke.

Infections have also been shown to promote the development of vascular disease. Even though atherosclerosis originates starting from the childhood, infection can also give rise to atherosclerosis and subsequent plaque rupture and ultimately stroke. Infection of the cells lining the arterial wall can lead to endothelial dysfunction and increased levels of proinflammatory cytokines thus leading to atherosclerotic plaque formation (Epstein et al., 2009). In some unstable atherosclerotic plaques, antigen from the microbial agents can activate specific T cells and might contribute to the plaque inflammation, rupture and stroke (Niessner et al., 2006). In addition to promoting atherosclerosis, infections can also damage the normal arterial walls and might cause coronary intimal thickening thus contributing to coronary artery disease or cryptogenic stroke (Grau et al., 2010).

Aging is one of the risk factors for infection. Pneumonia, most frequently occurring in elderly, is associated with higher morbidity and mortality rates when compared to younger population (Yoshikawa and Marrie, 2000). Likewise, urinary tract infections are also most common in the elderly population (Gavazzi and Krause, 2002). With aging, a big proportion of the elder populations have asymptomatic bacteriuria where they carry a significant number of bacteria in the urine without any usual symptoms (Gavazzi and Krause, 2002). Thus, aged infected patients have a bigger chance of suffering from stroke when compared to the young patients.

2.3.6 Aging and sex

Aging is a non-modifiable independent risk factor with aged people having higher incidence of stroke and risk of mortality when compared to young people (Weir and Dennis, 1997). Approximately, 88% of people who die from stroke are over 65 years old. Even though stroke can affect people of all ages, the risk of stroke increases rapidly after the age of 55 in both men and women. Women have a lower incidence of stroke between ages 55-74 when compared to an age-matched male. However, this trend reverses with advancing age with women above 80 having a higher risk for stroke when compared to men (Manwani and McCullough, 2011). Atherosclerosis, atrial fibrillation and hypertension are the main factors contributing to the

ischemic stroke in this group. Atrial fibrillation especially in women is the main risk factor for stroke after reachin the age of 75.

Irrespective of the sex, aging is associated with shrinkage of brain tissue. White matter atrophy is prominent in the brain compared to the gray matter as demonstrated by MRI (Guttmann et al., 1998). Regional changes in the brain have also been shown to be affected by aging. In Framingham heart study, elderly above the age of 50 showed greater decline in the volume of the frontal lobe (~12%) and temporal lobe (~9%) when compared to modest decline in parietal and occipital lobes (DeCarli et al., 2005). The physical changes in the brain have been suggested to occur due to a change in neuronal volume rather than the loss of the neurons themselves. In addition, changes in dendritic spines and synapses might contribute to the decline in neuronal volume which ultimately lead to cognitive decline (Peters, 2006). Likewise, glial cells thicken and increase with age such that the distance between the glia and shrinking neuronal process is preserved for glial-neuronal exchange (Glorioso and Sibille, 2011). In addition to the morphological changes at the cellular level, neurons also display increased DNA damage and ROS, mitochondrial dysfunction, calcium dysregulation, and inflammatory processes as evident in clinical and experimental studies (Yankner et al., 2008). When these neurons are injured, they release ATP, neurotransmittors, growth factors and cytokines, which ultimately activate microglial cells (Hanisch and Kettenmann, 2007). Upon activation, microglia releases a number of both pro- and anti-inflammatory molecules (Lucin and Wyss-Coray, 2009). Following ischemic stroke, the inflamed aged brain has been shown to react strongly with accelerated astrocytic and microglial activation (Badan et al., 2003). Several inflammatory chemokines (CCL2, CXCL-1) and cytokines (TNF-α, IL-1 and IL-6) are upregulated in aged rats upon ischemia. In addition inflammation related transcriptional genes like IL-6, transforming growth factor beta receptor I, ribosomal protein S2, Prostaglandin E synthase 3 were found to be upregulated in aged rats following transient ischemia (Buga et al., 2008). However, other studies point towards the attenuated inflammatory response of pro-inflammatory cytokines like TNF-α, IL-1β and IL-6 and chemokines like Mip-1α and IL-10 as a result of elevated cytokines in the aged brain preceding ischemia that may result in preconditioning (Sieber et al., 2011).

2.3.7 Apolipoprotein E

2.3.7.1 Overview

Apolipoprotein E (ApoE) is a class of lipoproteins that are primarily synthesized in the liver and are essential in maintaining lipid homeostasis through the catabolism of triglyceride-rich lipid components. ApoE was identified as an arginine rich protein constituent of very low density lipoprotein (VLDL) in 1973 (Shore and Shore, 1973). Subsequently, it was demonstrated to be present in chylomicrons, LDL and HDL (Zannis and Breslow, 1982). ApoE is incorporated into the surface of these four lipoprotein constituents where they act as ligands for lipoprotein receptors (Li et al., 1988). ApoE has been found to be present also in other tissues than liver, such as the brain, spleen, muscle and lungs (Mahley, 1988). In the central nervous system (CNS), ApoE is mainly synthesized by glia, particularly astrocytes but also microglia (Holtzman et al., 2012, Pitas et al., 1987). ApoE is primarily secreted in HDL-like particles although it is present in all forms of lipoproteins (Holtzman et al., 2012).

2.3.7.2 Genotypes and polymorphism

ApoE is polymorphic and exists in three human isoforms (ApoE2, ApoE3 and ApoE4) that are encoded by corresponding allele APOE ε2, ε3 and ε4. The polymorphism results from the difference in three alleles in single gene locus on chromosome 19. ApoE2 (cys112, cys158), ApoE3 (Cys112, Arg158) and ApoE4 (arg112, Arg158) differ from each other by only one or two amino acids at residue 112 and 158 (Holtzman et al., 2012). A single amino acid substitution among the isoforms alters the structure of the protein and hence influences its lipid association

and receptor binding (Hatters et al., 2006). ApoE2 have reduced affinity for its receptor, low-density lipoprotein receptor (LDLR), as a result of substitution of arginine with cysteine at position 158 and hence the disturbance in the conformation of α-helical structure. Thus, ApoE2 transport lipids less efficiently and results in hyperlipoproteinemia. Likewise, ApoE4 binds to large lipoproteins and is associated with increased risk of cardiovascular disease (Bu, 2009). APOE ε3 allele is the most common form of allele and is considered a neutral risk phenotype in the majority of the population. APOE ε3 is found in approximately 78% of the population whereas APOE ε4 and APOE ε2 are found in 14 and 7 percent of the population respectively (Davignon et al., 1988). These APOE genetic polymorphisms result in 6 different genotypes, three homozygous (ε2/ε2, ε3/ε3 and ε4/ε4) and three heterozygous (ε2/ε3, ε3/ε4 and ε2/ε4) with higher occurrence of ε3/ ε3 (55%), ε3/ε4 (25%) and ε2/ε3 (15%) (McColl, 2004).

2.3.7.3 Structure and function

The human ApoE protein is a 34kDa glycoprotein containing 299 amino acids and was originally identified as a main constituent of lipoproteins in plasma. ApoE contains an amphipathic α-helical lipid binding domain that can switch reversibly between the lipoprotein bound state and lipid free state (Hatters et al., 2006). In the lipid free state, ApoE contains two structural domains that are separated by a hinge region: the N-terminal domain containing four helical structures and C-terminal domain containing α-helical structure (Segrest et al., 1992, Wilson et al., 1991). The N-terminal domain (amino acids 1-191) contains a receptor binding region and binds to LDLR whereas a lipid binding domain (amino acid 224-272) has been shown to reside in C-terminal domain (Mahley, 1988, Mahley et al., 2009, Westerlund and Weisgraber, 1993).

ApoE is involved in a wide range of physiological functions, the most important being the lipid transport. ApoE is involved in transport of triglyceride and cholesterol from the intestine to the liver as a constituent of chylomicron. It also directs triglyceride and cholesterol from the liver to extrahepatic tissues as a constituent of VLDL. HDL-bound ApoE also take part in reverse cholesterol transport for efflux of cholesterol to the liver for excretion. ApoE2 is metabolically different from ApoE3 and ApoE4. ApoE4 binds selectively to VLDL that is rich in triglycerides where as ApoE2 and ApoE3 bind to HDL. APOE ε4/ε4 and APOE ε3/ε4 genotypes have been found to be associated with elevated systolic blood pressure that might result in myocardial infarction and atherosclerosis (Lenzen et al., 1986). In the brain, ApoE has also been proposed to be involved in lipid transport similar to that in plasma (McColl, 2004). In addition, ApoE is involved in neuronal sprouting and synaptogenesis (Masliah et al., 1995, Mauch et al., 2001).

2.3.7.4 ApoE receptors

ApoE is the critical component of various lipoproteins and serves as ligands for a group of receptors known as the LDL receptor family. Ten different types of receptors have been identified up to date and these include LDLR, VLDL Receptor (VLDLR), ApoE receptor 2 (APOER2), Multiple EGF-like domain 7 , LDLR related protein (LRP)1, LRP1B, LRP2, LRP5, LRP6) and Sortilin-related receptors with A-type repeats that are essentially unchanged during the course of evolution (Herz and Beffert, 2000). All the receptors have single membrane spanning structures followed by a cytoplasmic tail containing NPXY motif.

LDLR was first identified as a cell surface receptor that can uptake LDL from the circulating blood and mediate internalization and endocytosis via clathrin-coated pits (Brown and Goldstein, 1986). Inactivation of LDLR through loss-of-function mutation in *LDLR* gene is associated with elevated plasma levels and familial hypercholesterolemia and atherosclerosis. LDLR receptors are widely expressed in both periphery and brain. VLDLR and ApoER2 share structural similarities with LDLR but are involved in reelin signaling that are crucial for neuronal migration, dendritic spine development and synaptic plasticity (Beffert et al., 2005,

Niu et al., 2008, Trommsdorff et al., 1999). LRP1, another receptor, is also highly expressed in the liver and brain and serves as a receptor for more than 30 different types of ligands including APOE, tissue type plasminogen activator (tTPA) and Amyloid precursor protein (APP) (Bu, 2009). LRP1 is involved in cellular trafficking and processing of beta Amyloid (Aβ) that can have pathophysiological consequences in AD (Kounnas et al., 1995). LRP1 also functions similar to LDL receptors in internalization and endocytosis but at a faster rate (Li et al., 2001b). In addition to being the receptor for endocytosis in brain, LRP1 is also involved in synaptic transmission and motor functions.

2.3.7.5 Role of ApoE in Alzheimer's disease (AD)

AD, originally described by Alois Alzheimer in 1907, is a devastating progressive neurodegenerative disease that is often characterized by an irreversible loss of neurons leading to dementia. Approximately 26 million people are affected by this disease worldwide. Early-onset familial AD typically accounts for a small proportion of the population and is caused by a mutation in APP gene, Presenilin 1 or Presenilin 2 gene. Late-onset AD accounts for the majority of the population and is mainly attributed to the impairment in clearance of Aβ (Liu et al., 2013). Among the various susceptibility genes that have been discovered so far, APOE ε4 gene located on chromosome 19 was shown to be a risk factor for both sporadic and late-onset AD (Corder et al., 1993, Saunders et al., 1993, Strittmatter et al., 1993). However, APOE ε2 alleles have been suggested to have a protective role against AD (Corder et al., 1994). Increased risk of AD is approximately 12 fold in people with two ε4 alleles and 3 fold in people with a single ε4 allele when compared to people lacking ε4 allele at all (Holtzman et al., 2012). The onset of disease also shifts to earlier time points in people carrying one or two APOE ε4 alleles either in sporadic cases of APP or Presenilin 1 mutation or late-onset AD (Nacmias et al., 1995, Pastor et al., 2003, Saunders et al., 1993). In all the AD cases, Apo ε4 allele carriers account for 50% of cases. In addition to being one of the significant risk factors in development of AD, ApoE ε4 is also involved in production and aggregation of both Aβ and tau which are the hallmark of AD pathology (Verghese et al., 2011).

2.3.7.6 Role of ApoE in Stroke

ApoE has been shown to be associated with cholesterol metabolism, atherosclerosis, ischemic heart disease and cerebral amyloid angiopathy (CAA) which might ultimately influence the stroke outcome. Especially, the APOE ε4 allele is associated with an increased level of cholesterol that can accelerate atherosclerosis and hence ischemic heart disease (Sudlow et al., 2006). Thus, it is possible that APOE is also linked to ischemic stroke especially thrombotic stroke. Indeed, a meta-analysis linked the significant association between APOE ε4 allele and ischemic stroke (McCarron et al., 1999). Moreover, APOE ε4 allele has been demonstrated to be a risk factor for the recurrence of ischemic stroke (Kim et al., 2003). However, opposite association has been found in other studies, in case of ischemic stroke (Martínez-González and Sudlow, 2006).

Even though contradicting results exist between the ApoE polymorphism and ischemic stroke, there are a number of other studies that link ApoE polymorphism with increased risk of ICH and SAH. CAA is a form of angiopathy that is caused by deposition of Aβ peptide in the walls of blood vessels of CNS. Several studies have reported that CAA is an important cause of lobar cerebral hemorrhage in elderly and APOE ε4 allele enhances the amyloid deposition in blood vessels. In addition, the APOE ε2 allele also enhances the vasculopathic change that leads to the rupture of amyloid laden vessels (Greenberg et al., 1998, McCarron and Nicoll, 2000). Thus, carriers of APOE ε4 and APOE ε2 have an increased chance of having lobular ICH. Likewise, APOE ε4 alleles have been demonstrated as a risk factor for SAH and poorer outcome in number of studies (Guo et al., 2011, Kokubo et al., 2000, Lanterna et al., 2007, Lanterna and Biroli, 2009).

