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Approximated Control Affine Dynamics Mode For an Agricultural Field Robot Considering Wheel Terrain Interaction

Pablo Menendez-Aponte
University of Central Florida

NEUROLOGICAL PROFILE OF OLDER APOE-PON1 DOUBLE
KNOCKOUT MICE

by

CONNIE MITRA
Bachelor of Science, Bangalore University, India, 2005
Master of Science, Utkal University, India, 2007

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Major Professor: Sampath Parthasarathy

ABSTRACT

Atherosclerosis is a cardiovascular disease where plaques made up of lipids in the form of cholesterol ester build up in the carotid and innominate arteries that supply blood to the brain. Accumulation of the plaques limit the flow of blood and nutrients to the brain, leading to diminished oxygen supply, increased oxidative stress and cell death. All these have been implicated in Alzheimer's disease (AD). Alzheimer's disease, a chronic, progressive, age related neurodegenerative disorder is the most common form of dementia in the elderly accounting for 60-80% of the cases. Clinically, Alzheimer's disease is characterized by loss of memory, damage of brain tissues, and neuronal and synaptic loss. Pathologically, it is delineated by accumulation of amyloid beta and tau proteins forming senile plaques and neurofibrillary tangles respectively. Apolipoprotein E (ApoE) polymorphism, increased oxidative stress and products of lipid peroxidation are associated with atherosclerosis and Alzheimer's disease. ApoE is a glycosylated protein that mediates plasma lipoprotein metabolism. ApoE isoforms have differential effect on amyloid beta aggregation and clearance, thus playing an important role in Alzheimer's pathology. Serum paraoxonase 1 (PON1) is a lipoprotein associated antioxidant enzyme that prevents lipid peroxidation. S100B protein is a plasma biomarker, altered expression of which has been implied in AD. We propose the hypothesis that combined deficiencies in apolipoprotein E and antioxidant defense (established by the lack of PON1), together with dyslipidemia and development of carotid

atherosclerosis in aging mice would reflect Alzheimer's pathology. The brains of young and old ApoE-PON1 double knockout (DKO) mice and control C57BL/6J mice were harvested. Atherosclerotic lesions were quantified by Image J. RNA was isolated, cDNA was synthesized and quantitative RT-PCR was performed to detect mRNA levels of S100B. Blood levels of S100B protein was measured by ELISA. Brain tissues were stained with Hematoxylin and Eosin stain and 4G8 immunostain to detect histopathological changes. The blood brain barrier (BBB) is altered in AD resulting in increased permeability and vascular dysfunction. The vascular permeability of BBB was analyzed by Evans Blue Dye (EBD) assay.

The results showed that the older DKO mice had severe carotid atherosclerosis, increased levels of serum S100B protein and elevated mRNA levels of S100B. Histological examination showed the presence of characteristic hallmarks of AD. The leakage of EBD into brain parenchyma indicated disruption of BBB. The results suggest that diminished blood flow and nutrient supply to the brain due to atherosclerosis and increased oxidative stress might contribute to Alzheimer's pathology. We suggest that older ApoE-PON1 DKO mice may serve as a model of Alzheimer's disease and prevention of atherosclerosis might promote regression of Alzheimer's disease.

To the three P's of my life

Partha Sarathi Mitra

Pragnyadipta Sen

Sampath Parthasarathy

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CHAPTER 1: INTRODUCTION

1.1 Atherosclerosis

Atherosclerosis represents a major form of cardiovascular disease where plaques build up in the arteries that supply blood to the brain. These arteries include carotid and innominate arteries. Plaques made up of lipids in the form of cholesterol ester accumulates and narrows the arteries. Such atherosclerotic manifestation results in reduced supply of blood and nutrients to the brain, which leads to diminished oxygen supply (Kim *et al.* 2009), increased oxidative stress and cell death. This has been implicated in neurological diseases such as Alzheimer's disease (Dantoine *et al.* 2002, Cervellati *et al.* 2015, Irizarry 2004, Menini *et al.* 2014).

1.2 Rationale

There are a number of animal models to study atherosclerosis, of which two models have gained importance. They are the low density lipoprotein receptor (LDLR) knockout mice and the Apolipoprotein E (ApoE) knockout mice. These mice are used to study atherosclerosis when they are 4-24 weeks old. In our laboratory, we superposed Paraoxonase 1 (PON1) knockout in LDLR knockout and ApoE knockout mice. Lack of PON1 is suggested to increase oxidative stress. Our studies (RO1-HL088397-Parthasarathy-Novel Mechanisms by which Aspirin might protect against Atherosclerosis and RO1-HL123283-Parthasarathy-Role of Aldehyde oxidation in Atherosclerosis) have shown that

over time older (14 months old) ApoE-PON1 double knockout (DKO) mice developed increased plasma lipids and severe carotid atherosclerosis, with 70-80% stenosis in the carotid and innominate arteries, even on normal chow diet.
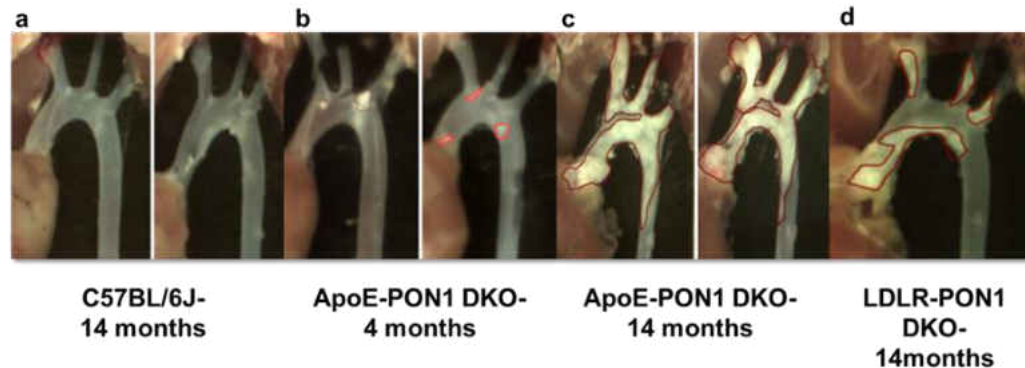
Figure 1: Atherosclerotic lesions in animal models under study.

The 14 months old C57BL/6J mice did not have any lesions (a); the 4 months old ApoE-PON1 DKO mice did not have any lesions (b); the 14 months old ApoE-PON1 DKO mice had severe atherosclerotic lesions (c); the 14 months old LDLR-PON1 DKO mice had atherosclerotic lesions (d), but to a lesser extent compared to age matched ApoE-PON1 DKO mice.

The younger (4 months old) animals with the same genetic background and the control C57BL/6J strain did not show any occlusion in the carotid arteries. The older LDLR-PON1 DKO mice had a lesser extent of atherosclerotic lesions than the age matched ApoE-PON1 DKO mice. While maintaining these animal colonies, we observed that the older ApoE-PON1 DKO mice suffered from seizures, neck and paw edema, and gait imbalance indicating that these animals might have other vasculature related neurological problems. Studies indicate that Alzheimer's disease patients, at later stages of the disease have 6-10 folds more risk of having seizures (Pandis and Scarmeas 2012). From these

observations, we suggest that older ApoE-PON1 DKO mice, with lesions in carotid and innominate arteries, that is, atherosclerosis, could develop neurological problems such as Alzheimer's disease.

1.3 Alzheimer's disease

Alzheimer's disease (AD), the most common form of dementia affecting older people, mostly over the age of 65 years (MacLeod *et al.* 2015, Liu *et al.* 2013, Rohn 2013) accounts for 75% of the cases of dementia (MacLeod *et al.* 2015, Sadigh-Eteghad *et al.* 2014, Qiu *et al.* 2009). It is a chronic, progressive, age related neurodegenerative disorder (Liu *et al.* 2013, Butterfield *et al.* 2013, Dubravka *et al.* 2001, Rohn 2013). Most cases of AD are sporadic. Approximately 5% have genetic disposition (familial) because of polymorphisms that affect amyloid beta secretion (MacLeod *et al.* 2015, Dubravka *et al.* 2001, Rohn 2013, Qiu *et al.* 2009). Clinically, AD is characterized by loss of episodic memory (Kim *et al.* 2009, Butterfield *et al.* 2013, Dubravka *et al.* 2001), cognitive and executive functions such as vocabulary and acumen (MacLeod *et al.* 2015, Kim *et al.* 2009, Dubravka *et al.* 2001, Sadigh-Eteghad *et al.* 2014) and depletion of neuronal and synaptic connections (MacLeod *et al.* 2015, Kim *et al.* 2009, Butterfield *et al.* 2013). AD is not part of normal aging (CDC). The disease initiates about 20 years before there are any apparent symptoms (Mikula *et al.* 2014, Rohn 2013, Sadigh-Eteghad *et al.* 2014). The symptoms of AD appear after the age of 60 years and the risk of disease advancement increases with age (Dubravka *et al.* 2001, CDC). So, older age is one of the greatest risk factors for

AD (CDC, www.alz.org, Qiu *et al.* 2009, Rohn 2013) affecting 13% of people over

the age of 65 years and 45% of people over the age of 85 years (Liu *et al.* 2013,

Hauser *et al.* 2013). Unfortunately, the onset of AD cannot be delayed, paused or

restrained (www.alz.org). Oxidative stress and neuroinflammation have also

been implicated in AD (Kim *et al.* 2009, Cervellati *et al.* 2015, Cirillo *et al.* 2015).