2.3.7.7 ApoE transgenic animal

Rodent models have been widely used to study the molecular and cellular basis of diseases. Several transgenic mice lines that either overexpress or underexpress several genes have been developed to study the molecular basis of various diseases including cancer, heart disease, obesity, stroke, aging and PD and hence develop therapeutic treatment regimen. In the context of this thesis, human APOE-targeted replacement mice are discussed. It is well known fact that rodent models express only one isoform of ApoE in contrast to the three isoforms found in humans. Thus, the generation of transgenic animals expressing human isoforms of ApoE is important in order to determine the effect of APOE polymorphism in context to human disease like stroke and AD.

APOE transgenic mice were initially developed using microinjection of allele-specific human genomic fragments to establish the founder mice that were then bred with APOE-knockout mice (Xu et al., 1996). In this model, ApoE levels were comparable to ApoE levels in WT mice. Later, transgenic mice expressing APOE ε3 and APOE ε4 under the control of GFAP promoters were developed to study the expression of astrocyte specific human ApoE isoforms (Sun et al., 1998). In addition, other transgenic lines where ApoE expression was restricted to neurons was developed driven by a neuron-specific enolase promoter.

APOE transgenic mice used in this thesis were generated by targeted replacement of the endogenous mouse APOE with human APOE without altering any endogenous regulatory sequences (Sullivan et al., 1997, Sullivan et al., 1998, Wang et al., 2005). The expression of ApoE mRNA in brain and other tissues were comparable to those found in WT mice since transgene expression in these mice is under the control of endogenous promoters and only the coding region is replaced. In addition, there were no differences in the cholesterol and triglyceride levels in fasted plasma between the APOE3-targeted replacement (ApoE3-TR) mice and WT counterparts.

2.3.7.8 ApoE in Preclinical Experimental Stroke

Transgenic mice expressing human ApoE isoforms have been utilized in ischemic stroke. Specifically, ApoE4 ε4 mice have been shown to have bigger infarcts compared to ApoE ε3 mice after transient focal ischemia (Sheng et al., 1998). In addition, ApoE4 isoforms have been shown to aggravate delayed infarction and reactive astrocytosis after permanent focal ischemia when compared to ApoE3 and ApoE2 isoforms and pharmacological modulation of astrocytosis was associated with better outcome (Mori et al., 2004, Mori et al., 2005). ApoE4 isoform was found to have a poorer outcome in global ischemia where significantly larger hippocampal damage was observed in the hippocampal area of ApoE4 mice (Horsburgh et al., 2000). In addition, ApoE4-targeted replacement mice showed increased prevalence of ICH, and it is associated with predominant vascular amyloid deposition (Sullivan et al., 2008). When ICH was induced by injection of clostridial collagenase in ApoE4-targeted replacement (ApoE4-TR) mice, they showed increased cerebral edema and poor functional outcome in rotarod (James et al., 2009). Taken together, it is evident that the APOE ε4 allele is associated with poorer outcome following stroke which coincides with the result that is often seen in clinical subjects.

The fact that the APOE ε3 allele is associated with smaller brain damage compared to APOE ε4 may be attributed to the attenuation of NMDA-mediated glutamate excitotoxicity (Aono et al., 2003, Aono et al., 2002, Buttini et al., 1999, Qiu et al., 2003). In addition, ApoE4 isoforms have been shown to reduce glutamate receptor function and synaptic plasticity that might affect the outcome after stroke (Chen et al., 2010). The antioxidant properties of ApoE are documented in a number of studies (Hayek et al., 1994, Kitagawa et al., 2002, Praticò et al., 1998). *In vitro* studies have demonstrated the isoform specific protection of neurons from hydrogen peroxide- and Aβ-induced oxidative damage in the order ApoE2 > ApoE3 > ApoE4 (Miyata and Smith, 1996). On the other hand, the ApoE4 isoform was shown to increase lipid peroxidation in post-mortem brain sample and elevated hydroxyl radical in blood (Jofre-Monseny et al., 2008).

Moreover, the ApoE4 isoform was shown to increase intracellular calcium and thus excitotoxicity when compared to the ApoE3 isoform further illustrating the negative effect of ApoE4 on neurodegenerative disease (Veinbergs et al., 2002). Overall, ApoE4 isoforms appear to modulate neurotoxicity while ApoE2 and ApoE3 are associated with neuroprotection.

The neuroinflammatory effect of ApoE4 was first demonstrated in human AD brains where microglial activation was demonstrated in frontal and temporal lobes (Egensperger et al., 1998). However, most of the evidence on the role of ApoE on inflammation comes from mice engineered to express human E2, E3 and E4 isoforms. Mice expressing ApoE ε4 allele were shown to have significantly higher levels of IL-6 and TNF-α in both brain and periphery in response to LPS administration (Lynch et al., 2003). Microglial activation but not astrocyte activation was observed in ApoE4 mice brains relative to ApoE3 mice brains. Subsequently, microglia from ApoE4 mice produce greater amounts of NO than ApoE3 mice (Jofre-Monseny et al., 2008). Even though the exact mechanism by which ApoE modulate these effect is not known, one study point towards the NF-kB activation (Ophir et al., 2005) while the other study hypothesizes that ApoE mediated effect in microglia are p38MAPK dependent(Maezawa et al., 2006).

2.3.7.9 Role of ApoE in other neurological disorders

APOE genotypes have been shown to be involved in a wide range of neurological disorders. In TBI, presence of the APOE ε4 allele has been linked with poorer neurological outcomes in a number of studies (Friedman et al., 1999, Verghese et al., 2011). One study has demonstrated that patients with APOE ε4 allele have unfavorable outcomes compared to the non-carriers (Teasdale et al., 1997). In addition, APOE ε4 in particular have been shown to predispose and reduce the age of onset of Down's syndrome-associated dementia in contrast to APOE ε2 that has a protective role (Verghese et al., 2011). In multiple sclerosis (MS), a higher frequency of APOE ε4 allele has been observed in the Danish population; however other studies have found no association with APOE ε4 allele frequency and MS susceptibility (Burwick et al., 2006, Ghaffar et al., 2010, Høgh et al., 2000). Likewise, APOE ε4 genotypes have been found to be associated in a number of diseases including Parkinson's disease (PD), Amyotrophic lateral sclerosis, Huntington's disease and Temporal lobe epilepsy even though controversies do exist (Verghese et al., 2011).

2.2.8 Other risk factors for stroke

Various other modifiable and non-modifiable risk factors influence the susceptibility and outcome of stroke. Heart disease and impaired cardiac function have strong interaction with stroke. People with ischemic heart disease, congestive heart failure and left ventricular hypertrophy have double the risk of stroke compared to a person lacking these disorders. Atrial fibrillation is also a primary cardiac condition and accounts for 1 of 6 ischemic stroke cases (Hart et al., 2002). Arterial fibrillation accounts for almost half of all cardioembolic stroke (Panel et al., 1997). Obesity is also linked with an increased risk of stroke since obese people have high blood pressure, blood glucose and atherogenic serum lipids.

2.4 EXPERIMENTAL MODELS OF CEREBRAL ISCHEMIA

A large number of approaches to study cerebral ischemia have been developed during the last 30-50 years with the aim to elucidate the molecular and cellular mechanism underlying stroke and hence explore the potential therapeutic options. *In vitro* models using primary cultures, cell lines and tissue culture are widely used to study the ischemia at cellular level and can provide valuable information of individual cellular components and their biological functions. On the other hand, *in vivo* models provide the information of the molecular and cellular interactions

and their physiochemical properties that ultimately lead to brain injury. *In* vivo animal models of stroke comprise models of: a) global ischemia, b) focal ischemia and c) hypoxia ischemia (Woodruff et al., 2011).

2.4.1 Global ischemia

Global ischemia is characterized by transient blockage of blood flow into the entire brain (Mohr et al., 2011). Global ischemia results in delayed selective neuronal loss and delayed neurological deficits leading to cognitive impairments (Lo et al., 2003). Only selected neuronal populations such as pyramidal neurons in hippocampal CA1, hilar neurons of dentate gyrus and Purkinje neurons of the cerebellum are vulnerable to ischemic damage (Harukuni and Bhardwaj, 2006, Mohr et al., 2011). Global ischemic models have significant clinical relevance as it resembles cardiac arrests and asphyxia in humans (McBean and Kelly, 1998). There are several ways to produce global ischemia; however, the most common method involves the occlusion of vertebral and common carotid arteries for short duration. The most commonly used models for global ischemia are (a) the four vessel occlusion in rats; (b) the two vessel occlusion or bilateral common carotid artery occlusion in gerbils or mice and (c) two vessel occlusion in combination with hypotension in rats (McBean and Kelly, 1998, Traystman, 2003, Woodruff et al., 2011). The four vessel occlusion involves a permanent coagulation of vertebral arteries and a temporary ligation of two common carotid arteries. In contrast, the two vessel occlusion model involves the temporary ligation of two common carotid arteries for a brief period. Gerbils serve as a relatively simple model for global forebrain ischemia because of the lack of posterior communicating arteries with highly reproducible hippocampal damage (Woodruff et al., 2011).

2.4.2 Focal ischemia

Focal ischemia is most commonly produced by the occlusion of MCA and can be either permanent or transient. Even though the MCA is occluded, collateral circulation can provide blood flow into the compromised area and hence reduce the impact of occlusion. Various animal models have been employed to study stroke-related injuries. Rodent models are extensively used because of their low cost, the relatively small size of the animal, and their consistent circulatory anatomy (Woolsey et al., 1996). There are a number of ways in which MCAo can be performed, and the occlusion can be either permanent or temporary at either distal or proximal part of the MCA (Traystman, 2003).

Transient MCA occlusion

The filament model originally described in 1986 has been routinely used to produce either permanent or transient MCAo in rodents (Koizumi et al., 1986). In this model, MCA is subjected to occlusion by ligating the common carotid and external carotid arteries and then inserting a nylon suture into the internal carotid artery and then advancing the thread to block the MCA (Koizumi et al., 1986, Longa et al., 1989). Later, a poly-L-lysine coated suture was found to produce more uniform infarct (Belayev et al., 1996). After occlusion, CBF was reduced to less than 15% in the core area while in penumbra area it was 40%. Removal of the suture allows the reperfusion of blood to the affected area and animal can then be used for assessment of behavioral outcome (DeVries et al., 2001). By varying the occlusion time, severity of the damage can be controlled.

Permanent MCA occlusion

Permanent MCA occlusion model was originally described in 1981 (Tamura et al., 1981). This model involves the permanent occlusion of the MCA and results in infarction of cortex and caudoputamen areas. In a distal model of permanent occlusion, the precise site and extent of MCA occlusion was demonstrated to influence the neurological outcome in addition to

inconsistent infarct size. In fact, avoiding lenticulostriate and small cortical branches from the proximal and distal sources allowed the formation of consistent lesion size (Bederson et al., 1986).

However, several modifications have been applied to the original method. For instance, occlusion of MCA via a burr hole made through temporoparietalis bone can produce consistent lesion (Koistinaho et al., 2002, Welsh et al., 1987). This proximal occlusion method involves the exposure of temporalis muscle, drilling of the temporal bone above the MCA at the level of inferior cerebral vein, removal of dura, and permanent cauterization of MCA using a thermocoagulator. Occlusion of MCA was also demonstrated using mechanical clips or ligature (van Bruggen et al., 1999). In permanent MCA occlusion, blood flow is not restored and has clinical significance since not all the human stroke cases are associated with reperfusion.

2.4.3 Hypoxia ischemia

This model involves the transient unilateral occlusion of common carotid arteries combined with hypoxia and is exclusively used in young or neonatal animals. Hypoxic condition can cause delayed neuronal death hippocampus, striatum and neocortex in neonatal mice (Adén et al., 2002). Hypoxic ischemic conditions have been suggested to contribute to brain damage through excitotoxic and apoptotic mechanisms (Beilharz et al., 1995, Northington et al., 2011).

2.5 BEHAVIORAL TESTING IN MOUSE MODELS OF FOCAL CEREBRAL ISCHEMIA

There are a number of different ways to produce ischemia in animal models with differences in the resulting lesion size and the specific site of the lesion. These differences ultimately affect the sensitivity and selectivity for various behavioral deficits (Mustafa et al., 2012). Finally, age, co-morbidities as well as strains of the animal used also influence the behavioral outcome (Fahlström et al., 2012, Manwani et al., 2011, Mustafa et al., 2012, Sweetnam et al., 2012). Thus, various parameters should be taken into account while designing the experiment that involves behavioural assessments following stroke. In fact, a large amount of behavioural testing has been successfully applied to mouse models of focal ischemia that were initially applied to rat models but later validated in mouse models of focal ischemia (Mustafa et al., 2012). Some of the most important behavioral tests are described below.

2.5.1 Rotarod

Rotarod was first described in rat and later successfully tested in mice for the evaluation of motor coordination and balance and has been applied in variety of stroke models and mouse strains with variable sensitivity (Dunham and Miya, 1957, Jones and Roberts, 1968, Mustafa et al., 2012). The setup consists of a rotating cylinder separated into several compartments. Mice are placed on a rotating rod with constant speed or with accelerated speed where they try to remain on the rod and avoid falling. The latency to fall is then used to evaluate motor functions. Rotarod shows short term sensitivity for up to 4 days in both transient and permanent proximal MCAo while no sensitivity was observed in distal MCAo. Mice should undergo preoperative testing and baseline determination to differentiate between actual learning and recovery after stroke (Mustafa et al., 2012).

2.5.2 Adhesive removal test

Adhesive removal tests were initially described in rats to determine sensorimotor deficits after unilateral lesions involving sensorimotor cortex and striatum (Schallert et al., 1982). This test shows sensitivity for both proximal and distal MCAo. In this test, circular adhesive patches of a certain diameter are attached to animal's forepaws in alternating sequences with equal pressure

(Bouet et al., 2009). The mouse is then released into the testing cubicle or testing cage and the latency of contact and removal of the patches is recorded. The maximum number of trials per day is 3 and duration of each trial is set to 2 min. In addition, preoperative testing is carried out before the ischemia to determine the baseline. In proximal MCAo, the sensitivity was reported to last for 4 to 6 weeks depending upon the duration of occlusion and animal models (Balkaya et al., 2013, Bouet et al., 2009, Bouët et al., 2007, Leconte et al., 2009) while in distal MCAo, the sensitivity has been shown to persist for up to 3 weeks in Swiss mice (Freret et al., 2009).

2.5.3 Corner test

The corner test is used to identify and quantify sensorimotor deficits, as well as postural asymmetries. The test was first developed in rats (Schallert et al., 1982) but later adopted in mice (Zhang et al., 2002). The apparatus consists of two boards placed together in 30 degree with a small slit at the junction to motivate the mice to move to the corner. The animal is then placed halfway facing the corner and as the animal approaches the corner, vibrissae on both sides will be simultaneously stimulated and animal will rear and turn either left or right. Intact or naïve mice show no preference on the direction to turn while animals with unilateral damage will prefer their ipsilateral side for rearing and turning (Li et al., 2004, Schaar et al., 2010). The corner test can detect both mild and chronic deficits in mice (Schallert, 2006). Preoperative testing to obtain baseline data and to compare pre-stroke and post-stroke values is important for the reduction of variability and identification of the preferential side. Corner tests have been successful in detecting the functional deficits at early time points following the proximal transient filament occlusion (Mustafa et al., 2012). However, no sensitivity was observed in the distal MCAo (Freret et al., 2009, Lubjuhn et al., 2009). Likewise, sensitivity was observed up to 90 days after embolic MCAo (Zhang et al., 2002). Thus, the corner test can detect the sensorimotor asymmetry at both early and late time points where striatal damage is involved.

2.5.4 Catwalk gait analysis

Gait impairment is one of the visible deficits in clinical stroke and is often the focus of post-stroke therapy (van de Port et al., 2007). Conventional gait analysis involves the use of ink on animal paws followed by walking over the length of paper (de Medinaceli et al., 1982). This technique allows evaluation of gait parameters like stride length and hind paws drag. Nowadays, a computer aided system with a catwalk is designed to detect gait impairment in rodent models. This system consists of a long transparent glass platform in which the animal walks, and the walking is recorded using a video camera underneath the glass plate. This system generates a vast amount of data and allows the detection of even slight deficits in gait. However, speed variation during the run and loss of body weight after stroke may hinder the analysis. There are only few papers that have reported the use of catwalk gait analysis in the mouse model of proximal MCAo with deficits in various parameters like stand duration, duty cycle, swing speed, stride length etc., (Balkaya et al., 2013, Encarnacion et al., 2011, Hetze et al., 2012, Lubjuhn et al., 2009).

2.5.5 Wire hang test

The wire hang test, used to evaluate grip strengths, endurance and balance, is a simple test in which mice are suspended using their front paws on a horizontal wire 50-60 cm above the ground (Hattori et al., 2000, Mustafa et al., 2012). The hind paws are covered with adhesive to prevent their use while hanging and latency to fall is used to access motor performance. In proximal filamentous MCAo, the wire hang test has shown sensitivity ranging from 1-3 weeks depending on the strain of mice used (Abe et al., 2009, Gertz et al., 2006). The wire hang test is suitable for detecting early and late post-stroke deficits where striatal damage is involved.

2.5.6 Other behavioral tests

In addition to the above described tests, there are a number of other options to detect behavioral deficits which include the pole test, an open field test, cylinder test, handedness test, staircase test, foot fault test, ladder rung test and grip strength (Balkaya et al., 2013). Ultimately, successful testing procedures involve the choice of the animal strain as well as the model of ischemia used. Some tests might be useful in some models and stains and not the other. Thus, important consideration should be taken into account when designing the experiment paradigm involving behavioral testing in mice.

2.6 CLUSTER OF DIFFERENTIATION (CD36) AS A THERAPEUTIC TARGET IN PERMANENT FOCAL CEREBRAL ISCHEMIA

2.6.1 Overview

CD36, or simply known as fatty acid translocase, is a transmembrane, highly glycosylated, 88KDa glycoprotein that belongs to class B scavenger receptors (Cho and Kim, 2009). CD36 was initially described as a platelet receptor for thrombospondin (TSP). Later it was found to be expressed on a wide variety of cells including neurons, microglia and astrocytes in brain, monocytes/macrophage, platelets, microvascular endothelium, skeletal muscle cells, adipocytes and dendritic cells in the periphery (Abumrad et al., 1993, Albert et al., 1998, Febbraio and Silverstein, 2007, Huh et al., 1996, Husemann et al., 2002, Medeiros et al., 2004, Swerlick et al., 1992). In the brain, CD36 is especially expressed in neurons on ventral CA1 hippocampal region, perirhinal cortex and ectorhinal cortex and brainstem nuclei (Glezer et al., 2009). CD36 has an extracellular domain flanked by two transmembranes and two cytoplasmic domains. The extracellular domain contains a hydrophobic region that interacts with the plasma membrane and a proline rich region (Collot-Teixeira et al., 2007). CD36 can bind to a wide array of ligands including collagen, TSP, oxidized LDL (ox-LDL), phospholipids, long chain fatty acids (LCFA) and fibrillar Aβ (Febbraio and Silverstein, 2007) and is involved in wide biological functions including angiogenesis, atherosclerosis, phagocytosis and inflammation (Febbraio and Silverstein, 2007, Hirano et al., 2003).

2.6.2 Role of CD36 in Ischemia induced inflammation

Under normal conditions, the brain expresses low levels of CD36. However, following cerebral ischemia, CD36 has been shown to be upregulated in infiltrating macrophages in the infarct area whereas astrocytic expression of CD36 has been reported on the peri-infarct area. Neuronal expression of CD36 was nearly absent (Cho and Kim, 2009). It is well known that CD36 acts as a receptor for extracellular matrix proteins TSPs, TSP-1 and TSP-2 and is involved in endothelial cell apoptosis (Jiménez et al., 2000). Following ischemic stroke, biphasic expression of TSP-1 was observed at 1 and 72 hours whereas TSP-2 was expressed during the recovery or resolution phase (Lin et al., 2003). Thus, TSP-1 and CD36 interaction may be involved in stroke pathogenesis during the early phase of ischemia through apoptosis mediated by caspase-3-like effector (Jiménez et al., 2000, Dawson et al., 1997). CD36 is a molecular sensor for innate immune response and accumulating evidence suggest that CD36 activation results in proinflammatory signaling. Therefore, it is not surprising that CD36 has a pathogenic role in brain ischemia. Following brain injury or tissue damage, innate immune response may mediate inflammatory responses against endogenous danger signals in the post-ischemic brain via pattern recognition receptors like CD36 (Matzinger, 2002, Cho and Kim, 2009). CD36 is involved in sterile inflammation where it contributes to tissue damage through free radical generation (Cho et al., 2005). CD36 deficient mice have been reported to develop smaller lesions compared to WT mice after stroke most probably through inhibition of NF-kB activation (Kunz et al., 2008, Kim et al., 2008, Cho et al., 2007). In addition, genetic deletion of CD36 has also been shown to

actuate brain injury following acute neonatal stroke (Woo et al., 2012). CD36 also contributes to exacerbated brain damage in co-morbid conditions like hyperlipidemia and diabetes (Cho and Kim, 2009). CD36 was reported to counteract with TLR2/1 heterodimers to induce neuroinflammation in post-ischemic brain (Abe et al., 2010). In addition, interaction of CD36 with fibrillar Aβ and integrins also results in the expression of proinflammatory cytokines and chemokines (Cho, 2012). Likewise, CD36 has been shown to contribute to inflammation through regulation of calcium influx in response to ER stress and formation of prostaglandin E2 *in vitro* (Kuda et al., 2011). Taken together, these data suggest an inflammatory nature for CD36 in cerebral ischemia and targeting CD36 might provide a therapeutic strategy for stroke.

There are only two published inhibitors for CD36. One such inhibitor, peptide SS31, has been shown to attenuate ischemia by downregulating CD36 mediated pathways *in vivo*. Most of the studies involving inhibition of CD36 have arised from *in vitro* studies (Angin et al., 2012, Geloen et al., 2012, Kuda et al., 2013, Min et al., 2013). Another potential inhibitor of CD36 *in vitro* is sulfosuccinimidyl oleate (SSO) (Coort et al., 2002). SSO has been shown to bind CD36 and thus inhibit fatty acid uptake and intracellular calcium signalling along with inhibition of ox-LDL uptake by macrophages (Kuda et al., 2013). In addition, it has been shown to inhibit complex III of the mitochondrial respiratory chain (Drahota et al., 2010). However, the potential therapeutic effect of SSO is yet to be explored and belongs to the focus of this study.

3 Aim of the study

Stroke mainly affects the elderly population affected with other co-morbidities. Even though recombinant tPA is the only approved drug for the treatment of stroke, its effectiveness is limited to a very narrow therapeutic time window. Moreover, hemorrhagic complications often occur following tPA administration and alternative treatment paradigms are being explored. Indeed, a number of compounds have proven to be beneficial in preclinical models of stroke. However, these treatment regimens have all failed in clinical studies. This might be attributed to the fact that while most of the treatment strategies in preclinical models have been performed in homogenous young cohorts, stroke typically affects elderly with accompanied co-morbidities. Thus, exploring the effect of co-morbidities like aging, diet, infections and apolipoprotein genotype in preclinical studies should provide the next level of understanding in the pathophysiology and mechanisms of ischemic stroke and ultimately lead to successful treatment paradigms. The main focus of this study is how co-morbidities and aging affect the outcome of ischemic stroke. Moreover, we aimed to perform initial trials for a new therapeutic strategy for experimental stroke. The main goals of this study were:

1. To explore the impact of HF diet on sensorimotor deficits and inflammation following pMCAo in aged male mice expressing human ApoE3 (ApoE3-TR) and (ApoE4-TR) isoforms in C57Bl/6j background.

2. To determine the effect of chronic peripheral Th1 shifted infection modelled by *Trichuris muris (T. Muris*) parasites in aged C57Bl/6j mice subjected to pMCAo

3. To investigate the effects of CD36 inhibitor, SSO, on the outcome following ischemic stroke in young Balb/CaBom mice.

4 Material and Methods

4.1 ANIMALS AND TREATMENT GROUPS (I-III)

All animal experiments were approved by National Animal Experiment Board of Finland and followed the Council of European Legislation and Regulation for Animal protection. ApoE3-TR and ApoE4-TR mice on a C57Bl/6j background with 8X generation backcross were obtained from Duke University Medical Center courtesy of Patrick M Sullivan. The transgenic mice were generated without altering any endogenous regulatory sequences as described elsewhere (Sullivan et al., 2008, Sullivan et al., 1997). These mice along with other strains including C57Bl/6j and Balb/cABom used in this study were housed in light and humidity controlled environment with free access to food and water available *ad libitum*. For the study involving human ApoE isoforms, thirteen month-old ApoE3-TR, ApoE4-TR and C57Bl/6j mice were divided into three subgroups: ischemic mice fed with HF diet (HF + Isch), ischemic mice fed with normal diet (ND + Isch) and sham operated mice fed with normal diet (ND + Sham). Western type HF diet (Teklad TD88137 containing 48.5 % carbohydrates, 21.2 % fat and 0.2 % cholesterol with energy density of 4.5 kcal / g, Harlan Laboratories, WI, USA) or normal diet (ND; Teklad 2016S containing 48.5 % carbohydrates, 4 % fat and 0 % cholesterol with energy density of 3.0 kcal / g, Harlan Laboratories) was fed to the corresponding groups for 15 weeks. Separate cohorts of young mice were also used for the study. In a separate study involving chronic infection, young C57Bl/6j mice aged 4 months and old C57Bl/6j aged 18-22 months were both subdivided into uninfected and infected groups. In this study, 10 infective *T.* Muris egg suspended in 50µl of phosphate-buffered saline were introduced by oral gavage into the stomach of the mice while those in uninfected groups received saline as a vehicle. These mice underwent ischemic surgeries after 35 days on per oral administration of infective eggs when the Th1 polarized immune response peaks (Dénes et al., 2010). In the study involving CD36 inhibition, 4 month-old Balb/cABom from Taconic was used.

4.2 ANIMAL RANDOMIZATION (I-III)

In all the experiments, animals were randomized into the respective treatment groups using Graph Pad QuickCalcs (GraphPad Software, Inc. La Jolla, CA, USA). All data were analyzed blinded to the treatment groups.

4.3 CEREBRAL ISCHEMIA (I-III)

All the animals used in this study underwent permanent occlusion of MCA as described previously (Koistinaho et al., 2002). The mice were anesthetized with 5% isoflurane (in 70% N_2O/30% O_2) and maintained at 1.8%-2% isoflurane during surgery. The body temperature of the mice was maintained at 36.7 ± 0.7 during the surgery using a thermostatically controlled rectal probe connected to a homeothermic blanket (PanLab, Harvard Apparatus, Barcelona, Spain). During the surgery, the temporal bone of the mouse was exposed, and a small hole of 1-mm diameter was drilled at the level of inferior cerebral vein. Saline was applied to the drilled area to prevent heat injury. The dura was then carefully removed to expose the MCA and the MCA was permanently cauterized using a thermocoagulator (Bovie Medical Corporation,

Clearwater, FL, USA). Following the electrocoagulation, the temporalis muscle was replaced and the wound was sutured. The mice were then allowed to recover from anesthesia. Sham-operated animals underwent the same procedure except the MCA occlusion. After the end of the experiment, the mice were anesthetized with 250mg/kg Avertin and transcardially perfused with heparinized saline (2500 IU/L). Alternatively, following anesthesia, the brain was removed and freshly frozen.