1.4 Epidemiology of Alzheimer's disease

At present, over 35 million people worldwide are affected by AD and its

ascendancy doubles every 5 years in patients over the age of 60 years (Mikula *et*

al. 2014, Sadigh-Eteghad *et al.* 2014, Qiu *et al.* 2009, CDC). It is the sixth leading

cause of death in the United States (www.alz.org, CDC, Rohn 2013). More than

5 million Americans aged 65 years or more suffered from AD in 2013

(www.alz.org, CDC, Butterfield *et al.* 2013, Rohn 2013) and this number is

predicted to inflate to 14 million by 2050 which is almost a 3-4-fold increase

(www.alz.org, CDC). The healthcare cost of AD is estimated to be $172 billion a

year (Reitz *et al.* 2014).

1.5 Characteristics of Alzheimer's disease

Pathologically, AD is delineated by the aggregation of two proteins, the

amyloid beta or Aβ and tau. The amyloid beta forms senile plaques surrounded

by glial cells and damaged neurons outside and around nerve tissues of the brain

(MacLeod *et al.* 2015, Kim *et al.* 2009, Paulson *et al.* 2008, Serrano-Pozo *et al.*

2011, Perl 2010), and tau proteins are associated with microtubules, which

forms neurofibrillary tangles (NFT) within nerve cells or neurons, especially in the hippocampus (MacLeod *et al.* 2015, Kim *et al.* 2009, Paulson *et al.* 2008, Lasagna-Reeves *et al.* 2012, Serrano-Pozo *et al.* 2011). The NFTs are formed due to hyperphosphorylation of twisted filaments of tau (Paulson *et al.* 2008, Serrano-Pozo *et al.* 2011). Other characteristics of AD include diffuse and neuritic plaques (Reitz *et al.* 2014), ventricular enlargement and degeneration (MacLeod *et al.* 2015).

Histological hallmarks, shown in research with other animal models of AD encompass neuronal and synaptic loss, neuritic dystrophy, loss of pyramidal cells (http://www.scholarpedia.org/article/Pyramidal_neuron) in the cornu ammonis (CA) region, loss of granule cells in the dentate gyrus of hippocampus, granulovacuolar degeneration (Paulson *et al.* 2008, Serrano-Pozo *et al.* 2011, Perl 2010), presence of deeply stained nuclei, neuropil threads (Serrano-Pozo *et al.* 2011, Dong *et al.* 2012), and eosinophilic rod-like bodies known as Hirano bodies (Serrano-Pozo *et al.* 2011, Perl 2010). Cortical dysfunction and dystrophic neurites are also noticed (Serrano-Pozo *et al.* 2011).

Hippocampus is the main area of the brain affected by AD and the histological markers are primarily seen in this region. It is located under the cerebral cortex (http://neuroscience.uth.tmc.edu/s4/chapter05.html) and plays a central role in brain functions such as learning, short and long term memory and spatial memory (Jonas *et al.* 2015). The hippocampus is divided into two major

parts, the cornu ammonis (CA) region and the dentate gyrus (DG) (Hartley *et al.* 2014). The CA region is subdivided into the CA1, CA2, CA3 and CA4 regions (Hartley *et al.* 2014). The preeminent types of cells in CA and DG areas are the pyramidal and granule cells respectively (Amaral *et al.* 2007).

Pyramidal cells have a triangular shaped body called soma, a single axon that extends towards the grey matter and thick, multiple, branching dendrites extending towards the cortex (http://www.scholarpedia.org/article/Pyramidal_neuron, Elston 2003). Pyramidal cells send nerve impulses to cerebral cortex, hippocampus and amygdala parts of the brain and helps in cognitive functions (http://www.scholarpedia.org/article/Pyramidal_neuron). The pyramidal cells of CA1 region are susceptible to neurodegeneration.

The CA4 region underlies the dentate gyrus. Neurogenesis occurs at the DG region and new memories are formed (Amaral *et al.* 2007, Jonas *et al.* 2015, Lopez-Rojas *et al.* 2016). Granule cells are formed in the subgranular zone of DG (Lopez-Rojas *et al.* 2016) and they receive excitatory neuronal input from the entorhinal cortex and send excitatory neuronal output to CA3 region (Amaral *et al.* 2007, Jonas *et al.* 2015). Loss of granule cells in the DG result in memory loss.

Neuropil threads are found with NFTs that comprise of hyperphosphorylated tau and ubiquitin in the dendrites (Lasagna-Reeves *et al.*

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2012, Serrano-Pozo *et al.* 2011, Dubravka *et al.* 2001). They are segments of axons, dendrites and presynaptic terminals (Serrano-Pozo *et al.* 2011, Dubravka *et al.* 2001). In the neuropil they are present as argentophilic circuit of paired helical fibers (Dubravka *et al.* 2001).

Granulovacuolar degeneration (GVD) are cytoplasmic lesions in the pyramidal cells of hippocampus (Serrano-Pozo *et al.* 2011, Dubravka *et al.* 2001), between the CA1 and CA2 regions (Perl 2010). They are argentophilic double membraned vacuolar structures within the neurons with a granular basophilic core (Serrano-Pozo *et al.* 2011, Perl 2010, Dubravka *et al.* 2001).

Hirano bodies are eosinophilic inclusions surrounding the neurons in the CA1 region of hippocampus (Serrano-Pozo *et al.* 2011, Perl 2010, Dubravka *et al.* 2001). Hirano bodies are rodlike structures composed of braided parallel fibers (Serrano-Pozo *et al.* 2011, Perl 2010, Dubravka *et al.* 2001) and are composed of actin and actin associated proteins in neurons (Maselli *et al.* 2003).

Due to altered chromatin conformation, which are pathological markers of AD (Crapper *et al.* 1979), deeply stained nuclei are identified in AD brain. They are small, deeply stained irregular nuclei due to fragmentation and contain chromatin granules.

1.6 Parts of brain affected by Alzheimer's disease

The parts of the brain that are affected by Alzheimer's disease are hippocampus, cerebral cortex and olfactory lobes (MacLeod *et al.* 2015, Dubravka *et al.,* Sadigh-Eteghad *et al.* 2014). Morphologically in AD brain, the cerebral cortex and hippocampus are shrunken due to death of neurons and loss of connection between them and the ventricles are amplified due to neuronal tissue degeneration (MacLeod *et al.* 2015, Perl 2010) and cerebrospinal fluid accumulation.

1.7 Stages and Symptoms of Alzheimer's Disease

AD develops in seven stages (www.webmd.com, www.alzheimers.net) as shown in Figure 2. These stages are: Pre-clinical, Very Mild Cognitive decline, Mild Cognitive Impairment (MCI), Moderate decline, Moderately Severe decline, Severe decline and Very Severe decline (Butterfield *et al.* 2013, Rohn 2013, www.webmd.com, www.alzheimers.net). AD can be classified as early onset AD (<65 years) (Butterfield *et al.* 2013, Reitz *et al.* 2014, www.hopkinsmedicine.org) and late onset AD (>65 years) based on the age of onset (Liu *et al.* 2013, Butterfield *et al.* 2013, Reitz *et al.* 2014).
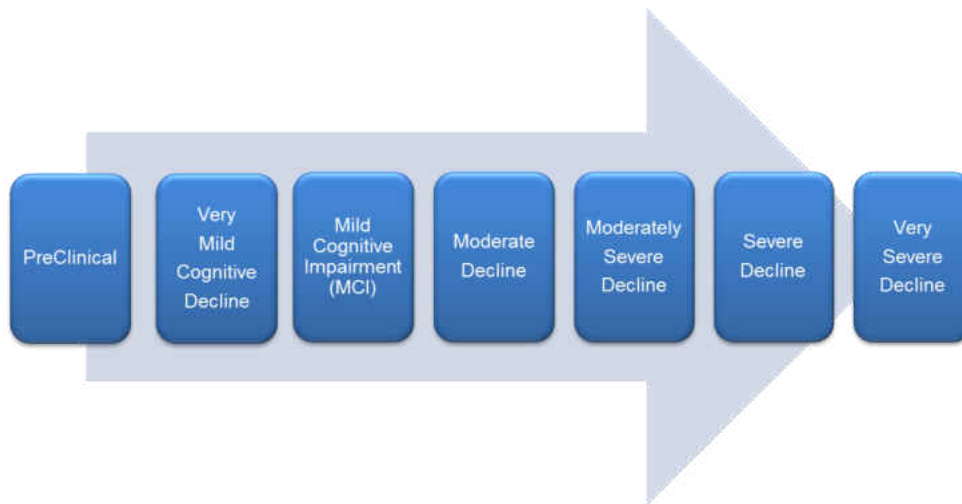
Figure 2: The seven stages of Alzheimer's disease progression.

The seven stages in which Alzheimer's disease develops are Pre-clinical, Very Mild Cognitive decline, Mild Cognitive Impairment (MCI), Moderate decline, Moderately Severe decline, Severe decline and Very Severe decline.

Memory impairment, incompetence to learn, alterations in personality and behavior, judgmental errors, ineptitude to communicate, hardship to perform normal tasks, inadequacy in intellect and aptitude are crucial symptoms of AD that result in abatement of the quality of life and longevity (Sadigh-Eteghad *et al.* 2014, Rohn 2013, www.healthline.com, www.hopkinsmedicine.org). Early onset AD is indicated by failure to remembering recently acquired knowledge or task, dates, time of year and places, issues with vision and speech, problems with decision making, regularly losing things, enquiring about the same thing repeatedly and inability to backtrack (Rohn 2013, www.healthline.com, www.hopkinsmedicine.org, http://www.alz.org/alzheimers_disease_stages_of_alzheimers.asp). Symptoms of

late onset AD encompass severe loss of memory, diminished language and communication proficiency, minimal motion, inability to respond, insensitivity, difficulty in swallowing (Dubravka *et al.* 2001, www.curealz.org, www.alz.org/alzheimers_disease_stages_of_alzheimers.asp, www.hopkinsmedicine.org, www.webmd.com/alzheimers/guide/understanding-alzheimers-disease-symptoms). Symptoms also include confusion (www.webmd.com/alzheimers/guide/understanding-alzheimers-disease-symptoms, www.alz.org/alzheimers_disease_stages_of_alzheimers.asp), changes in mood (www.hopkinsmedicine.org), and severe weight loss (Dubravka *et al.* 2001).