4.4 PHYSIOLOGICAL PARAMETERS (I-III)

Physiological parameters were measured immediately after the onset of ischemia from blood samples drawn from the saphenous vein. Blood glucose levels were measured using the free style glucose monitoring system (Abbott, Alameda, CA, USA). The partial pressure of carbon dioxide and oxygen and pH were measured using an i-STAT analyzer (Abbott, Abbott Park, Il, USA).

4.5 CHOLESTEROL MEASUREMENT (I)

Blood samples collected via cardiac puncture 10 days post-surgery were used to quantify the serum total cholesterol levels. Right before perfusion, blood samples were collected in nonheparinized state and allowed to stand at room temperature for 30-90 min and centrifuged at 3500 rpm for 15 minutes. The serum samples that were frozen until further analysis were thawed at room temperature and cholesterol measurement was done using a cholesterol measurement kit according to manufacturer's instruction (Cayman Chemicals, Ann Arbor, MI, USA). Data were collected using a Wallace 1420 workstation (Perkin Elmer, Waltham, MA, USA).

4.6 ASSESSMENT OF ISCHEMIC DAMAGE (I-III)

The volume of the infarct size in ischemic animal was assessed either *ex vivo* or *in vivo* using a vertical 9.4 T Oxford NMR 400 Magnet (Oxford Instrument Plc, Abingdon, UK) interfaced to a Varian DirectDrive console (Varian Inc, Palo Alto, CA, USA). For the *in vivo* measurement of infarct size, the mice were anesthetized and maintained with isoflurane as describe in section 3.3. A quadrature volume RF coil was used for transmission and reception (Rapid Biomedical GmBH, Rimpar, Germany). Scout images were obtained to locate the area of the brain, and the axial sections were acquired right from the start of olfactory bulbs. Multislice T2 weighted images (repetition time 3,000 ms, echo time 40 ms, matrix size 128×256, field of view 19.2×19.2 mm^2, slice thickness 0.8 mm and number of slices 12) were obtained with double spin-echo sequence with adiabatic refocusing pulse. For the *ex vivo* measurement of the infarct size, brain submerged in 4% paraformaldehyde (PFA) 24 hours following perfusion was used. The procedure remained the same as in *in vivo* infarct measurement except the repetition time of 2.5 s and 15 consecutive slices. The obtained images were analyzed by defining the region of interest using in-house made software (Aedes) under the Matlab environment (Math-works, Natick, MA, USA). The total infarction volume and the volumes of left and right healthy hemispheres were calculated from either 12 or 15 consecutive slices. The lesion volume was determined by multiplying the number of pixel with pixel size and slice thickness. Alternatively, the relative percentage of the infarction volume was determined using the formula as described previously (Shuaib et al., 2002).

4.7 ADHESIVE REMOVAL TEST (I)

Adhesive removal test was used to determine the sensorimotor deficits following cerebral ischemia in study involving aged ApoE3-TR, ApoE4-Tr and C56Bl/6j mice. This study involves the use of circular adhesive patches of 6.5mm diameter (Bel-Art products, Wayne, NJ, USA). The mouse is taken from the home cage and adhesive patches were placed on front paws in an alternate pattern as describe in section 2.5. The mouse was then placed in a transparent cubical box and the latency from sensing to removal of the patches was recorded. Each mouse underwent 3 trials per day with duration of each trial being 120 seconds. Animals were tested 3 days prior and 3 and 7 days post-ischemia. Testing and evaluation was carried out by the same people in a blinded fashion (Bouet et al., 2009).

4.8 IMMUNOHISTOCHEMISTRY (I-III)

For immunohistochemical analysis, mice were anesthetized with 250mg/kg of Avertin and perfused transcardially with heparinized (2500IU/L) saline. The brains were dissected out and post-fixed in 4% PFA for 24 h followed by cryoprotection in 30% sucrose for 48-72 hours. The brains were then snap frozen in liquid nitrogen and cut into 20 μm thick coronal sections using a cryostat (Leica Microsystems GmbH, Wetzlar, Germany). For each staining, a set of six sections that are 400 μm apart spanning through the lesion area were taken.

For the detection of astrogliosis, microgliosis, neurogenesis, neutrophils and COX-2, brain sections were reacted against appropriate antibodies overnight at room temperature Following overnight incubation, the sections were washed with PBS containing 0.5% Tween20 (Sigma-Aldrich, St. Louis, MO, USA). The sections were then incubated with appropriate biotinylated secondary antibody for 2 hours and subsequently reacted against avidin-biotin complex reagent according to manufacturer's instructions (1:200 dilution; Vector, Burlingame, CA, USA). Alternatively, Alexa 568 conjugated secondary antibody (1:200 dilution; Invitrogen, Eugene, OR, USA) was used. The bound immunoreactivity was visualized by development with nickel-enhanced 3,3'-diaminobenzidine. Immunoreactivity was imaged using an AX70 microscope (Olympus Corporation, Tokyo, Japan) on 10X magnification with an attached digital camera (Soft Imaging System, Munster, Germany). All immunoreactive areas were quantified blinded to the study group using ImagePro Plus Software (Media Cybernetics, Rockville, MD, USA) at a predefined range and represented as percentage of positively strained immunoreactive area.

4.8.1 Ionized calcium binding adapter molecule-1 (Iba-1) (I-III)

Microgliosis was analyzed using Iba-1 for detecting microglia along with infiltrating macrophages either at day 1, 3 and 10 days post-ischemia. The sections were reacted against primary Iba-1 antibody (1:250 dilution; Wako Chemicals GmbH, Neuss, Germany) and followed the procedure described above. For the quantification of Iba-1 immunoreactivity in the peri-ischemic area, a 712μm*512μm or 720μm*530μm area adjacent to the infarct border was imaged from the six consecutive sections. In addition, the corresponding area in the contralateral side was also imaged.

4.8.2 GFAP (I & III)

Astrogliosis was analyzed using GFAP to detect reactive astrocytes at day 3 and 10 days post-ischemia. Primary GFAP antibody (1:500 dilution; DAKO, Glostrup, Germany) was reacted against the brain sections and essentially followed the step as described above. GFAP immunoreactivity was visualized from 712μm*512μm area adjacent to the ischemic area. In addition, images from the corresponding contralateral hemisphere were also taken.

4.8.3 Cycloxygenase-2 (COX-2) (I & III)

COX-2 immunostaining was performed using primary COX-2 antibody at day 3 post-ischemia (1:500 dilution, Cayman Chemicals) which then followed the procedure as above. For visualizing COX-2 immunoreactivity, an area of 712µm*512µm adjacent to the ischemic area on the ventral side of the brain was taken in addition to the corresponding side on the contralateral hemisphere.

4.8.4 Neutrophils (II)

For detecting neutrophils, sections were incubated with primary anti-neutrophil antibody (1:5000 dilution; Serotec, Kidlington, UK) and followed the process described above. However, an area of 720µm*530µm right on the lesion site was used to quantify the neutrophils. Since the contralateral side was devoid of neutrophils, no images were taken from the contralateral side.

4.8.5 Doublecortin (DCX)

Neurogenesis was analyzed from the brain sections of animals sacrificed 10 days post-ischemia. For detecting neurogenesis, sections were incubated with primary DXC antibody (1:200 dilution; Cell signaling, Danvers, MA, USA). DCX immunoreactivity was visualized from six consecutive sections in 400µm intervals from the subventricular area of 718µm*532µm adjacent to the wall of lateral ventricles from both hemispheres.

4.9 CYTOKINES (I & II)

Cytokines were measured from plasma samples as well as from freshly frozen brain samples. For the measurement of cytokines from the plasma sample, blood was collected from either saphenous vein or cardiac puncture using 1:10 volume of sodium citrate (3.8%) as an anticoagulant. The blood samples were then centrifuged at 1500g for 10 min and plasma was withdrawn and snap frozen in liquid nitrogen until further analysis. In the study using targeted replacement mice, cytokines including IL-6, IL-10, MCP-1, TNF, IFN-γ and IL12p70 were analyzed from the blood drawn via cardiac puncture at day 3 post-ischemia using Cytometric Bead Array (CBA) and Mouse inflammation kit (BD Biosciences, San Jose, CA, USA) according to manufacturer's instructions. The samples were run on FACS Caliber flow cytometer (BD Biosciences) and analyzed using FCAP Array 2.0 software (Soft Flow Hungary Ltd, Pecs, Hungary). The concentration of MCP-1 was further analyzed by MCP-1 ELISA kit (Thermo Fischer Scientific, Rockford, IL, USA).

Likewise, arrays of cytokines were measured from plasma samples taken 1 and 4 after ischemia as well as from freshly frozen brain section 24h post-ischemia. Cytokines including RANTES, MCP-1, Keratinocyte chemoattractant (KC), IL-6, IL-1β, IL-1α, IL-17α, IL-10, IFN-γ and GCSF in brain and plasma were measured using CBA kit as described above. To analyze the brain cytokines, brain areas were dissected into three regions: a) lesion area clearly visible as a white area consisting of dead tissue, b) peri-ischemic area as 1-mm wide area surrounding the lesion area and c) corresponding contralateral area. The brain samples were snap frozen in liquid nitrogen until further analysis. The frozen brain samples were homogenized in lysis buffer at 4°C as described earlier (Dénes et al., 2010). Plasma level of RANTES was also measured 1 day prior to ischemia. In addition, selected cytokines like IL-1, IL-4, IL-6, IL-10, IL-17α, IFN-γ and TNF-α was also measured from the plasma sample taken at the end time point of 24 hours using CBA Flex Sets (BD Biosciences).

4.10 ASSESSMENT OF ATHEROSCLEROTIC LESIONS (I)

For the determination of atherosclerotic lesion, the heart was removed and fixed in 4% paraformaldehyde for 4-6 hours after perfusion at day 3 post-ischemia. Following fixation, the heart was stored in PBS and the apex of the heart was removed and the base of the heart was casted in paraffin with ventricles down and aorta up. Five micrometer sections were collected from the aortic valves perpendicular to the lumen towards the base of the heart. Every fifth section and a total of 6-8 sections were stained with hematoxylin-eosin to observe the possible lesions.

4.11 IN VITRO NO RELEASE ASSAY AND LATEX BEAD PHAGOCYTOSIS (III)

To determine if the application of the SSO reduced the phagocytosis activity of BV-2 microglial cells, BV-2 microglial cells were plated in 96 well plates (9000 cells/well) in a 100µl of RPMI-1640 medium (Sigma-Aldrich, Cat# R0883) containing 10% fetal bovine serum and 5µg/ml gentamicin. Twenty-four hours after plating, the medium was replaced with serial dilution of 50µl of SSO and 50µl of 100ng/ml lipopolysaccharides (LPS) (Sigma) and incubated for the next 24 hours. After incubation, 50 µl of medium was used for NO release assay using Griess reagent. For the remaining 50µl media, additional 50µl of fluorosphere fluorescent bead suspension diluted to 1:500 was added and incubated for an additional 5 hours. Following incubation, the culture medium was removed and the cells were washed with PBS and lysed. Extent to phagocytosed beads was assayed by measuring fluorescence at 485nm/590nm excitation/emission wavelength on vector II multilabel reader (Perkin-Elmers). Cell viability was measured separately where treatment media was removed after 24 h and replaced with 100µl of 10µM pre warmed resazurin. The plates were then incubated for 2h at 37°C in 5% CO_2 and resazurin fluorescence measured again.

4.12 STATISTICAL ANALYSIS AND EXCLUSION CRITERIA (I, II, III)

Statistical analysis was performed using either GraphPad prism or SPSS 19 software (IBM SPSS Inc., Chicago, IL, USA) when appropriate. The effect of treatment and genotype as dependent variable was analyzed by two way analysis of variance followed by Sidak *Post-hoc* test and logarithmic transformation was applied to normalize the data when appropriate. One way analysis of variance with Bonferroni post-hoc test was also applied where appropriate. Data are either expressed as mean±SD or mean±SEM and $P<0.05$ was considered statistically significant. Exclusion criteria were set prior to the experiment and included: statistical outliers as analyzed by Graph Pad Quickcalcs, mice with bleeding during surgery, hemorrhage visible as black spots in ischemic hemisphere in magnetic resonance imaging (MRI) image were excluded from the experiments.

5 Results

5.1 PHYSIOLOGICAL PARAMETERS WERE UNALTERED BETWEEN THE STUDY GROUPS (I & II)

Following ischemic stroke, several physiological parameters including body temperature, blood glucose, and partial pressure of oxygen and carbon dioxide maybe altered and might affect the outcome after ischemic stroke. We, therefore, analyzed these parameters immediately after MCA occlusion (Sorce et al., 2010). We observed no significant differences in pH, glucose, pCO_2 and pO_2 following ischemia in any of the study groups (Study I, table 1).

5.2 APOE ALLELE MODIFIES INFLAMMATORY RESPONSE IN BOTH CNS AND PERIPHERY AND CONTRIBUTES TO FUNCTIONAL DEFICITS FOLLOWING ISCHEMIC STROKE (I)

5.2.1 HF diet accompanies increased body weight and cholesterol in both ApoE-TR and ApoE4-TR mice

Body weight of the mice was monitored before and after the HF diet. Prior to the HF diet, ApoE3-TR mice reveled 20% greater body weight compared to ApoE4-TR and WT mice. ApoE3-TR mice weighed on average 38.1 ± 6.0 g whereas ApoE4-TR and WT mice weighed 31.4 ± 3.4 g and 30.5 ± 3.1 g respectively. This significant difference in weight ($p<0.001$) between ApoE3-TR mice versus ApoE4-TR mice and WT mice lasted throughout the HF diet period. The weight of all the mice peaked at 13 weeks and reached a stable plateau thereafter. At the end of 15 weeks on HF diet, the average weight of ApoE3-TR mice was 46.2 ± 2.55, ApoE4-TR mice 39.3 ± 1.49 g and WT mice 40.7 ± 2.10 g (mean±SEM). Compared to mice fed on ND diet, serum cholesterol levels were increased to a significant extent in all strains. However, the serum cholesterol level on WT mice on HF was significantly lower compared to ApoE3-TR mice on the same diet. Intriguingly, ApoE3-TR mice fed with normal chow exhibited significantly higher cholesterol compared to ApoE4-TR mice on same diet (Study I, Figure 1).