1.8 Risk factors of Alzheimer's disease

The definite etiology of AD is unidentified. Both genetic and non-genetic factors are implicated in the risk of developing AD (Kim *et al.* 2009, Menini *et al.* 2014, Rohn 2013, Qiu *et al.* 2009, Reitz *et al.* 2014). Non-genetic factors incorporate metabolic and environmental factors (Kim *et al.* 2009, Rohn 2013, Qiu *et al.* 2009, Reitz *et al.* 2014), which encompass cerebrovascular disease, elevated blood pressure, type 2 diabetes, increased body weight, plasma lipid levels, metabolic syndrome such as hypercholesterolaemia, hyperhomocysteinaemia, smoking, dyslipidemia and traumatic brain injury (Qiu *et al.* 2009, Reitz *et al.* 2014, Breteler *et al.* 2000, Iadecola *et al.* 2004). Various risk factors contributing to AD are shown in Table 1 (Rohn 2013, Qiu *et al.* 2009, Reitz *et al.* 2014, Breteler *et al.* 2000, Iadecola *et al.* 2004).

Table 1: Various risk factors affecting Alzheimer's disease.

APP: Amyloid Precursor Protein, PSEN: Presenilin, ApoE: Apolipoprotein E, BIN1: Bridging Integrator 1, CLU: Clusterin, ABC: ATP Binding Cassette, A7: Annexin 7, CR1: Complement Receptor 1, PICALM: Phosphatidylinositol Binding Clathrin Assembly Protein, A2M: Alpha-2-Macroglobulin, SORL1: Sortilin-related receptor gene, TREM2: Triggering receptor expressed on myeloid cells 2.

Risk factors of Alzheimer's disease

Genetic

Early onset AD
- APP
- PSEN1
- PSEN2
- ApoE
- Down's syndrome

Late onset AD
- BIN1
- CLU
- ABCA7
- CR1
- PICALM
- A2M
- SORL1
- TREM2
- ApoE4

Non-Genetic

Metabolic
- Hyperhomocysteinemia
- Hypercholesterolemia
- Hyperinsulinemia
- Proinflammatory cytokines
- Vitamin D deficiency
- Altered adipokines
- Type 2 diabetes
- Micronutrients deficiency

Environmental
- Traumatic brain injury
- Aluminium toxicity
- Hypertension
- Vascular injury
- Smoking
- High blood pressure
- Obesity
- Psychological stress
- Education
- Physical activity

1.9 Atherosclerosis and Alzheimer's disease

Atherosclerosis and AD are two of the most crucial threats to public health. Atherosclerosis of cerebral arteries is one of the major risk factors associated with the onset and advancement of Alzheimer's disease (Gupta *et al.* 2015, Scacchi *et al.* 2003, Breteler *et al.* 2000). Clinical, epidemiological, pathological and experimental evidence suggest that atherosclerosis and AD are associated (Bell *et al.* 2009, Gupta *et al.* 2015) and collectively affect brain's

structure and functions, as well as amyloid beta (Aβ) production and clearance (Gupta *et al.* 2015). Data from Cardiovascular Health Study demonstrated the risk of developing cardiovascular diseases such as atherosclerosis with AD (Qiu *et al.* 2009). Blockage in carotid and innominate arteries due to atherosclerosis alters perfusion of oxygen to the brain by diminishing the flow of blood and nutrients. This enhances the activities of β and γ secretase, which forms more Aβ, contributing to Alzheimer's pathology. Increased Aβ, in turn leads to vasoconstriction of arteries and reduced vasodilation (Gupta *et al.* 2015). The diminished blood flow also causes intermittent ischemia and reperfusion that would deprive nutrients and induce cell death. Ischemia augments AD (Iadecola *et al.* 2004). Atherosclerosis and AD also have common risk factors, such as diabetes, hypercholesterolemia and aging (Gupta *et al.* 2015, Austin *et al.* 2010, Iadecola *et al.* 2004).

1.10 Biomarkers of Alzheimer's disease

There is no definitive ante-mortem diagnostic test for AD. Precise diagnosis can be done by examining the histological markers in the brain specimen (Perl 2010). Diagnosis is based on core clinical and clinical research as drugs that can arrest the disease furtherance are unavailable (MacLeod *et al.* 2015). Core clinical diagnosis is not reliable because the pathology of AD is concealed within the brain (Butterfield *et al.* 2013). Diagnosis by clinical research includes biomarkers. Biomarkers or biological markers are aspects or

components in an organism that are inspected squarely and analyzed (Ahmed *et al.* 2014). They usually indicate the normality of an entity or pathogenicity of a disease and also points out its response to therapies (Ahmed *et al.* 2014, Sadigh-Eteghad *et al.* 2014, Sen *et al.* 2007). Biomarkers determine the risk of a disease and endow diagnosis (Reitz *et al.* 2014). Characteristics of an ideal biomarker include stability, sensitivity, specificity, and reproducibility (Ahmed *et al.* 2014, Sen *et al.* 2007). A biomarker should also be rapid and non-invasive (Irizarry 2004). A number of biomarkers are probed for the diagnosis of Alzheimer's disease. The identification of proper biomarkers for AD is essential to differentiate it from other forms of dementia. Different types of AD biomarkers based on their classification are depicted in Table 2.

Table 2: Alzheimer's disease biomarkers

CRP: C-Reactive Protein, TNF: Tumor Necrosis Factor, IL: Interleukin, SOD: Superoxide Dismutase, PON: Paraoxonase, APP: Amyloid Precursor protein, ApoE: Apolipoprotein E, TOMM40: Translocase of Outer Mitochondrial membrane 40, Aβ: Amyloid beta, ICAM: Intercellular adhesion Molecule, VCAM: Vascular Cell Adhesion Molecule.

Types of Biomarkers	Genes and proteins involved
Blood	S100B, γ-secretase
Histological	Aβ protein, tau protein
Inflammatory	CRP, TNF-α, IL-6, IL-1β
Oxidative stress	Catalase, SOD, PON1, PON2
Genetic	APP, ApoE, Presenilin, TOMM40
Cerebrospinal fluid	Aβ-40, Aβ-42
Mitochondrial dysfunction	Reactive Oxygen Species
Microvascular injury	ICAM1, VCAM1, Selectins

For this study, focus will mainly be on S100B and Amyloid beta protein.

1.10.1 S100B

S100B is a calcium-binding brain specific protein (Lam *et al.* 2013, Mikula *et al.* 2014, Sen *et al.* 2007). It belongs to the S100 protein family (Mikula *et al.* 2014), which are present in the cytoplasm and nucleus of a number of cells and plays an important role in cellular growth, proliferation and differentiation, progression of cell cycle, signal transduction, calcium homeostasis and structure and function of cytoskeleton (Lam *et al.* 2013, Sen *et al.* 2007). In the central nervous system, S100B is produced mainly in the Schwann cells and astroglial cells (Lam *et al.* 2013, Mikula *et al.* 2014, Sen *et al.* 2007). It is an astrocytic cytokine and acts as a plasma biomarker for Alzheimer's disease (Peskind *et al.* 2001, Mikula *et al.* 2014, Sen *et al.* 2007). S100B can be both neurotrophic and neurotoxic based on its concentration (Lam *et al. 2013*, Mikula *et al.* 2014). At low concentrations, S100B abets survival, outgrowth and extension of neurons, functioning as a neurotrophic factor (Peskind *et al.* 2001, Lam *et al.* 2013, Mikula *et al.* 2014, Cirillo *et al.* 2015, Sen *et al.* 2007). In neurons and neurites, β amyloid precursor protein (βAPP) is generated by S100B (Peskind *et al.* 2001). S100B is composed of 92 amino acids (Guo et al. 2013) and exist in cells as a 21 kDa homodimer (Lam *et al.* 2013). S100B levels increase due to damage in nervous system, inducing inflammation and apoptosis in neurons, thereby being neurotoxic (Lam *et al.* 2013, Cirillo *et al.* 2015). Neurodegenerative diseases such as Alzheimer's, cerebrovascular diseases and traumatic brain injuries have

elevated S100B levels (Lam *et al.* 2013, Mikula *et al.* 2014, Peskind *et al.* 2001, Sen *et al.* 2007). Alzheimer's patients have higher S100B level in areas of brain with more neuritic plaques (Lam *et al.* 2013, Mikula *et al.* 2014). Increased S100B level is due to cell stress and neuroinflammation, which is associated with AD (Lam *et al.* 2013, Cirillo *et al.* 2015). S100B is overexpressed in activated astrocytes and forms diffused Aβ deposits, dystrophic neurites leading to apoptosis, thus contributing to neurotoxicity (Peskind *et al.* 2001, Lam *et al.* 2013, Mikula *et al.* 2014, Sen *et al.* 2007). There are epidemiologic and experimental evidence that implicates the role of S100B in Alzheimer's (Peskind *et al.* 2001, Sen *et al.* 2007). Increased level of S100B in serum is an indication of blood brain barrier (BBB) disruption (Lam *et al.* 2013).

1.10.2 Amyloid beta plaques

Amyloid beta or Aβ plaques are one of the most important pathophysiological features of AD (Irizarry 2004, Sadigh-Eteghad *et al.* 2014). Aβ is a 4kDa protein that plays a major role in the commencement and development of the disease (MacLeod *et al.* 2015, Kim *et al.* 2009, Butterfield *et al.* 2013, Sadigh-Eteghad *et al.* 2014). Aβ acts as a plasma and cerebrospinal fluid (CSF) biomarker for AD (Irizarry 2004). Proteolytic cleavage of Amyloid Precursor Protein (APP) by β and γ secretases generates Aβ (MacLeod *et al.* 2015, Kim *et al.* 2009, Butterfield *et al.* 2013, Sadigh-Eteghad *et al.* 2014). APP is a type 1 single-pass transmembrane protein (MacLeod *et al.* 2015, Butterfield *et al.* 2013, Sadigh-Eteghad *et al.* 2014, O'Brien *et al.* 2011) found in many human tissues

like heart, skin, liver and lung (MacLeod *et al.* 2015). In the central nervous system (CNS) it is concentrated in the dendrites, cell bodies, axons and synapses of neurons (MacLeod *et al.* 2015, Kim *et al.* 2009, O'Brien *et al.* 2011). APP is not neurotoxic and functions in cellular and neurite growth (MacLeod *et al.* 2015, Butterfield *et al.* 2013, O'Brien *et al.* 2011), formation of synapse, neuronal protein transport and neural plasticity. APP is cleaved at N and C terminals by β and γ secretases respectively (MacLeod *et al.* 2015). α secretase, on the other hand, furcate APP within the Aβ sequence generating incomplete Aβ fragments (MacLeod *et al.* 2015). Other proteins formed are α-synuclein, ubiquitin, apolipoprotein E, presenilins, and alpha antichymotrypsin. Different isoforms of Aβ are formed due to APP cleavage by β and γ secretases that agglomerate to form plaques (MacLeod *et al.* 2015, Sadigh-Eteghad *et al.* 2014, Rohn 2013). The most common isoforms of Aβ are 40 and 42 amino acids long Aβ40 and Aβ42 respectively differing at the C-terminal (MacLeod *et al.* 2015, Irizarry 2004). The amount of Aβ40 is more (MacLeod *et al.* 2015, Kim *et al.* 2009, Butterfield *et al.* 2013), however Aβ42 is toxic and is more in AD brain (Butterfield *et al.* 2013, Irizarry 2004) and susceptible to accumulation (MacLeod *et al.* 2015, Butterfield *et al.* 2013). Elevated soluble Aβ oligomer levels cause synaptic deterioration and neurodegeneration (MacLeod *et al.* 2015, Liu *et al.* 2013, Serrano-Pozo *et al.* 2011). Based on pathological, genetic and functional studies, it is postulated that Aβ plaques accumulate in the brain because of inequality in its production and disposition from brain during aging (MacLeod *et*

al. 2015, Bell *et al.* 2009, Sadigh-Eteghad *et al.* 2014, Liu *et al.* 2013). Impaired degradation, removal of Aβ from brain by cellular uptake or transport through blood brain barrier might regulate sporadic AD (Kim *et al.* 2009, Bell *et al.* 2009). Aβ is generated by all cells, mostly by neurons (MacLeod *et al.* 2015, Kim *et al.* 2009). Under certain conditions, Aβ, which is synthesized in normal individuals aggregate into β sheets forming oligomers and fibrils which accumulate extracellularly to form toxic senile plaques (MacLeod *et al.* 2015, Liu *et al.* 2013, Sadigh-Eteghad *et al.* 2014, Rohn 2013,) resulting in neuronal dysfunction (MacLeod *et al.* 2015, Sadigh-Eteghad *et al.* 2014, Rohn 2013), synaptic injury and hyperphosphorylation of tau forming NFTs, thus contributing to Alzheimer's pathology (MacLeod *et al.* 2015, Liu *et al.* 2013, Rohn 2013).

<u>1.11 Apolipoprotein E</u>

Association between genes and single nucleotide polymorphisms (SNP) (Kim *et al.* 2009) with the risk of developing AD have been noted. Autosomal dominant mutations in three genes, amyloid precursor protein (APP), presenilin 1 (PSEN1) and presenilin 2 (PSEN2) have been implicated in early onset AD (Liu *et al.* 2013, Irizarry 2004, Rohn 2013, Reitz *et al.* 2014). Apolipoprotein E polymorphism has been implicated in late onset AD and is of significant importance (Liu *et al.* 2013, Choi *et al.* 2004, O'Brien *et al.* 2011). Apolipoprotein E is a 34kDa glycosylated protein consisting of 299 amino acids (Kim *et al.* 2009, Liu *et al.* 2013, Holtzman *et al.* 2000, Hauser *et al.* 2013, Rohn 2013). In the peripheral tissues, it is primarily synthesized in the liver and macrophages and

helps in cholesterol metabolism (Kim *et al.* 2009, Liu *et al.* 2013, Holtzman *et al.* 2000, Hauser *et al.* 2013). In the CNS it is also expressed in the brain by neurons, astrocytes and microglia (Kim *et al.* 2009, Liu *et al.* 2013, Hauser *et al.* 2013, Rohn 2013). ApoE binds lipids (Reitz *et al.* 2014) plays an important role in neuronal function (Hauser *et al.* 2013) and carries cholesterol in brain by ApoE receptors that results in transport of lipids, synaptogenesis, synaptic plasticity and repair of damages in the brain (Liu *et al.* 2013, Rohn 2013, Bell *et al.* 2009). Diminished ApoE functions results in oxidative stress, changes in neuronal functions and synaptic loss (Choi *et al.* 2004). Epidemiological studies show a link between Apolipoprotein E alleles and Alzheimer's disease (Kim *et al.* 2009, Holtzman *et al.* 2000). Human ApoE, encoded by *ApoE* genes is polymorphic with three isoforms: ApoE2, ApoE3 and ApoE4, which differ by substitution of a single amino acid cysteine by arginine at position 112 and 158 (Kim *et al.* 2009, Holtzman *et al.* 2000, Choi *et al.* 2004). ApoE2 allele abates the risk of development of AD, hence being protective (Kim *et al.* 2009, Holtzman *et al.* 2000, Hauser *et al.* 2013, Rohn 2013, Bell *et al.* 2009), while ApoE3 allele does not influence one's risk of developing AD (Hauser *et al.* 2013, Rohn 2013). ApoE4 variant increases the risk of AD and has been indicated to be the largest known genetic risk factor for late onset Alzheimer's disease (Hauser *et al.* 2013, Rohn 2013, Kim *et al.* 2009, Holtzman *et al.* 2000, Choi *et al.* 2004). Table 3 shows the three major isoforms of Apolipoprotein E.

Table 3: Apolipoprotein E allele sequence.

The three major isoforms of ApoE depicting the change in allele sequence, shown in italics.

ApoE: L*s*kelqaaqa rlgadmedvr grlvqyrgev qamlgqstee lrvrla*s*

ApoE2:   V*c*grlvqyrg evqamlgqst eelrvrlash lrklrkrllr dadalqk*c*

ApoE3:   V*c*grlvqyrg evqamlgqst eelrvrlash lrklrkrllr dadalqk*r*

ApoE4:   V*r*grlvqyrg evqamlgqst eelrvrlash lrklrkrllr dadalqk*r*

AD develops due to Aβ deposition in the brain because of ApoE4 allele

(Kim *et al.* 2009, Holtzman *et al.* 2000, O'Brien *et al.* 2011). Aβ peptides are

formed at normal levels in AD patients, but are not transported or cleared from

the brain to blood due to its association with ApoE4 (Sadigh-Eteghad *et al.* 2014,

Liu *et al.* 2013). This increased level of Aβ leads to loss of synaptic function and

neuronal network, ultimately leading to loss of cognitive functions. Deposition of

Aβ forms plaques that result in death of neurons. Typically, ApoE forms high-

density lipoprotein (HDL) (Kim *et al.* 2009, Holtzman *et al.* 2000, O'Brien *et al.*

2011), which proteolytically degrades Aβ peptides. However, due to mutated

ApoE gene, this degradation does not happen, leading to the deposition of Aβ

peptides. Evidences show that ApoE4 plays an important role in the metabolism

of APP by increasing its cleavage, thereby elevating Aβ production (Kim *et al.*

2009, Austin *et al.* 2010). ApoE and Aβ interact with each other and impacts the

clearance of Aβ from the brain by uptake via cells or through the blood brain

barrier (Kim *et al.* 2009, Liu *et al.* 2013, Hauser *et al.* 2013, Bell *et al.* 2009).

There are differences in the structures and functions of different isoforms of ApoE that impact the ApoE- Aβ interaction leading to neurodegeneration (Kim *et al.* 2009, Liu *et al.* 2013, Hauser *et al.* 2013). Due to mutation of ApoE, the transport of Aβ bound to ApoE4 is hindered (Kim *et al.* 2009, Bell *et al.* 2009). Also, the predisposition of ApoE4 to undergo cleavage generating neurotoxic N and C terminal fragments leads to its loss of function and impairs its binding with Aβ (Rohn 2013). ApoE4 is correlated with high LDL levels, hyperlipidaemia or hypercholesterolemia and therefore impacts cardiovascular diseases such as atherosclerosis (Kim *et al.* 2009, Liu *et al.* 2013). Therefore, formation of ApoE4, its proneness to cleavage resulting in its inability to transport cholesterol and clear amyloid beta may justify it being a risk factor for AD (Rohn 2013).