5.2.2 Sensorimotor deficits were pronounced in ApoE4-TR mice on HF diet

The distal pMCAo model used in this study produces a small cortical infarct. Here, we examined the effect of ApoE isoform alone or in combination of HF diet on the infarct size using MRI images obtained 3-day post-ischemia. Data from the MRI analysis revealed no differences between the study groups even though there was a mild tendency towards increased infarct size in WT and ApoE4-TR mice fed with a HF diet (Study I, Figure 2A). Since the lesion in our pMCAo model develops in the somatosensory cortex, we examined the adhesive removal test to determine the effect of ApoE isoforms and HF diet on the behavioral outcome. Irrespective of lesion size, long term intake of a HF diet resulted in clear sensorimotor deficits in ApoE4-TR mice when compared to ApoE3-TR mice on the same diet at day 3- and 7 post-ischemia (Study I, Figure 2B and 2C). An interesting observation in this study was the ability of sham-operated ApoE3-TR mice on ND to remove the adhesive patches faster when compared to sham-operated mice of other genotypes on the same diet. Stroke induced sensorimotor deficits were observed in ApoE3-TR mice at both 3 and 7 days post-ischemia. In contrast, stroke induced sensorimotor deficits were apparent in WT mice at day 7 post-ischemia.

5.2.3 Altered inflammatory response in brain and periphery of mice fed on a HF diet

Altered glial activation is a hallmark of cerebral ischemia. We thus analyzed the brain glial activation at day 3 and 10 post-ischemia by using Iba-1 and GFAP antibodies. Following ischemia, there was significant upregulation of both glial markers in the peri-ischemic area when compared to the contralateral side in all treatment groups at day 3 and 10. Ischemia induced GFAP upregulation in the peri-ischemic area to a similar extent in all strains fed on a ND at 3 days post-ischemia (Study I, Figure 3A). However, HF diet further actuated the GFAP upregulation to a significant level in ApoE3-TR and ApoE4-TR mice but not in WT mice at 3 days post-ischemia. In contrast, HF diet-accompanied upregulation in astrocytic activation was not observed at 10 days post-ischemia. Microglial activation as revealed by Iba-1 immunoreactivity did not differ between the genotypes regardless of the dietary intervention at either 3 or 10 days post-ischemia (Study I, Figure 3B). Because of plethoric inflammatory response occurring in the brain following ischemia, glial activation does not necessarily reveal all the events occurring following ischemia. We thus examined the effect of genotypes and HF diet on ischemia induced peri-ischemic COX-2 activation. Quantification of COX-2 immunoreactivity revealed ischemia induced significant upregulation of COX-2 in the peri-ischemic area of all treatment groups except ApoE3-TR mice on ND. Interestingly, a HF diet was found to cause significant upregulation of COX-2 in ApoE4-TR mice in contrast to other genotypes on a HF diet (Study I, Figure 4A).

Experimental stroke has been shown to rapidly activate the peripheral immune system with concomitant upregulation of several cytokines and chemokines (Ferrarese et al., 1999, Offner et al., 2006). Taking into account the contribution of peripheral inflammatory cells and their release of toxic inflammatory molecules in ischemic stroke, we sought to determine a panel of cytokines that might be affected following ischemia. In fact, we analyzed various cytokines (Il-6, IL-10, MCP-1, TNF, IFN-γ and IL-12p70) and only IL-6 and MCP-1 were found within the detectable limits in the plasma samples analyzed 3 days post-ischemia. CBA assay revealed a significant upregulation of IL-6 in plasma of ApoE4-TR mice on HF diet compared to ApoE4-TR on normal chow (Study I, Figure 4H). In addition, the plasma level of IL-6 on ApoE4-TR on HF diet was significantly higher compared to WT mice on the same diet. ELISA of MCP-1 did not reveal any significant differences between the study groups (Study I, Figure 4I).

Atherosclerosis is accompanied by inflammation in concurrent to lipid accumulation in the artery wall. ApoE3-TR and ApoE4-TR mice have been shown to develop atherosclerotic plaques following an atherogenic diet (Knouff et al., 1999, Sullivan et al., 1997). We thus sought to determine if the mice in this study developed atherosclerosis accompanied by inflammation and whether this contributed to the poor behavioral outcome. However, none of the study groups showed foam cell or fatty streak formation on the endocardial surface of the valve leaflets indicating that the intake of a HF diet was unable to induce atherosclerosis in any of the strains.

5.2.4 Isoform specific ischemia-induced induction of neurogenesis

During the late phase of ischemia, neurogenesis is one of the endogenous mechanisms associated with improved recovery. Since ApoE ε4 allele has been shown to dampen neurogenesis following ischemia (Crawford et al., 2009, Li et al., 2009), we hypothesized that poorer outcome in behavioral test in ApoE4-TR mice might be accompanied by decline in ischemia-induced neurogenesis. Quantification of DCX staining revealed that ischemia failed to upregulate significant neurogenesis in WT and ApoE4-TR mice but not in ApoE3-TR mice fed on a ND (Study I, Figure 6). However, a HF diet did not have any additional impacts on endogenous neurogenesis.

5.3 CHRONIC PERIPHERAL INFECTION IN AGED ANIMALS EXACERBATE BRAIN DAMAGE (II)

5.3.1 Brain damage in aged infected mice correlates with pre-ischemic plasma RANTES

Assessment of the ischemic damage was carried out using ex vivo MRI. Quantification of MRI images revealed that the infection specifically in aged mice contributes to the exacerbated brain damage when compared to young mice (Study II, Figure 1). However, aging alone had no effect of the size of lesion volume. Likewise, young mice with or without infection did not show any difference in lesion size suggesting that infection has no detrimental role in young cohorts. Administration of *T. muris* has been shown to cause Th-1 polarized immune response and significant upregulation of RANTES that peaks at 4-5 weeks post infection (Dénes et al., 2010). We thus determined the pre-ischemic plasma level of RANTES and checked the correlation of circulating plasma RANTES level with infarct size. Indeed, we observed significant upregulation of RANTES in both young and old infected mice when compared to their uninfected counterparts (Study II, Figure 2A). We also found a significant correlation exists between the plasma RANTES level and infarct size in old ischemic infected mice but not young ischemic infected mice (Study II, Figure 2B).

5.3.2 Levels of plasma GCSF-1, KC and IL-6 increased within hours after stroke

Dysregulation of immune system and inability to respond to the pathological insults are hallmarks of aging as described previously (Dorshkind et al., 2009, Plackett et al., 2004, Raynor et al., 2012). An altered immune system following aging increases the susceptibility of people to infections thus increasing the risk of various insults including ischemic stroke. Thus, we sought to determine the effect of aging and chronic infection on various inflammatory mediators in both plasma and brains of young and old infected mice at 1 and 4 hours post-ischemia. An array of cytokines were analyzed including TNF-α, RANTES, MCP-1, KC, IL-6, IL-1β, IL-1α, IL-17α, IL-10, IFN-γ and GSCF-1 but only plasma levels of GCSF-1, KC and IL-6 was significantly upregulated at 4 hours following ischemia when compared to 1 hour post-ischemia in all study groups irrespective of age and infection.

5.3.3 Brain microgliosis remained unaltered while neutrophil infiltration was highly upregulated in aged infected mice

Brain microgliosis as assessed by immunohistochemical staining using Iba-1 antibody was found to cause a significant upregulation of ischemia induced Iba-1 immunoreactivity in the ipsilateral side when compared to the contralateral side. However, no difference in Iba-1 immunoreactivity was observed in the peri-ischemic area between treatment groups (Study II, Figure 3). Neutrophil infiltration as analyzed by anti-neutrophil antibody revealed that aging with preceding chronic infection causes a massive infiltration of neutrophils in the lesion site (Study II, Figure 4A). However, infection did not alter the level of infiltrated neutrophils in young mice. Aging alone was insufficient to cause increased infiltration of neutrophils. Moreover, the size of the infarction also correlated with neutrophil infiltration (Study II, Figure 4B). Contralateral hemisphere was devoid of any neutrophils.

5.3.4 Peri-ischemic levels of GCSF-1 and MCP-1 was compromised in aged infected mice

Cortical brain samples were dissected 24 hours post-ischemia from all treatment groups and were further analyzed to determine the levels of various cytokines and chemokines including TNF, RANTES, MCP-1, KC, IL-6, IL-1β, IL-1α, IL-17α, IL-10, IFN-γ and G-CSF using CBA kit. Infection alone (independent of aging) was able to upregulate the peri-ischemic levels of G-CSF, MCP-1 and KC in all the study groups even though differential effects were observed in IL-1 α and RANTES in both young infected and young uninfected mice (Study II, Figure 5). Moreover,

ischemia upregulated the cytokines to a greater extent in aged mice when compared to young mice. However, the levels of GCSF-1 and MCP-1 were significantly attenuated in aged mice. Likewise, cytokine levels in plasma were also analyzed 24 h post-ischemia. Of various cytokines analyzed, significant alterations were found in the levels of TNF-α and IL-17α (Study II, Figure 6A and 6B). Infection along with aging was found to cause significant upregulation of TNF-α when compared to young infected or uninfected mice. Aging alone had no effect on the plasma level of TNF. Likewise, old infected mice also exhibited a higher plasma level of IL-17α when compared to young infected but not old or young uninfected.

5.4 SSO PROTECTS AGAINST CEREBRAL ISCHEMIA INDUCED BRAIN DAMAGE (III)

5.4.1 Regulation of phagocytosis and NO release in BV-2 microglial cells by SSO

Microglia are immunocompetent cells with the ability to phagocytose cellular debris following acute insult. However, they also release a number of potential proinflammatory molecules that can harm the surrounding cells during their activation phase. Thus, suppression of microglial activation can be beneficial following ischemic stroke. Here, we tried to determine if the treatment with SSO can reduce the activation of microglial cells following insult or stimulation by LPS *in vitro*. Indeed, SSO was able to reduce the microglial activation at varying concentrations without affecting the cell viability (Study III, Figure 4D and 4F). In addition, microglial stimulation resulted in a rapid increase in NO production (Figure 4E). SSO was able to significantly reduce the release of NO even at low concentrations.

5.4.2 Ischemia induced brain damage in mice is attenuated by SSO

To evaluate the efficacy of SSO on neuroprotection after focal brain ischemia, Balb/CABom mice were subjected to permanent electrocoagulation of MCA and the brain damage was assessed using T2-weight MRI 3 days post-ischemia. Quantification of *in vivo* MRI images revealed that ischemic brain damage was significantly reduced in mice treated with 50mg/kg SSO when compared to the control mice treated with vehicle. Percentage of the infarction volume in treated and untreated mice was 16.5±1.7 and 14.9±1.3 respectively (Study III, Figure 1A and 1B).

5.4.3 Peri-ischemic COX-2 and Iba-1 immunoreactivity were significantly reduced in SSO treated mice

Brain astrogliosis and microgliosis are induced upon ischemia and contribute to secondary ischemic damage. We thus analyzed the brain microgliosis and astrogliosis using Iba-1 antibody and GFAP antibody respectively in both SSO treated and control mice. We found significant upregulation of ischemia induced Iba-1 immunoreactivity in the peri-ischemic area on the ipsilateral side in both treatment groups when compared to the the contralateral side (Study III, Figure 2B and 2G-2J). However, peri-ischemic Iba-1 immunoreactivity was significantly reduced in SSO treated mice indicating the efficacy of the drug in reducing the ischemia induced microgliosis and subsequent production of various inflammatory mediators. Likewise, GFAP immunoreactivity for astrocytosis was also significantly upregulated in the peri-ischemic area on the ipsilateral side when compared to contralateral side in both groups (Study III, Figure 2A and 2C-2F). SSO was unable to alter astrogliosis following stroke. Due to the deleterious role of COX-2 in ischemia, we sought to determine if the levels of COX-2 immunoreactivity are upregulated upon ischemia and whether SSO has a downregulating effect. In fact, following ischemia, levels of COX-2 immunoreactivity were significantly upregulated in the peri-ischemic area of both SSO and vehicle treated mice. In addition to these observations, we detected significant downregulation of COX-2 immunoreactivity in the peri-

ischemic area in SSO treated mice when compared to the same area in vehicle treated mice (Study III, Figure 3A-3E).