1.12 Paraoxonase 1

Increased oxidative stress (Dantoine *et al.* 2002, Butterfield *et al.* 2013, Cervellati *et al.* 2015, Irizarry 2004, Menini *et al.* 2014), decreased antioxidant content and antioxidant enzyme activity (Butterfield *et al.* 2013) have been noted in Alzheimer's brain. Defective paraoxonase activity leads to the development of diseases associated with oxidative stress and lipid peroxidation (Cervellati *et al.* 2015). Paraoxonase, an esterase (Dantoine *et al.* 2002, Menini *et al.* 2014, Litvinov *et al.* 2012) present with high-density lipoprotein (HDL) (Scacchi *et al.* 2003, Cervellati *et al.* 2015, Menini *et al.* 2014) imparts antioxidant and anti-inflammatory competence to the particle (Menini *et al.* 2014, Litvinov *et al.* 2012). PON reduces the peroxidation of LDL (Dantoine *et al.* 2002, Cellini *et al.* 2006,

Wingo *et al.* 2012) which induces atherogenesis and dementia (Cellini *et al.* 2006). It is an antioxidant enzyme and plays a significant role in atherosclerosis by providing protection against the disease as shown in epidemiological studies (Scacchi *et al.* 2003, Wingo *et al.* 2012, Menini *et al.* 2014, Litvinov *et al.* 2012). It modulates paraoxon hydrolysis (Scacchi *et al.* 2003, Menini *et al.* 2014). Mutation in the PON gene may increase the risk of developing AD, the strongest association being PON1 (Cellini *et al.* 2006, Wingo *et al.* 2012, Menini *et al.* 2014). There are 3 genetic variants of PON gene: PON1, PON2, PON3 (Cellini *et al.* 2006, Scacchi *et al.* 2003, Litvinov *et al.* 2012). PON1 is primarily produced in the liver with HDL (Menini *et al.* 2014, Litvinov *et al.* 2012) and is implicated in causing neurodegenerative diseases, such as AD (Wingo *et al.* 2012). Diminished PON1 activity is linked to risk of causing cardiovascular diseases and Late Onset of AD or LOAD (Cellini *et al.* 2006, Cervellati *et al.* 2015, Menini *et al.* 2014). PON1 gene might be competent to treat AD as it can respond to cholinesterase inhibitors (Cellini *et al.* 2006). Thus, PON, lipid peroxidation, atherosclerosis and AD are closely related (Cellini *et al.* 2006, Wingo *et al.* 2012).

PON2 is an antioxidant and anti-inflammatory enzyme present in various cells including heart, lung, kidney, liver, small intestine, pancreas, macrophages and brain. In the brain, it is found in astrocytes. Increase in PON2 expression reduces lesions formed in atherosclerosis. PON2 polymorphism increases the risk of developing AD (Costa *et al.* 2014).

1.13 Blood Brain Barrier and Evans Blue Dye

Blood brain barrier (BBB) is a vascular system partitioning blood in circulation and CSF in the brain (Jaffer *et al.* 2013, Manaenko *et al.* 2011). It is made up of endothelial cell tight junctions, glial cells and basal lamina (Jaffer *et al.* 2013, Manaenko *et al.* 2011, Iadecola *et al.* 2004). BBB is permeable to oxygen, carbon dioxide, glucose, water and ions and inhibits the flow of proteins, bacteria, toxins, drugs and solutes between brain and blood (Bell *et al.* 2009, Montagne *et al.* 2015, Marques *et al.* 2013). Disarticulation of this barrier increases permeability of neurotoxic proteins (Montagne *et al.* 2015) and other proteins present in the serum (Rákos *et al.* 2007) resulting in vascular dysfunction, as seen in several diseases, such as brain tumors, infection in CNS, epilepsy, and neurological diseases such as Alzheimer's (Jaffer *et al.* 2013, Montagne *et al.* 2015, www.todaysgeriatricmedicine.com, Marques *et al.* 2013, Bell *et al.* 2009). The vascular permeability of BBB in animal models can be analyzed by Evans Blue dye (EBD) assay (Jaffer *et al.* 2013, Manaenko *et al.* 2011, Wang *et al.* 2014). Disruption of BBB is indicated by leakage of EBD into brain parenchyma (Wang *et al.* 2014).

Evans Blue Dye (EBD) is a non-toxic, azo dye that binds to serum albumin promptly after being injected intravenously into the bloodstream, and functions as a fluorescent marker of serum albumin (Jaffer *et al.* 2013, Radu *et al.* 2013, Rákos *et al.* 2007, Wang *et al.* 2014). Since brain endothelium is impervious to albumin (Radu *et al.* 2013), serum albumin is unable to cross blood brain barrier

restricting EBD to blood vessels under normal state (Jaffer *et al.* 2013, Radu *et al.* 2013, Manaenko *et al.* 2011). However, during pathological conditions, the endothelium becomes porous allowing extravasation of EBD bound to albumin in the vascularized tissues (Radu *et al.* 2013, Rákos *et al.* 2007). Since the BBB is disarticulating in neurological diseases, in mice suffering from AD, EBD will be more in the brain tissues. EBD fluoresces with excitation peaks at 520 nm and emission peaks at 680 nm. The level of vascular permeability can be assessed by detecting the presence of blue color due to EB fluorescence in the tissues.
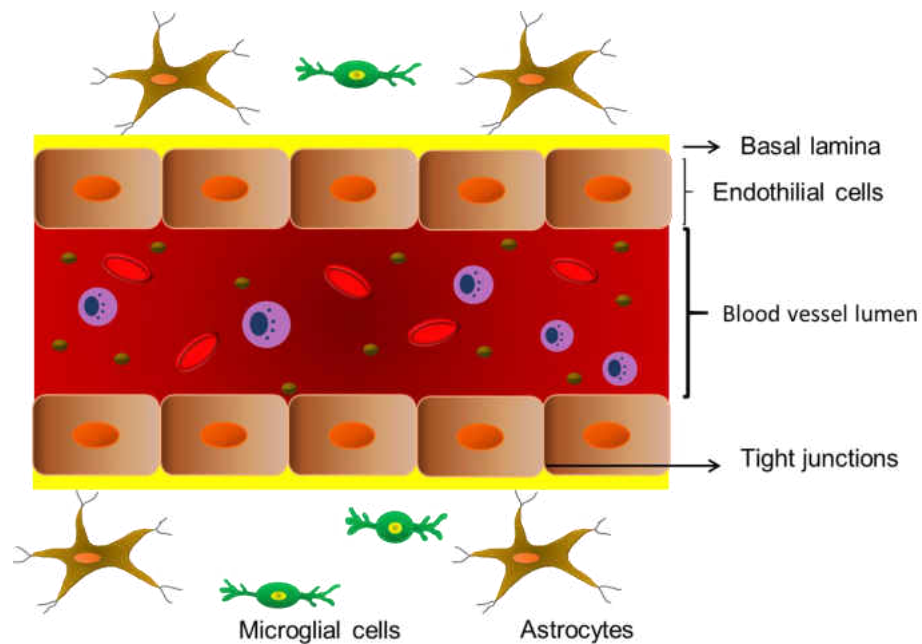

Figure 3: Blood brain barrier

The blood brain barrier is made up of endothelial cells connected by tight junctions, basal lamina and astrocytes.

CHAPTER 2: MATERIALS AND METHODS

2.1 Reagents and Antibodies

The standard reagents were purchased from Sigma (St. Louis, MO). The PCR primers were bought from Invitrogen (Carlsband, CA). The TrizolTM reagent was bought from Invitrogen (Carlsband, CA). ELISA kits for S100 Calcium Binding Protein B were obtained from Cloud-Clone Corp (Houston, TX). Evans Blue dye (Lot # MKBX1555V) was purchased from Sigma (St. Louis, MO).

2.2 Ethical statement

All animals used were maintained at the University of Central Florida vivarium accredited by Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC). All the protocols were in compliance with University of Central Florida Institutional Animal Care and Use Committee (IACUC) and followed the NIH guidelines.

2.3 Animal models and development of strains

Animal experiments were performed on 14 months old C57BL/6J mice, and 4 months and 14 months old ApoE-PON1 double knockout (DKO) mice. The C57BL/6J mice were used as controls. All mice analyzed in the study were of C57BL/6J background. Mice were purchased from Jackson laboratories. ApoE-PON1 double knockout mice were developed by crossing ApoE knockout and PON1 knockout mice for 12 generations. The mice were genotyped by

polymerase chain reaction (PCR) of tail DNA and agarose gel electrophoresis following the instructions in supplier's protocol (QIAGEN DNeasy Blood and Tissue Kit).

2.4 Plasma lipid profile

6 animals each of 14 months old C57BL/6J mice, 4 months and 14 months old ApoE-PON1 DKO mice were fasted overnight and anesthetized with isoflurane (1-2% inhaled) the following day. Fasting blood samples were collected by heart puncture. Red Blood Cells and plasma was separated by centrifugation (3000 rpm for 20 minutes). Plasma lipid profiles of TRG (triglyceride), TC (total cholesterol), HDL-c (high density lipoprotein-cholesterol), LDL-c (low density lipoprotein-cholesterol) and vLDL-c (very low density lipoprotein-cholesterol) were determined using a Cholestec L*D*X analyzer (Cholestec Corp, Hayaward, CA).

2.5 Analysis of lesions

The aorta of 6 animals each of 14 months old C57BL/6J mice, 4 months and 14 months old ApoE-PON1 DKO mice were washed with cold Phosphate Buffer Solution (PBS) through the left ventricle. The aortas were dissected from iliac bifurcation to heart including beginning of carotid and subclavian arteries under a stereomicroscope. Excess fat and connective tissues were removed. Images of aorta were taken using digital camera and the lesions were marked under the microscope. Size of the atherosclerotic lesions were analyzed by the

software Image J. Rulers were used to mark out the pixel to mm^2 conversion factor.

2.6 Isolation of brain

14 months old C57BL/6J mice, 4 months and 14 months old ApoE-PON1 DKO mice were fasted overnight and anesthetized with 1-2% isoflurane. Blood samples were collected in vacutainer tubes by heart puncture. The blood was centrifuged at 3000 rpm for 20 minutes. Serum was separated and stored at 80ºC. Brains were collected by opening the cranium, washed with saline and weighed. Few samples were stored in 4% paraformaldehyde to be used in histology and rest were snap frozen in liquid nitrogen for RNA isolation and gene expression studies.

2.7 Histological analysis

Brain samples were fixed in 4% paraformaldehyde. The tissues were processed and embedded with paraffin in blocks. 5-10 μm sections were cut using rotary microtome (Leica). The sections were stained with Hematoxylin and Eosin stain and 4G8 immunostaining. Images were taken using a Leica DM2000 microscope using 5X, 10X and 40X objectives.

2.7.1 Hematoxylin and Eosin staining

The Hematoxylin and Eosin staining of the slides were done in the pathology core laboratory of The Children's Hospital of Philadelphia Research

Institute, Philadelphia, PA and Sanford Burnham Prebys Medical Discovery

Institute, Orlando, FL. The cells were quantified by the software Stereologer

2000.

2.7.2 4G8 immunostaining

The 4G8 immunostaining for amyloid beta plaques of the slides were done

in the pathology core laboratory of The Children's Hospital of Philadelphia

Research Institute, Philadelphia, PA.

2.8 Isolation of RNA

Isolation of RNA from mice brains was performed using Trizol^{TM} reagent

(Invitrogen #15596018). 800 µL Trizol reagent was added to the brain samples in

tubes kept on ice. The tissue was homogenized using tissue homogenizer

(PowerGen700) and transferred to a 1.5 ml Eppendorf tube. 160 µL of chloroform

was added to the tubes and vigorously shaken for 10-15 seconds. The tubes

were then centrifuged at 12,400 x g for 15 minutes at 4ºC. The aqueous phase

was pipetted out and transferred to a new 1.5 ml eppendorf tube. The lower

organic phase was stored in -80ºC for protein isolation. For precipitating RNA,

400 µL of 100% isopropanol was added to the aqueous phase and incubated at

room temperature for 10 minutes. The tubes were centrifuged at 12,000 x g for

10 minutes at 4ºC and kept on ice for 10 minutes. The supernatant was aspirated

and the tubes were air dried. Excess liquid was removed by capillary action with

the help of pipette tips. To the RNA pellet, 1 ml of 75% ethanol was added. This

is the washing step. The sample was mixed gently and centrifuged at 7,400 x g for 5 minutes at 4ºC. The tubes with RNA were air dried for 10 minutes and resuspended in 50 µL of RNase free water. The concentration of RNA was determined using nanodrop instrument (Nanodrop 8000 spectrophotometer, Thermo Scientific) and calculated to use 1 µg of RNA for cDNA synthesis.

2.9 cDNA synthesis

Synthesis of cDNA by reverse transcription was performed using SuperScript III First-Strand Synthesis SuperMix for qRT-PCR kit from Invitrogen (Life Technologies #11752-050). The reagents were thawed and mixed well. The master mix was prepared in an eppendorf tube on ice by using the following kit components.

Table 4: cDNA synthesis mix preparation.

Component	Amount per Reaction
2X RT Reaction Mix	10 µL
RT Enzyme Mix	2 µL
RNA (1 µg)	X µL
DEPC-treated water	Volume made up to 20 µL
Total Volume	**20 µL**

The RT Enzyme Mix constitutes of SuperScript III RT and RNaseOUT. 2X RT Reaction Mix comprises of oligo(dT)$_{20}$(2.5 µM), random hexamers (2.5 ng/µL), 10 mM MgCl$_2$, and dNTPs. The contents of the microcentrifuge tubes were mixed gently and incubated in a thermos cycler (Bioer Gene Pro Thermal Cycler/VWR) at 25ºC for 10 minutes and again at 50ºC for 30 minutes. The reaction was terminated by incubation at 85ºC for 5 mins. The samples were kept on ice for 5 minutes. 1 µL (2 U) of *E.coli* RNase H was added to the tubes and further incubated for 20 minutes at 37ºC. The samples were stored in -20ºC until future use.

2.10 Real-Time PCR

Quantitative Real-Time PCR was set up using SYBR Green [ER] qPCR SuperMix for iCycler (Invitrogen, Carlsbad, CA # 11761-500). The RT-PCR run was performed in Bio-Rad iQ5 Multicolor Real-Time PCR Detection System using a 96 well PCR plate (Bio-Rad #2239441). For each gene analyzed, a master mix was prepared using the following components.

Table 5: PCR components master mix preparation.

Component	Amount per reaction tube
2X SYBR GreenER Supermix	10 µL
Forward Primer, 10 µM	1 µL
Reverse Primer, 10 µM	1 µL
DEPC-treated water	7 µL
Total Volume	**19 µL**

19 µL of master mix was loaded in each well of PCR plate and 1 µL of cDNA was added. The plate was sealed with a microseal optical adhesive film (Bio-Rad#MSB1001) and centrifuged at 12,000 rpm for 10 minutes at 4ºC. The PCR plate was placed in a CFX 96 iCycler Multicolor Real-Time PCR Detection System (Bio-Rad, Hercules, CA). Specific primers were used. GAPDH primers were used as reference. PCR was programmed for 1 cycle of 50ºC for 2 minutes followed by 1 cycle of 95ºC for 10 minutes. This was followed by 40 cycles of 95ºC for 20 seconds each and 20 seconds at 60ºC. Melt curves were generated for the reactions at 95ºC for 1 minute, 55ºC for 1 minute and 80 cycles of 55ºC $\pm$ 0.5 ºC /cycle for 10 seconds till the temperature reached 95ºC. The Ct values and melt curves were analyzed using Iq5 Optical System Software provided by Bio-Rad. By using 2-ΔΔCt method, normalized fold expression was determined. The following primers for mouse targets were used:

Table 6: Primer sequences for Real-Time PCR.

Primer	Sequence (5' to 3')
S100B	Forward: AACCGCAGCTCCCCTTTCTT Reverse: AGCCACCCCCAAACCTTCAT
GAPDH	Forward: ACCCAGAAGACTGTGGATGG Reverse: CACATTGGGGGTAGGAACAC

The mRNA levels were normalized corresponding to GAPDH gene expression levels.

2.11 Enzyme Linked Immunosorbent Assay (ELISA) for S100B

ELISA for S100B (Commercially available kits, Cloud-Clone Corp. Houston, TX) was carried out on blood samples collected from 8 mice each of 14 months old C57BL/6J, 4 months old and 14 months old ApoE-PON1 DKO mice according to the supplier's protocol. Optical density was measured at 450 nm by a microplate reader (Bio-Rad, Hercules, CA). Correction absorbance was measured at 540 nm. S100B levels were determined using Standard curves which were generated using Sigma plot and concentration of samples were determined from them.

2.12 Evans Blue Dye perfusion

5µg of Evans Blue dye solution was prepared by dissolving it in 1ml of sterile phosphate buffer solution and sterilized by passage through 0.22µm filter

(Fisherbrand Cat# 097203 22µm, PVDF sterile filter, Pittsburgh, PA). 100 µl of the dye was injected intravenously through the tail of 3 numbers each of 4 months old C57BL/6J and ApoE-PON1 DKO mice and 14 months old ApoE-PON1 DKO mice placed in a restraint device. The dye was allowed to circulate for 2 hours. After 2 hours the brain tissues were harvested. The intensity of the of the dye was evaluated by taking Fluorescence image using excitation wavelength 520nm and emission of 680 nm long pass filter. Fluorescence image of the brains were taken with 2 seconds exposure time and Reflectance image of brains were taken with 0.5 sec exposure time. Fluorescence and Reflectance *ex-vivo* images were taken in Bruker Xtreme in-vivo machine overlayed using Bruker MI software.

2.13 Statistical Analysis

The results were expressed as mean $\pm$ SD. Comparisons using paired or unpaired Student's t-test and two-way ANOVA followed by Student's t-test within groups were made between two or more than two groups. Statistical significance was assessed by Student's t-test and differences with $p < 0.05$ were considered significant.

CHAPTER 3: RESULTS

3.1 Weight of the animals

An increase in the body weight of older ApoE-PON1 DKO mice (14 months old) were observed compared to younger ApoE-PON1 DKO mice (4 months old) and older controls C57BL/6J (14 months old) as shown in Figure 4. Apolipoprotein E is involved in lipid metabolism. Therefore, lack of ApoE would reduce the clearance of triglyceride from the body and result in increased body weight as seen in older ApoE-PON1 DKO mice.
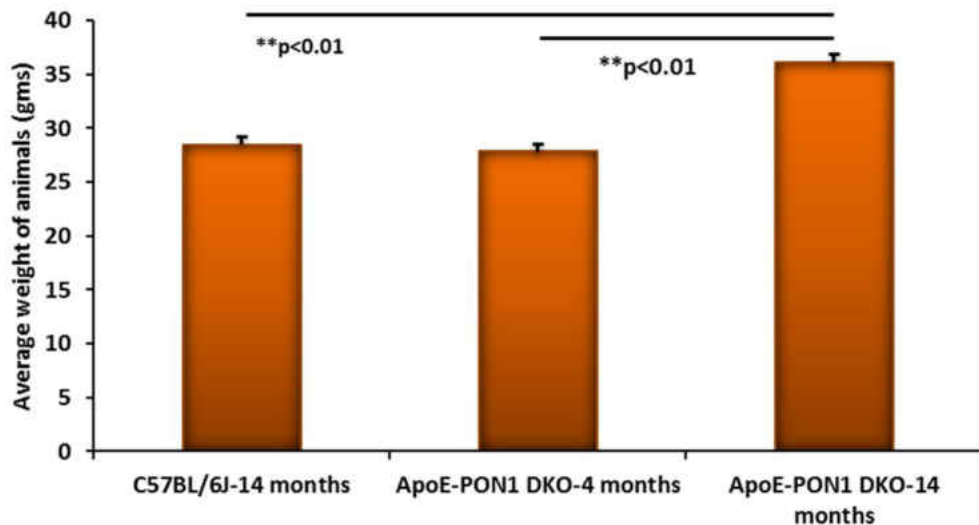

Figure 4: Average weight of animals in grams.