5.5 MAIN FINDING OF THE THESIS

Paper	Main Findings
1	a) Aged mice expressing human ApoE4 are more susceptible to sensorimotor deficits upon ischemia when fed on a HF diet. b) In brain, HF diet was able to increase COX-2 activity in ApoE4-TR mice but not ApoE3-TR and WT mice. c) In periphery, plasma level of IL-6 was significantly upregulated in a HF diet fed ApoE4-TR mice when compared to the mice on normal chow. There was no alteration in IL-6 levels in both ApoE3-TR and WT mice. d) Ischemia induced endogenous neurogenesis was impaired in ApoE4-TR mice compared to ApoE3-TR mice that displayed higher neurogenesis.
2	a) Th-1 polarized chronic infection renders the aged animal more vulnerable to ischemic insult when compared to young animals. b) Pre-stroke level of RANTES correlated with infarct size in aged mice. c) Chronic peripheral infection in aged mice was accompanied by massive influx of neutrophils into the lesion site. d) Aged infected mice exhibit attenuated levels of GCSF and MCP-1 in the brain. e) Aged infected mice showed increase plasma levels of IL-17 and TNF-α and this increase might have resulted in rapid influx of neutrophils in lesion area
3	a) SSO was able to reduce the phagocytic activity of BV-2 microglial cells *in vitro* without altering cell viability b) Massive release of NO by phagocytic BV-2 cells following LPS stimulation was significantly attenuated by SSO even at lower concentration. c) Following pMCAo, SSO was found to attenuate the infarct size to a significant extent when compared to vehicle group. d) In addition, SSO was able to reduce the inflammation in the brain by reducing Iba-1 and COX-2 activity

6 Discussion

Stroke is a complex neurodegenerative disease which often leads to long-term hospitalization and in extreme cases the death of the patient. The mortality rate of 1.1 million per year in Europe alone signifies the economic burden caused by the disease. Considering the vast amount of resources that need to be mobilized for both treatment and long-term rehabilitation, a great deal of research has focused on the discovery of therapeutic drugs. To this date, only tPA has been proven to be safe and effective in the treatment of stroke but often only in a limited number of patients. Moreover, the therapeutic time window for the treatment of stroke using tPA is limited to 3-4 hours that hinders the treatment of the patient who arrives at the hospital too late. So, strategies for prolonging the efficacy of the tPA beyond 4 hours' time or alternatives to tPA in the treatment of stroke patients are currently under intensive investigation. In fact, a number of drugs with potential therapeutic efficacy in pre-clinical stroke studies have undergone clinical trials. However, none of the drugs has been able to achieve the safety and efficacy in humans. The reason for the failure might stem from using young homogenous cohorts of animals in preclinical trials while stroke typically affects aged humans suffering from accompanying co-morbid conditions like atherosclerosis, infections, diabetes and obesity (Cheng et al., 2004). The present study aimed to clarify the impact of co-morbid conditions on the pathophysiology of ischemic stroke in preclinical settings with the attempt to better understand how stroke modelling needs to be improved. Specifically, in this study, we studied the impact of ApoE4 on aged mice fed on a HF diet and assessed both behavioral and inflammatory outcome following ischemic stroke. Likewise, the effect of pre-ischemic infection by common gut parasite *T. muris* on the outcome after stroke was also studied. Finally, the efficacy of CD36 inhibition on the mouse model of ischemic stroke outcome was assessed.

6.1 FUNCTIONAL OUTCOME IS ALTERED IN AGED APOE4 MICE FED ON A HF DIET (I)

APOE has an important role in lipoprotein metabolism. However, existence of three different isoforms of ApoE (ApoE2, ApoE3 and ApoE4) has various pathological consequences. Among these isoforms ApoE4 has been extensively studied in various models of neurodegeneration. In this study, we showed that the combination of co-morbid conditions like aging and HF diet renders transgenic mice harboring human APOE ε4 allele prone to sensorimotor deficits following cerebral ischemia compared to those mice fed on a normal diet. In fact, ApoE4 isoforms have been shown to confer the greater risk of developing various neurodegenerative disease including AD, ICH, TBI and CAA (Verghese et al., 2011). A number of studies have demonstrated the relationship of *APOE ε4* with cognitive decline in clinical subjects (Albert et al., 2007, Caselli et al., 1999, Mayeux et al., 2001, Schiepers et al., 2012). In addition, ApoE4 has been linked to a rapid decline in motor function in aged human subjects (Buchman et al., 2009). Considerable efforts have been put forth in understanding the cause of cognitive decline in human subjects harboring ApoE4 using targeted replacement mice in which human ApoE isoforms are replaced by human isoforms. In pre-clinical models, ApoE4-TR mice exhibit significantly fewer and shorter dendritic spines in cortical pyramidal neurons when compared to ApoE3-TR and C57Bl mice suggesting reduced synaptic plasticity underlying learning and memory (Dumanis et al., 2009). In addition, decreased spine density in old age can be attributed to increased spine elimination which further impairs the synaptic plasticity.

However, in the hippocampus, no difference was observed in the spine density among different isoforms. In line with these observations, a recent article by Rodriquez and colleagues found significantly fewer and shorter dendritic spines in the medial enthorinal cortex in ApoE4-TR mice when compared to ApoE3-TR mice. This was associated with poor spatial learning and memory in ApoE4-TR mice at both young and old age as assessed by the Barnes maze (Rodriguez et al., 2013). Moreover, ApoE4-TR mice were also shown to possess lower excitatory synaptic activity in amygdala when compared to ApoE3-TR, C57Bl6, ApoE-ko and ApoE2/4-TR mice thus leading to cognitive impairment (Klein et al., 2010).

The associations between stroke subtypes and ApoE4 have also been studied in both pre-clinical and clinical subjects. In clinical subjects, *APOE ε4* allele has been shown to be associated with poor neurological outcome after SAH and ICH (Lanterna et al., 2005, Martínez-González and Sudlow, 2006, McCarron et al., 2003) but not ischemic stroke. However, other studies point towards the impairment of cognition in the early phase after stroke in carriers with either one or two alleles of *APOE ε4* (Wagle et al., 2009). The progression of the cognitive decline is also faster in aged stroke patients with early cognitive impairment (Ballard et al., 2004, Dik et al., 2000). Thus, ApoE4 can be attributed to poor cognition following stroke.

Western type diet has been implicated in the development of atherosclerosis, obesity, neurodegenerative disease and cardiovascular disease (Francis and Stevenson, 2013). A number of studies have linked the possible relationship between HF diet and cognitive impairment in clinical studies (Kalmijn et al., 2004, Morris et al., 2004). Moreover, Aging and HF diet have been shown to accelerate the decline in cognitive functions (Okereke et al., 2012) even though conflicting data do exist where no association between aging and cognition was reported following high saturated fat diets (Naqvi et al., 2011). However, in animal models, HF diet along with aging has been found to increase hippocampal oxidative stress and cognitive impairment. The cognitive function mainly affected by HF diet involves hippocampus and prefrontal cortex which are the major brain areas involved in memory, attention and learning. HF diet has been found to reduce hippocampal brain derived neurotropic factors, increase oxidative stress and increase the inflammatory process thus affecting neuronal plasticity, learning and memory (Francis and Stevenson, 2013, Molteni et al., 2002, Pistell et al., 2010).

Even though a large number of studies point towards the impairment in memory and cognition following stroke in ApoE4-TR mice, the combined effect of co-morbidities like aging and HF diet is poorly understood in relation to motor function. Given that motor cortex is involved in processing the cognitive information related to sensorimotor function (Bonnard et al., 2004), we aimed to establish the underlying effect of co-morbid conditions in sensorimotor functions following ischemic stroke such that it bears clinical relevance to a sub population of patients. Here, we modelled the impact of HF diet in the context of different ApoE genotypes by feeding 16-month-old ApoE3-TR, ApoE4-TR and C57Bl/6j mice on a HF diet and subjected the mice to pMCAo. For the first time, we observed significant sensorimotor deficits in ApoE4-TR mice fed on a HF diet compared to ApoE3-TR mice on the same diet independent of the infarct size. Thus, the expression of ApoE4 isoforms along with HF diet is more likely to interfere with the recovery following brain infarction. The superior performance of ApoE3-TR mice when compared to ApoE4-TR mice and WT C57Bl mice on the adhesive removal test might be due to the complex branching pattern of dendrites and enhanced excitatory activity of ApoE3-TR which is not reduced even upon aging (Klein et al., 2010). ApoE4-TR mice exhibit reduced long term potentiation and poor spatial learning and memory (Rodriguez et al., 2013). Now we show that they also display poor sensory-motor outcome upon stroke. Endogenous repair mechanisms are activated following stroke to compensate the lost neuronal function. It has been shown that ApoE4 isoforms dampen neurogenesis in the hippocampus (Li et al., 2009). Specifically, neuronal maturation and development of hippocampal neurons in ApoE4-TR mice is impaired and this impairment was shown to occur due to the reduced survival and function of GABAergic interneurons in the hippocampus (Li et al., 2009). Thus, we sought to explore

whether neurogenesis is impaired in ApoE4-TR animals. To our knowledge, the neurogenesis was also reduced in ApoE4-TR mice compared to ApoE3-TR mice in the sub ventricular zone suggesting a better ability of ApoE3-TR mice to cope with ischemia induced brain damage.

In preclinical studies, ApoE4 genotype has been found to increase brain damage following pMCAo and ischemia-reperfusion model (Mori et al., 2004, Sheng et al., 1998). However, in our model we were not able to find any differences in the infarct size between the genotypes. The reason for the contradiction between these studies and our study is most likely due to the model. In fact, our model of pMCAo produces a small and strictly cortical lesion while the model used by Mori and colleagues involved transient occlusion of CCA and thus resulted in bigger ischemic damage. In order to validate and exclude the effect of aging, we also used young ApoE4-TR and C57BL/6j mice and subjected them to pMCAo. However, young ApoE4-TR animals also showed similar infarct size compared to the old animals used in this study. In addition, the infarct size in young ApoE4-TR and WT mice was relatively the same.

Overall, our main finding in this study was that aged ApoE4-TR mice fed with a HF diet showed increased deficits in motor coordination compared to ApoE3-TR mice as assessed by the adhesive removal test and this observation is supported by a number of studies showing detrimental effects of ApoE4 genotype in neurodegenerative diseases.

6.2 CNS INFLAMMATION IS INFLUENCED BY CO-MORBIDITIES (I, II)

Following ischemic stroke, a number of molecular and cellular inflammatory mediators are either upregulated or downregulated. Inflammatory cells, mainly microglia and astrocytes, get activated in the early phase of stroke and contribute to the outcome of stroke. Microglial activation occurs within hours following stroke and they transform to a phagocytic phenotype in order to clear dead cells and debris (Perego et al., 2011). In addition, they also release a number anti-inflammatory cytokines and growth factors for maintenance and removal of injured neurons. In addition, microglia secrete pro-inflammatory cytokines, ROS and NO, thereby exacerbating secondary brain damage (Gehrmann et al., 1995). In addition to microgliosis, astrogliosis is an important contributor of the ischemic outcome through secretion of various pro-inflammatory mediators (Lakhan et al., 2009).

In our study with ApoE-TR mice (I), we found significant induction in microgliosis in the peri-ischemic area in all genotypes when compared to the contralateral side according to the published literature. However, HF diet had no effect on the peri-ischemic activation of microglia in any of the phenotypes tested; however, it was indeed interesting to find higher levels of microglial activation in ApoE4-TR when compared to C57Bl mice. Aging is accompanied with the atrophy of both white and gray matter due to the loss of neurons. In contrast, glial cells get activated in an aging brain. ApoE4-TR mice have been shown to have higher production of NO through utilization of arginine by microglia when compared to ApoE3-TR mice thus adversely affecting the nearby neurons (Brown et al., 2002, Czapiga and Colton, 2003). Astrocytosis was also significantly induced in the peri-ischemic area of all the genotypes tested at both 3 and 10 day post-ischemia. However, HF diet further actuated the astrogliosis in ApoE3-TR and ApoE4-TR but not in WT mice at 3 days post-ischemia with no difference at 10 days post-ischemia. In addition to its protective role, astrocytes are known to release a variety of neurotoxic inflammatory molecules and even glutamate which might potentially contribute to the neuronal damage (Zhao and Rempe, 2010). Our results showing that HF induced astrocytic activation are in agreement with a study showing that high cholesterol diet increased astrogliosis in the cerebral cortex of naïve mice (Crisby et al., 2004). In addition, Badan *et. al.* showed that generation of glial scar was accelerated in the aged rats and correlated with reduced functional recovery (Badan et al., 2003). Their results together with

data obtained from our study suggest that aging and HF diet typical of western cuisine leads to the altered glial activation after stroke in animals carrying different ApoE isoforms.

In addition to microgliosis and astrogliosis, inflammatory enzymes like COX-2 are also involved in the development of brain damage. Following ischemic injury and other forms of neuronal injury in the brain, COX-2 catalyzes the conversion of arachidonic acid to prostaglandins which together with prostaglandin receptor mediate the toxic effects in the brain (Andreasson, 2010). In addition, a HF diet has been found to promote neural inflammation in the cerebral cortex in naïve mice through dramatic increase in expression of COX-2 and thus prostaglandin E_2 (Zhang et al., 2005). We thus tried to elucidate the effect of COX-2 on HF diet and cerebral ischemia in the context of APOE background. We found ischemia induced upregulation of COX-2 immunoreactivity in the peri-ischemic area in all treatment groups except ApoE3-TR mice on a normal diet. HF diet significantly actuated the COX-2 immunoreactivity in ApoE4-TR mice but not in WT and ApoE3-TR mice even though there was a trend towards increased COX-2 activation on a HF diet fed treatment group. We thus hypothesize that COX-2 is involved in HF diet induced neural inflammation in ApoE4-TR mice following ischemia.

Pre- and post-ischemic infections are also co-morbid risk factors that negatively affect the outcome after stroke. Pre-ischemic infections increase the risk of stroke while post-ischemic infections are associated with aggravated brain damage and hence poor neurological outcome. Stroke incidence is increased during the season with high occurrence of respiratory and urinary tract infections and both acute and chronic infections originating from bacteria, virus and fungus are thought as possible triggers for acute ischemic stroke (Grau et al., 2010). Chronic infections by *H. Pylori*, *C. pneumoniae* and influenza virus in clinical subjects are associated with stroke risk and the risk increased with the number of chronic infections (Emsley and Tyrrell, 2002, Grau et al., 2010). The contribution of chronic pre-stroke infection by influenza virus and *T. muris* in the exacerbation of brain injury has been demonstrated in pre-clinical models as well (Dénes et al., 2010, Muhammad et al., 2011). These studies showed the impact of peripheral infections and inflammation in young animals. When modelling a disease of the elderly, one should bear in mind that aging is a non-modifiable risk factor for stroke and is known to be associated with altered immune response following stroke (Popa-Wagner et al., 2007, Sieber et al., 2011). We thus aimed to investigate the impact of aging on ischemic stroke following chronic infection (II). In this study, chronic peripheral infection was modelled by administration of *T. muris* parasite eggs to young and agedC57Bl/6j mice. This model induces Th-1 polarized immune response. Here, we demonstrated that infarct size in aged mice was similar to young mice and that chronic peripheral infection markedly increased ischemic brain damage specifically in aged mice. Aging is associated with a dysregulated immune system and chronic infection and inflammation are more often present in older people. Thus, the aggravated ischemic damage seen in this study bears clinical relevance.