Average weight of 14 months old C57BL/6J, 4 months and 14 months old ApoE-PON1 DKO mice. The weight of older ApoE-PON1 DKO mice were significantly higher than age matched C57BL/6J mice and young ApoE-PON1 DKO mice. p value is less than 0.01. Values are represented as mean $\pm$ SD. Differences with $p<0.05$ were accepted significant.

3.2 Plasma lipid profile

A significant increase in the levels of triglycerides (TRG), total cholesterol (TC), Low density lipoprotein (LDL-c) and very Low Density Lipoprotein (vLDL-c) was observed in both young (4 months old) and old (14 months old) ApoE-PON1 DKO mice compared to older (14 months old) controls C57BL/6J (Figure 5B). The increase in total cholesterol level was due to deficiency in Apolipoprotein E. The plasma sample of the control mice was transparent compared to the younger and older ApoE-PON1 DKO mice as seen in Figure 5A. The turbidity of the plasma is due to the increased level of TRG, TC and LDL.
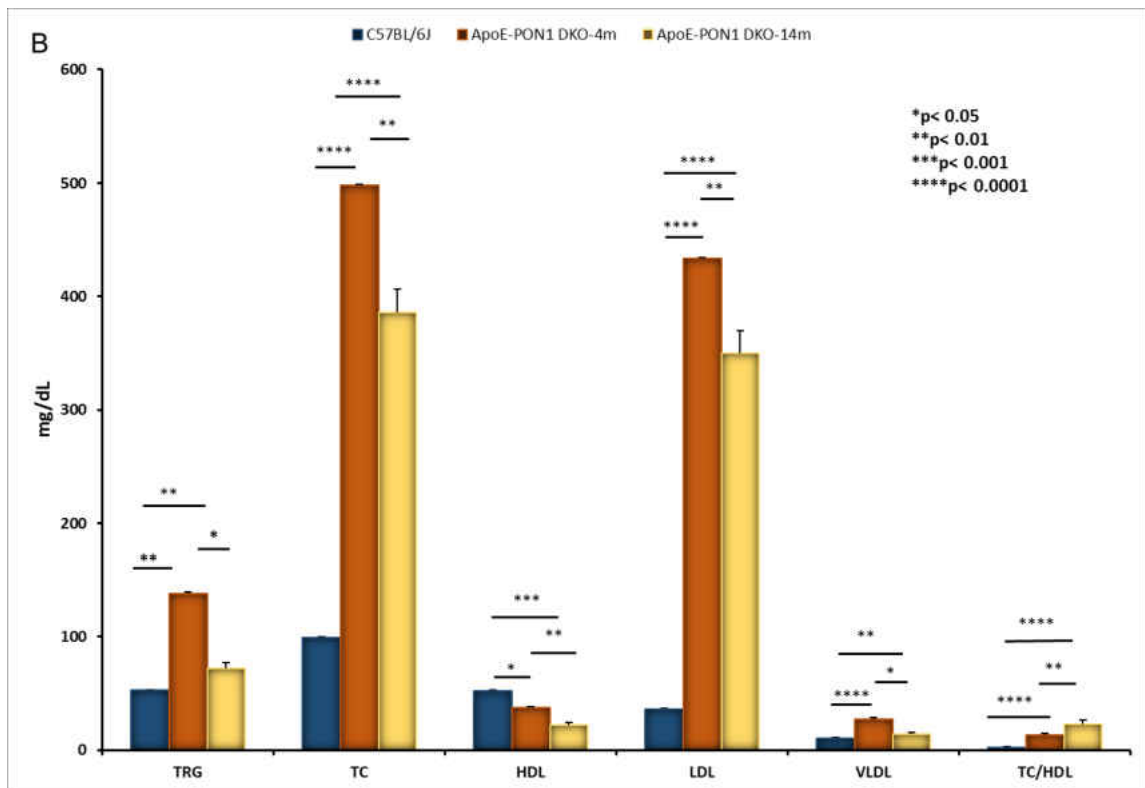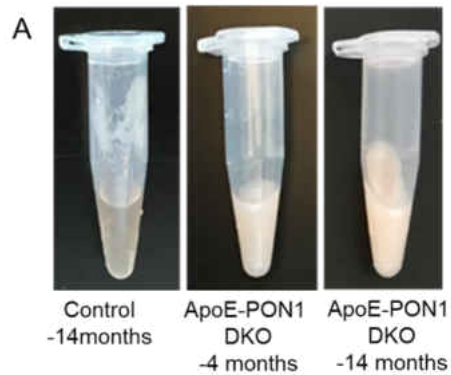
Figure 5: Plasma samples and Plasma Lipid profiles

Representation of the plasma samples of C57BL/6J, 4 months and 14 months old ApoE-PON1 DKO mice. The plasma of ApoE-PON1 DKO mice were more turbid than C57BL/6J mice due to lack of ApoE which led to increase in TRG, TC and LDL levels (A). Plasma lipid levels of 14 months old C57BL/6J, 4 months and 14 months old ApoE-PON1 DKO mice. Values are represented as mean $\pm$ SD. Differences with $p<0.05$ were accepted significant (B).

3.3 Weight of the brains

The brains of the older ApoE-PON1 DKO mice (14 months old) had shrinkage. There was lack of blood in supply in the brains due to lesions in the carotid arteries. No differences in the weights of older (14 months old) control C57BL/6J mice and younger (4 months old) ApoE-PON1 DKO mice were observed (Figure 6B). The brain weight of older ApoE-PON1 DKO mice were significantly lower than the control and younger ApoE-PON1 DKO mice (Figure 6B) because in Alzheimer's brain there is death of neurons and degeneration of neuronal tissue.
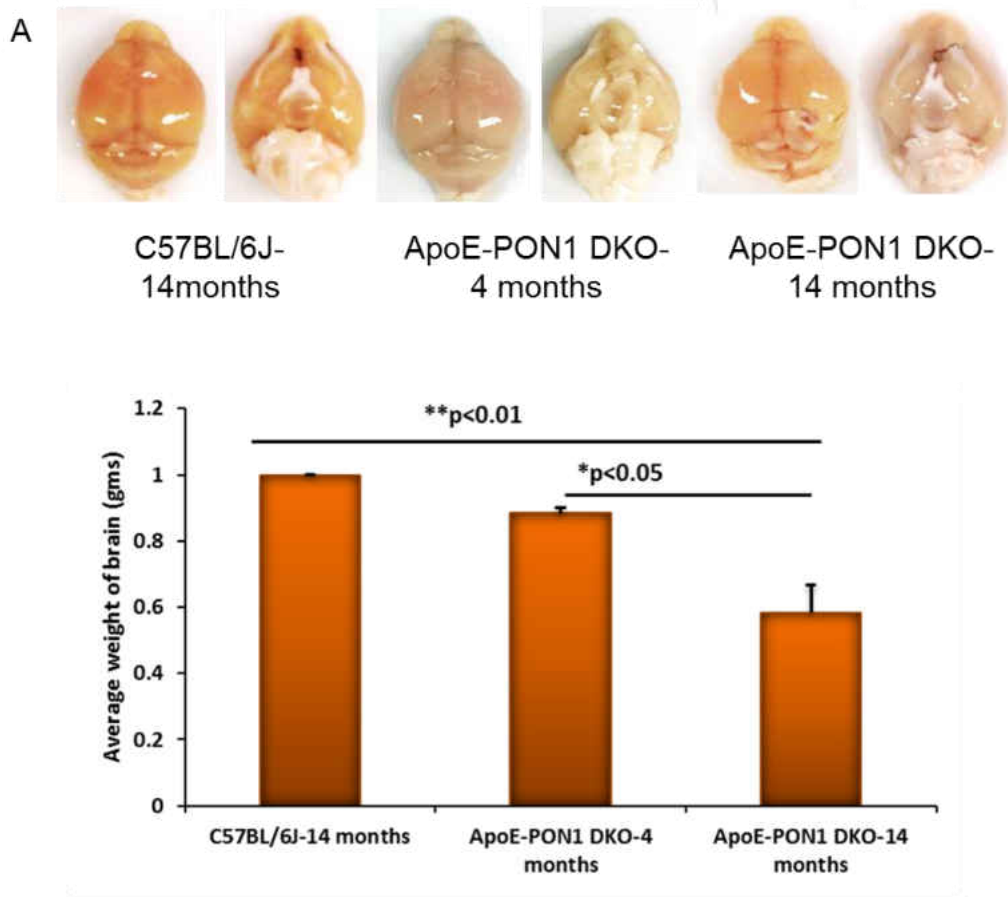
Figure 6: Images and average weight of the brains.

Representation of the brain samples of 14 months old C57BL/6J, 4 months and 14 months old ApoE-PON1 DKO mice (A). The brain weights of 4 months old ApoE-PON1 DKO mice and 14 months old ApoE-PON1 DKO mice were significantly lower than the control C57BL/6J mice (B). The values are represented as mean $\pm$ SD. Differences with $p<0.05$ were accepted significant.