As discussed earlier, neuroinflammation after stroke is associated with glial cell activation and upregulation of a number of cytokines and chemokines that are often produced by these inflammatory cells. However, the effect of chronic infection and aging on neuroinflammation following ischemic stroke is not well documented. We thus investigated the glial activation and activation of various inflammatory mediators in aged ischemic mice subjected to chronic peripheral infection. In this study, brain microgliosis was significantly upregulated in the peri-ischemic area of all treatment groups when compared to contralateral sides. However, brain microgliosis was not altered between any of the treatment groups. Glial cells are not the sole source of inflammation in the brain following neurodegeneration and other types of cells are also responsible for the production of various inflammatory mediators. Moreover, quantification of stroke induced Iba-1 and GFAP immunoreactivities do not necessarily reflect subtle changes that may occur at the cellular level and may still have a significant impact on the outcome of stroke. We thus analyzed the inflammatory profile of the ischemic brains by using

CBA at 24 hours post-ischemia. In this analysis, the levels of G-CSF, MCP-1, KC, IL-1α and RANTES were elevated after ischemia in aged mice when compared to their young counterparts. Surprisingly, aged mice with chronic peripheral infection failed to upregulate G-CSF and MCP-1 to similar levels as seen in aged uninfected mice suggesting the dysregulation of an immune response in aged infected mice. G-CSF is a member of the cytokine family of growth factors that was used initially to treat neutropenia (Nguyen, 1994) but was later shown to be upregulated in the peri-ischemic area following stroke (Schneider et al., 2005b). In an experimental model of stroke, G-CSF was shown to reduce infarct size and early neurological outcome most likely through inhibition of apoptosis mediated by caspase 3 and prevention of pro-inflammatory cytokine expression (Görgen et al., 1992, Solaroglu et al., 2009). G-CSF was shown to confer neuroprotection directly by binding to its receptors G-CSFR which in turn activates signal transducer and activator of transcription 3 (STAT-3) proteins. STAT-3 protein then mediates anti-apoptotic functions by activating Bcl-2 (Schäbitz et al., 2003a, Schäbitz et al., 2000). In addition, G-CSF confers neuroprotection through stem cell mobilization and promotion of neurogenesis (Schneider et al., 2005a, Sehara et al., 2007). Given these facts, a lower level of G-CSF in the peri-ischemic area of the brain found in aged infected mice may correlate with increased neutrophils infiltration into the brain. We thus performed immunohistochemical staining for neutrophil and found that there is a massive infiltration of neutrophils in the lesion area in aged infected mice. In addition, we also saw decreased levels of G-CSF in the peri-ischemic area. Thus, we can speculate that dysregulation of G-CSF in the brain following ischemic stroke in aged infected mice is accompanied by massive neutrophil infiltration and thus aggravated brain damage in our model. Neutrophils are amongst the first leukocytes to infiltrate into the brain parenchyma in response to ischemic stroke and are shown to exacerbate ischemic neuronal damage through MMP-mediated BBB damage, release of variety of pro-inflammatory cytokines and chemokines and excessive production of ROS (Jin et al., 2010). MCP-1 that is secreted by neurons and astrocytes following ischemic stroke is a known contributor for neuronal damage (Che et al., 2001). MCP-1 deficiency has been demonstrated to impair the expression of G-CSF in a mouse model of transient MCAo (Strecker et al., 2011) and thus we can hypothesize that the decreased levels of MCP-1 and G-CSF together with increased neutrophil infiltration in aged infected mice leads to aggravated brain damage.

6.3 CO-MORBIDITIES ALTER THE SYSTEMIC INFLAMMATORY STATUS IN THE PERIPHERY BOTH PRE- AND POST-ISCHEMIA (II)

It is a well-known fact that central inflammatory responses play an important role in ischemic brain damage. Pre-existing peripheral inflammation induced by co-morbid conditions such as hypertension, infection, atherosclerosis and diabetes also increase the risk of brain damage. Inflammatory conditions associated with atherosclerosis and rupture of atherosclerotic plaques is amongst the leading causes of stroke. In addition, preceding infections of bacterial, viral and fungal origins modify the systemic inflammatory status in the periphery and hence contribute to the ischemic brain damage. In fact, acute infection of bacterial origin mostly affecting respiratory and urinary tract has been shown to increase the risk for stroke (Clayton et al., 2008, Smeeth et al., 2004). Likewise, post-ischemic peripheral inflammation also contributes to the aggravated brain damage and neurological outcome (Kwan et al., 2013, Wartenberg et al., 2011, Westendorp et al., 2011). Following cerebral ischemia, a number of inflammatory cells of peripheral origin including neutrophils and monocytes infiltrate into the brain parenchyma and produce an array of cytotoxic mediators involved in exacerbation of the brain damage (Buck et al., 2008, Emsley et al., 2003). In addition, adhesion molecules like VCAM-1, ICAM-1, selectins and integrins that are required for the transmigration of leukocytes are also upregulated

following ischemia. Moreover, plasma CRP, IL-6 and leukocyte counts are elevated following stroke and correlated with the outcome after stroke (Basic Kes et al., 2008, Muir et al., 2007, Waje-Andreassen et al., 2005).

Peripheral infections accompanied with systemic inflammation have been shown to exacerbate brain damage in the rodent model of focal ischemia (Dénes et al., 2010, Muhammad et al., 2011). Both these studies utilizing young animals point to RANTES as the mediator of chronic infection-induced stroke exacerbation. In this study, we found a significant elevation in plasma levels of RANTES in both young and old infected mice 1 day pre-ischemia in agreement with previous studies. In addition, we also found a significant correlation between circulating pre-ischemic RANTES levels and the infarct size in aged infected mice. Levels of RANTES were also elevated in the brain following ischemia in both old infected and uninfected mice when compared to the contralateral hemisphere. Both aged infected and uninfected mice exhibited higher levels of RANTES following ischemia thus encompassing the adverse effect of RANTES following ischemic events.

In addition, we evaluated the impact of aging and infection on peripheral cytokines and chemokines in plasma at 1 and 4 hours post-ischemia. Of various cytokines analyzed, we found that the levels of G-CSF, KC and IL-6 were significantly induced at 4h post-stroke when compared to 1h post-stroke in all study groups indicating the insignificance of aging and infection at these time points. Indeed, IL-6 was found to be sharply increased following stroke in a rodent model of cerebral ischemia independent of infection (Dénes et al., 2010). We can thus speculate that IL-6 levels sharply rise following ischemia irrespective of the preceding infection or that preceding infection has no effect on the levels of IL-6. IL-6 has been demonstrated to be upregulated in the acute phase of stroke in human subjects and this upregulation correlates with the larger infarct size and poorer neurological outcome (Emsley et al., 2003, Smith et al., 2004, Whiteley et al., 2009). Thus, an increase in level of IL-6 following stroke is related to ischemic insult itself and not infection.

Twenty-four hours after ischemia, plasma levels of proinflammatory cytokines such as TNF-α and IL-17A were significantly increased in aged infected mice. TNF-α is a well characterized proinflammatory mediator implicated in the pathogenesis of ischemic stroke (Hallenbeck, 2002). TNF-α levels in the periphery have been shown to be increased in both preclinical and clinical subjects following ischemic stroke and might serve as a potential inflammatory biomarker (Sotgiu et al., 2006, Yousuf et al., 2013). IL-17A, on the other hand, is secreted by multiple cell types including CD4+ $\delta\beta$ T cells, $\gamma\delta$ T cells, natural killer cells and neutrophils (Roark et al., 2008). IL-17A has been shown to be a critical mediator of neutrophil infiltration and recruitment in ischemia reperfusion injury in hepatic injury (Kono et al., 2011) as well as in a cardiac transplantation model (Zhu et al., 2013). In cerebral ischemia, IL-17A mainly produced by $\gamma\delta$ T cells in the brain has been shown to be involved in neutrophil infiltration to the site of ischemic damage and the blockage of IL-17A pathway is associated with reduced brain damage and improved clinical outcome (Gelderblom et al., 2012). This effect is mainly mediated by astrocyte derived CXCL-1. In addition, $\gamma\delta$ T cells have been shown to infiltrate into the ischemic brain parenchyma from the periphery at the later stages of the injury where they contribute to the increased levels of IL-17A in the brain (Li et al., 2005, Shichita et al., 2009). In our study, we detected increased plasma levels of IL-17A in aged infected mice when compared to young infected mice possibly reflecting increased activation of $\gamma\delta$ T cells. Indeed, one study has reported that aging together with infection in mice is associated with a rapid increase of peripheral IL-17A and neutrophil activation in a case of liver injury (Stout-Delgado et al., 2009). Even though we didn't examine $\gamma\delta$ T cells in the brain and periphery, we can hypothesize that increased plasma levels of IL-17A in aged infected mice may be one of many reasons leading to increased infiltration of neutrophils in these mice when compared to other treatment groups.

Other co-morbid conditions like atherosclerosis and increased cholesterol levels in plasma also alter the outcome before and after stroke. ApoE, especially ApoE4, is pro-inflammatory and

is associated with increased risk of atherosclerosis that ultimately determines stroke outcome. Inclusion of HF diet to this already pre-existing genetic risk factor for atherosclerosis in aged mice has been the focus of our study. In study I, we assessed the levels of cholesterol after HF diet in ApoE3-TR, ApoE4-TR and WT mice. We found that ApoE4-TR in general has lower levels of total serum cholesterol compared to ApoE3-TR mice. These results are in contrast to the previous reports where VLDL cholesterol have been found to be lower in young female ApoE3-TR mice compared to ApoE4-TR mice of the same sex (Knouff et al., 1999). The levels of total cholesterol were elevated to a similar extent in both ApoE3-TR and ApoE4-TR mice but not in WT mice. In agreement with previous observations, ApoE3-TR had a significantly higher amount of cholesterol compared to WT mice (Sullivan et al., 1997). However, the increase in serum cholesterol did not correlate with the atherosclerosis plaque or foam cell formation in ApoE-TR mice fed on a HF diet in contrast to previous observations (Knouff et al., 1999, Sullivan et al., 1997). This might be attributed to differences in sex, diet and age of the animals used in these studies. In fact, the diet used in our study is better representative of western type diet typical to human cuisine where the diet used by them was high in cholate. Thus, clear atherosclerotic changes did not play any role in our study. However, following stroke, a number of peripheral cytokines and chemokines are upregulated that might have affected the outcome. We determine the cytokine levels in plasma using CBA assay. Of various cytokines analyzed, we detected a clear increase in IL-6 in ApoE4-TR mice fed on a HF diet compared to a normal diet. Peripheral IL-6, unlike brain IL-6, has been shown to be associated with a worse cognitive outcome in elderly people and in APOE ε4 carrier (Economos et al., 2013, Schram et al., 2007). Thus, we can hypothesize that APOE ε4 can modify peripheral inflammatory response and affect cognition in CNS diseases.

6.4 CD36 CAN BE A POTENTIAL THERAPEUTIC TARGET FOR TREATING ISCHEMIC STROKE

Most of the clinical trials involving a single drug to target a single pathway in the treatment of ischemic stroke have often failed due to the heterogeneity of stroke pathophysiology. However, a strategy to target a single molecule that is involved in multiple pathogenic mechanisms in stroke is not well understood. CD36 is one such receptor that is involved in atherosclerosis, lipid metabolism, inflammation and ischemic stroke (Cho and Kim, 2009). Following ischemia, various ligands for CD36 including oxLDL, TSP and LCFA are produced which might contribute to brain injury (Cho and Kim, 2009). Thus utilizing therapeutic drugs with an aim to inhibit CD36 might provide overall protection against ischemic damage. The deleterious role of CD36 in cerebral ischemia was first shown by Cho *et. al.*. They observed a 50% reduction in infarct volume in CD36 null mice compared to WT mice in a transient filament occlusion model. However, a therapeutic drug to suppress the activity of CD36 has not been addressed up to now and is the focus of this study. SSO, a LCFA, is a specific inhibitor of CD36 and is involved in the inhibition of fatty acids uptake by adipocytes (Harmon et al., 1991). In addition, SSO was found to inhibit signaling for intracellular calcium and restrict uptake of ox-LDL by macrophages (Kuda et al., 2013). Given the inhibitory effect of SSO on CD36 and lack of studies using pharmacological inhibition of CD36 in cerebral ischemia, we aimed to determine if SSO could, in fact, attenuate ischemic brain injury following pMCAo.

In this study, we found that oral administration of SSO following ischemia was neuroprotective against brain damage as analyzed by MRI. Our results are in agreement with the previous findings showing that CD36 deletion can have a neuroprotective effect following ischemia even though the approach was different. Several studies have linked microglia and ROS production in cerebral ischemia via NADPH oxidase, cytokines and MMP-9 (del Zoppo et al., 2007, Jin et al., 2010, Pun et al., 2009). Considering that CD36 is predominantly expressed in

microglia/macrophage in the ischemic territory at early phase after stroke, Cho et al. proposed that CD36 mediates ischemic damage via ROS production (Cho et al., 2005). We also assessed whether treatment with CD36 inhibitor SSO would reduce the stroke induced activation of microglia and astrocytes. We show that the microgliosis was significantly attenuated in SSO treated mice compared to control mice. Thus, the neuroprotection seen in our study might be due to reduced microglial activation and subsequent ROS production even though we did not analyze ROS production in our study. We also assessed the phagocytic activity of microglial BV-12 cells *in vitro* using latex bead phagocytosis and the capacity of SSO to reduce microglial activation. As expected, phagocytic capacity of microglial cells was reduced by SSO. In addition, there was a massive decrease in the release of NO by these cells in higher concentrations of SSO. Thus, we can stipulate that ROS alongside reactive nitrogen species produced by microglial cells might contribute to the brain damage that we see *in vivo*. In addition to glial cell mediated inflammation, we also analyzed whether inflammatory enzyme COX-2 is involved in the mediation of ischemic brain damage. Here, we found that COX-2 immunoreactivity was significantly higher in control mice compared to SSO treated mice. These results indicate that SSO may restrict formation of proinflammatory eicosanoids by inhibiting COX-2 activity. In fact, CD36 has been shown to contribute the generation of proinflammatory eicosanoids *in vitro* and our observations *in vivo* further verify the fact (Kuda et al., 2011). *In vitro*, CD36 has also been linked to regulation of membrane calcium influx in response to ER stress and SSO was shown to inhibit calcium signaling by binding to CD36 lysine 164 (Kuda et al., 2011, Kuda et al., 2013). Taken together, we show that CD36 is involved in a number of pathological processes taking place upon ischemic insults and inhibition of CD36 by SSO may provide a treatment paradigm for ischemic stroke

6.5 ADVANTAGES AND DISADVANTAGES OF ANIMAL MODEL USED IN THIS STUDY

There are a number of ways in which both permanent and transient ischemia can be induced in rodent models of stroke. Examples include insertion of the suture, electrocoagulation, endothelin-1 injection, and photothrombosis etc. However, none of these models reflectsthe true clinical situation. Permanent occlusion where there is no recanalization occurs in a very small proportion of population (Jin et al., 2010). Likewise, transient ischemic models involve occlusion of the artery for a short duration whereas human ischemic stroke is of longer duration before spontaneous recanalization (Fisher et al., 2009). Thus, the use of animal models depends on the specific question raised. For example, effect of a thrombolytic drug can be studied using thromboembolic stroke; anti-inflammatory drugs can be tested in permanent focal ischemia; and a free radical scavenger can be used to test in transient ischemia (Macrae, 2011). In addition, the mouse model should reflect the heterogeneous risk factors associated with clinical stroke to test the efficacy of neuroprotective compounds. This can be achieved by inducing co-morbidity in aged animals.

In this study we used permanent methods to occlude MCA. This model provides good reproducibility of the lesion size and the successful occlusion of the MCA can be visually confirmed. In addition, mortality rate of the animals is very low thus reflecting the need to use less animals compared to transient models where mortality rates are often high depending on the duration of occlusion (Macrae, 2011). In addition, the surgical procedure is relatively quick thus minimizing the amount of time the animal spends under neuroprotective anesthetics (Seto et al., 2014). The major drawback of this model is that the ischemic surgery requires craniotomy.

The Stroke Therapy Academy Industry Roundtable (STAIR) has emphasized the use of animal models with accompanying co-morbid risk factors before testing the efficacy of drugs in clinical subjects (Fisher et al., 2009). However, there are only a handful of studies that have been

published so far focusing on heterogeneity of ischemic stroke. In this thesis, we aimed to do that by modelling co-morbidities that are typically present in humans. We have shown that the inflammatory status in the brain and periphery of aged animals with predisposing risk factors are completely different than observed in young animals. In fact, this might be an important determinant for the clinical failure of several neuroprotective drugs. Even though we tried to mimic some aspect of the human stroke in preclinical model, the model used in this study reflects only two of the many conditions. For example, some cases of human stroke are often presented with predisposing infections prior to the ischemic event while other cases might present diabetes, hyperlipidemia and other risk factors. Thus, there is no universally accepted animal model of cerebral ischemia, and the usability of the models depends upon the compromise and specific questions that are often asked before designing the experiment (Howells et al., 2010).

7 Summary and Conclusions

The present study was carried out to assess the role of various co-morbid conditions on the outcome of ischemic stroke. The influence of western-type diet on aged mice expressing E3 and E4 isoforms of human ApoE was assessed. The effect of pre-disposing infection using gut parasite was studied in both aged and young mice with an emphasis on inflammation. In addition, the therapeutic potential of SSO in ischemic stroke was explored. The results obtained from these studies are listed below.

I. In study I, we demonstrated that aged ApoE4 mice fed a HF diet are more susceptible to sensorimotor deficits when compared to aged ApoE3 and -WT mice on the same diet following pMCAo. Those deficits were most likely due to the increased peripheral inflammation contributed by IL-6. In addition, impaired neurogenesis, increased COX-2 enzyme and increased astrogliosis in the CNS may have contributed to the worse outcome after stroke.

II. In the second study, we demonstrated that aged mice subjected to predisposing chronic infection by gut parasite *T. muris.* displayed larger ischemic damage and this damage correlated with the pre-stroke level of plasma RANTES. Infected aged mice also displayed significant infiltration of neutrophils into the infarct core along with increase of plasma levels of IL-17α and TNF-α when compared to the young infected and uninfected animals.

III. In study III, we assessed the effect of SSO administration on the outcome of ischemic stroke in young mice. We observed that per oral treatment of mice immediately after pMCAO protected the brain from ischemic damage. This protection might be due to reduced microgliosis and COX-2 expression in the brain. In addition, the protection may be explained by the reduction in NO as demonstrated by *in vitro* experiments.

In summary, we have demonstrated that the inflammatory status in the brain and periphery in aged mice under co-morbid conditions are completely different to those of a homogenous cohort of young mice (Figure 6). We show that ApoE allele modifies the inflammatory status in the brain and the periphery thus contributing to functional outcome after stroke. In addition, our results show that chronic peripheral infection in aged animals renders the brain more susceptible to ischemic damage possibly through altering the inflammatory status in both brain and periphery. Finally, SSO was able to protect the brain from damage following ischemia in young mice by modifying the inflammatory status in the brain. Based on these results we see that the age of animals used for preclinical experiments affects the results obtained. We also know that aging along with other co-morbid conditions carries the highest risk of stroke. Therefore, this study bears clinical significance when designing drug studies in the future.

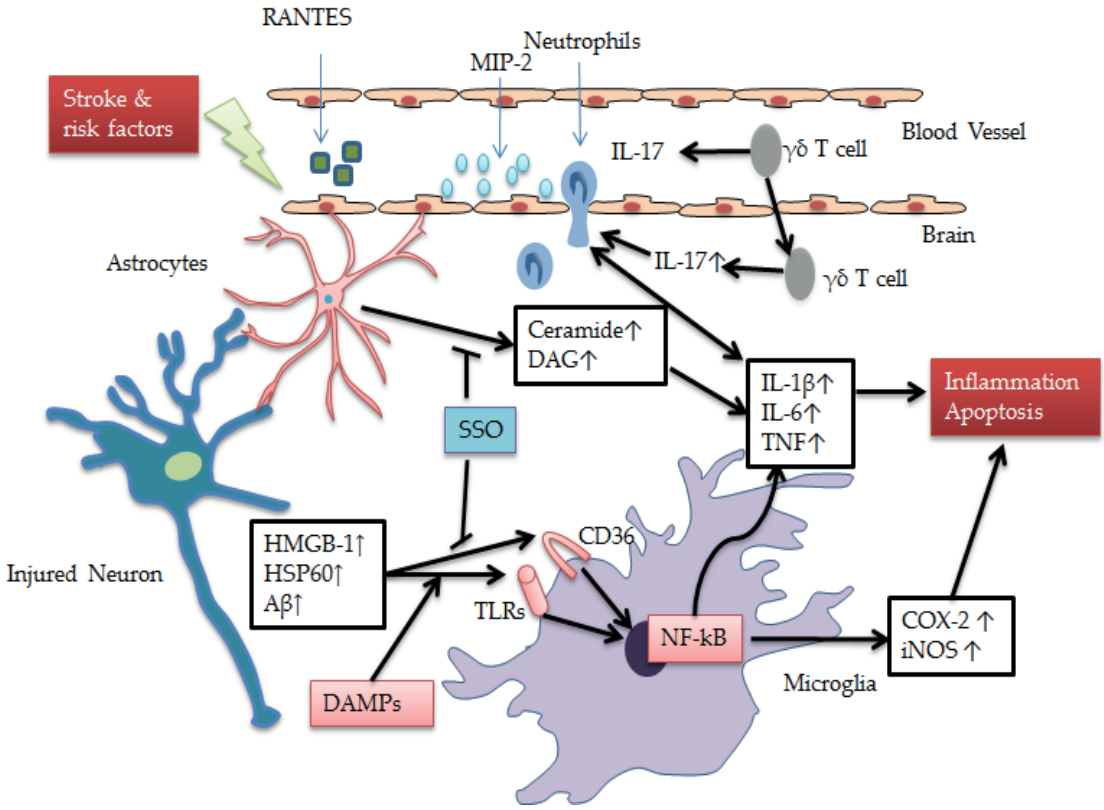

Figure 6: Role of stroke associated risk factors on inflammation and proposed therapeutic target of SSO. Stroke accompanied by infections and other risk factors increases RANTES level in the peripheral circulation. The circulating RANTES then induces MIP-2 and ultimately facilitates the transmigration of neutrophils into the ischemic territory. At the ischemic site, microglia, astrocytes, neurons and infiltrating leukocyte secrete various mediators thus promoting inflammation and apoptosis. Astrocytes secrete ceramide and diacylglycerol following early ischemic event that can increase the amount of proinflammatory cytokines via NF-kB pathway. In addition, injured neurons promote CD36 induction. SSO is proposed to provide the neuroprotection following ischemic stroke via inhibition of CD36 and decreased production of ceramide. Abbreviations: RANTES, Regulated on activation, normal T cells expressed and secreted; MIP-2, Macrophage inflammatory protein 2; DAG, Diacylglycerol; IL, Interleukin; HMGB-1. High-mobility group protein B1; HSP60, Heat Shock Protein 60; Aβ, Amyloid beta; DAMPs, Danger associated Molecular Patterns; TLRs, Toll like Receptors; CD36, Cluster of differentiation 36; NF-kB, Nuclear factor kappa beta; COX-2, Cyclooxygenase 2; iNOS, inducible nitric oxide synthase; TNF, Tumor necrosis factor

8 Future Perspective of Stroke Modelling and Neuroprotective Treatment Remedies

Preclinical trials are the basis for translation of therapeutic strategies into clinical subjects. During the past 20 years or so, many drugs targeting various pathways in the ischemic cascade have been carried out in animal models and later tested in humans. However, they have often failed with little or no success (Minnerup et al., 2012). We can point out many reasons for the failure in the translation. First, the pathophysiological mechanism contributing to the ischemic damage in humans might be more complex than that of those in animal models (Endres et al., 2008). Second, stroke typically occurs in old age when patients often suffer from various co-morbid conditions like obesity, diabetes, atherosclerosis, and infections just to name a few. However, most of the preclinical research is still being carried out in young, homogenous cohorts of animals with no accompanying risk factors. Third, the model of stroke that we use to limit the blood flow to the brain may not be good when considering the heterogeneous nature of human stroke.

Then how to model stroke properly? There is no particular answer to this question. Ischemic damage in humans comes in many shapes and sizes considering the heterogeneity of the cerebral vasculature. However, occlusion of MCA is the most common type of human ischemic stroke. In animal models, this is achieved by either permanent ligation of MCA or transient occlusion of the blood flow for a desired duration as discussed elsewhere (Howells et al., 2010). In addition, the thromboembolic model in which murine thrombin is pneumatically injected into MCA of the mouse is also slowly gaining traction (Orset et al., 2007). The fact that thrombin is used to clot the blood right at the MCA bifurcation is a very relevant approach as it better represents the clinical situation. However, all these animal experiments are done at the expense of anesthetics. There are a number of studies where neuroprotective effects of various anesthetics have been demonstrated, and these are sure to affect the final outcome after stroke. So, an approach to keep the time of anesthesia to a minimum or development of occlusion model in which the animal can be awake without being invasive can provide the direction for the future (Seto et al., 2014). However, the ultimate occlusion method used for any study depends on the specific question that is addressed.

In addition, to better model human stroke, co-morbid conditions should be taken into account and it is often a neglected fact in most preclinical studies (Casals et al., 2011, Howells et al., 2010, Mergenthaler and Meisel, 2012). Aging is one of the non-modifiable risk factors for stroke that is often accompanied by various pathological complications. In this thesis, we tried to model these complexities in aged mice and came to the conclusion that discrepancies do exist between aged and young mice when using the same method of occlusion. Thus, failure to include the risk factors often seen in clinical conditions will ultimately result in a failure in translational research. So, a better approach to study the therapeutic efficacy of a drug would be to include the animal model of co-morbidities.

Finally, most of the drugs that are used in the preclinical trial only target a single ischemic cascade whereas ischemic stroke involves multiple overlapping ischemic cascades. So the use of combinational therapy targeting different mediators of the ischemic event might be a suitable treatment strategy for stroke treatment (Culmsee et al., 2004). Alternatively, molecules capable of targeting the multiple cellular mechanisms involved in the ischemic event can also be helpful. In this regard, CD36 is one of the receptors involved in various pathophysiological processes ranging from atherosclerosis, inflammation to ischemic stroke (Cho and Kim, 2009). In this study, we also aimed to inhibit CD36 using its inhibitor, SSO, in an attempt to modulate

the inflammatory response and have it shown that targeting this receptor may provide neuroprotection following ischemia.

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Hiramani Dhungana

Modelling of Ischemic Stroke: Focus on Co-morbidities and Therapeutic Intervention

Stroke is characterized by sudden disruption of blood flow into the brain resulting in disability and death. This thesis focuses on modelling the heterogenic conditions of human stroke in preclinical settings. This study demonstrates that preceding infection, diet and genetic composition of the animal models alter the ischemia induced inflammatory status especially in aged mice. Our data pinpoint the need to consider heterogeneity of human stroke in preclinical settings when testing new potentially neuroprotective drugs.

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