3.4 Analysis of lesions

Atherosclerosis was examined in the older (14 months old) control

C57BL/6J mice and older ApoE-PON1 DKO mice (14 months old) as well as

younger ApoE-PON1 DKO mice (4 months old). The extent of lesions was

documented by measuring the lesion surface area. As shown in Figure 7A, older

ApoE-PON1 DKO mice had more atherosclerotic lesions compared to younger

ApoE-PON1 DKO mice and older control C57BL/6J mice. The increase in lesions

suggest that atherosclerotic burden increased with age even on normal chow

diet.
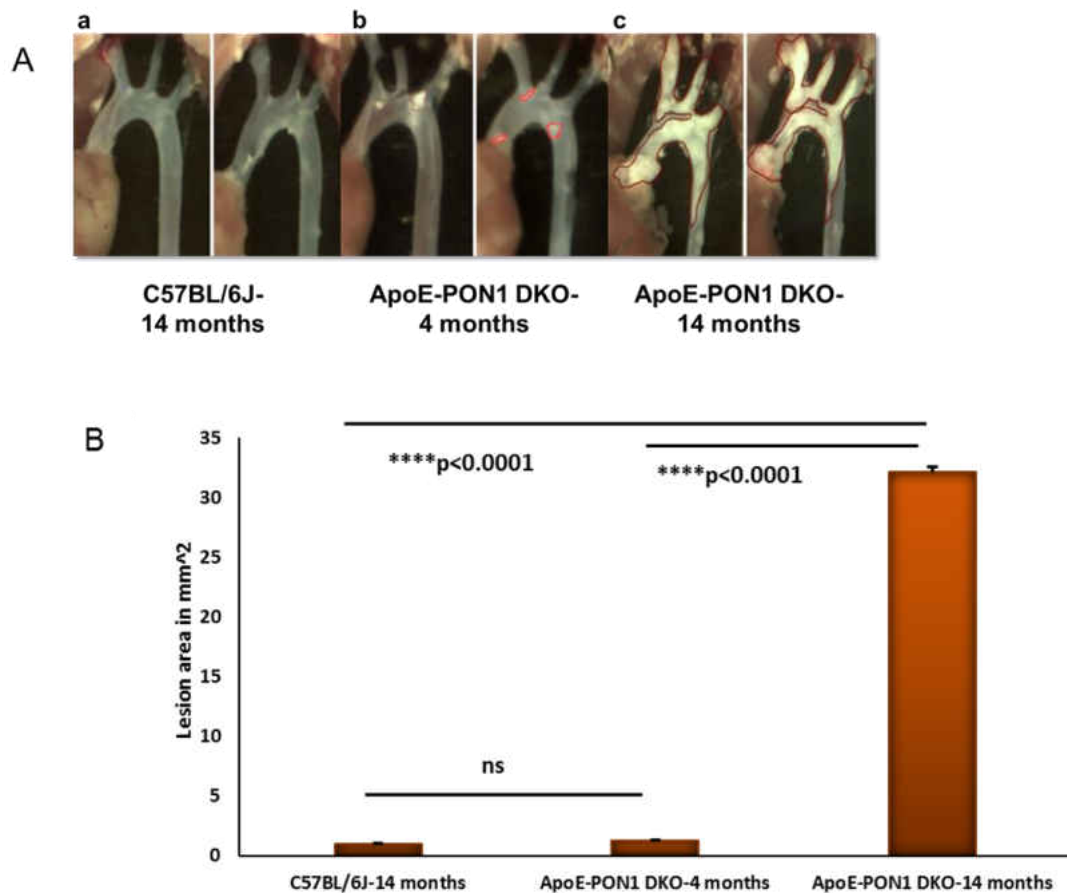
Figure 7: Atherosclerotic lesions.

Representative aortic lesions of 14 months old C57BL/6J, 4 months and 14 months old ApoE-PON1 DKO mice (A). Lesion area quantified by Image J. The 14 months old ApoE-PON1 DKO mice had significantly higher atherosclerotic lesions compared to age matched control C57BL/6J mice and 4 months old ApoE-PON1 DKO mice (B). The values are expressed as mean $\pm$ SD. Differences with p<0.05 were accepted significant.

3.5 Histological analysis

Histological examination reveals the evidence of the characteristic hallmarks of AD and helps in diagnosis of AD by identification of morphological abnormalities. Our main area of interest is the hippocampal region of the brain. Tissue sections were stained with Hematoxylin and Eosin (H and E) stain and 4G8 immunostain. Hematoxylin stains blue/violet. It stains basic/ +ve in nucleus, binds DNA/RNA that are -vely charged and acidic. Eosin stains pink. It stains acidic/ -ve, binds proteins in cytoplasm. 4G8 is a primary mouse monoclonal antibody to Aβ. It illustrates central core and peripheral amyloid halo of plaque in brown. Histologically, the brains showed the following differences.

In the H and E stained slides we see that the 14 months old ApoE-PON1 DKO mice has disorganized granule cells, neuronal loss, loss of pyramidal cells and granule cells (Figure 8). Quantitative analysis of the pyramidal and granule cells showed a decrease in the cell numbers in the older ApoE-PON1 DKO mice compared to age matched control C57BL/6J mice and younger ApoE-PON1 DKO mice (Figure 9). Deeply stained nuclei and Hirano bodies were also observed in the brain tissues of older ApoE-PON1 DKO mice (Figure 10).
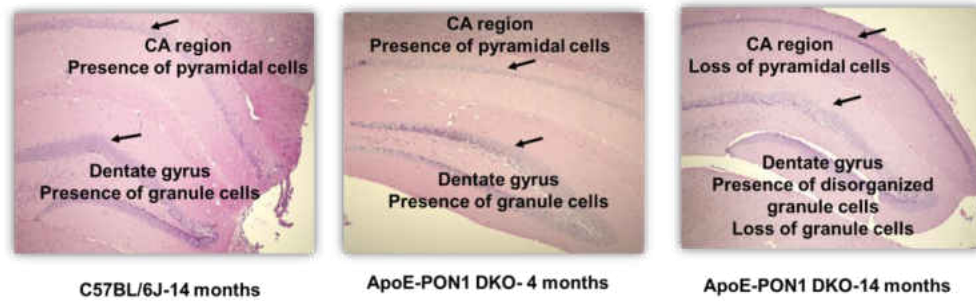
Figure 8: Hematoxylin and Eosin staining of pyramidal and granular cells (5X magnification).

The images shown are from the hippocampal section of the mice stained with H and E staining. The arrows represent disorganized granule cells and loss of pyramidal cells in the 14 months old ApoE-PON1 DKO mice.
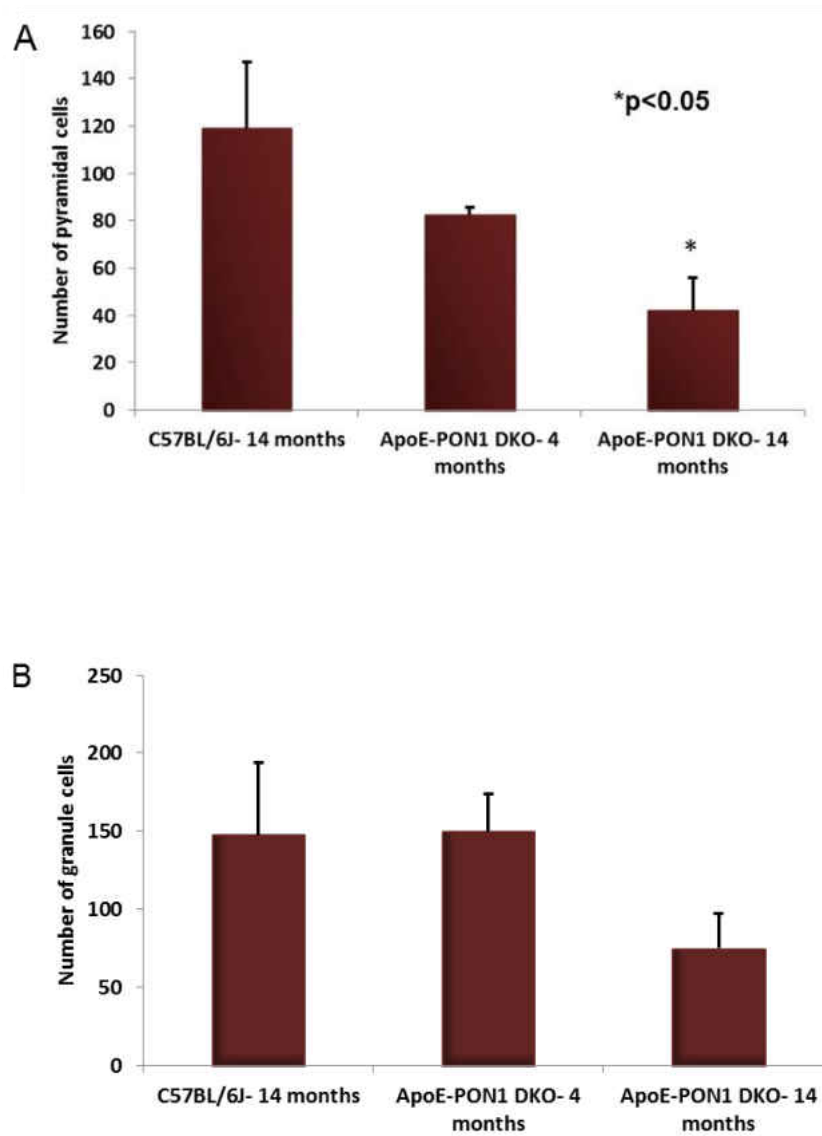
Figure 9: Number of pyramidal cells and granule cells.

There is a decrease in the number of both pyramidal (A) and granule cells (B) in the 14 months old ApoE-PON1 DKO mice.
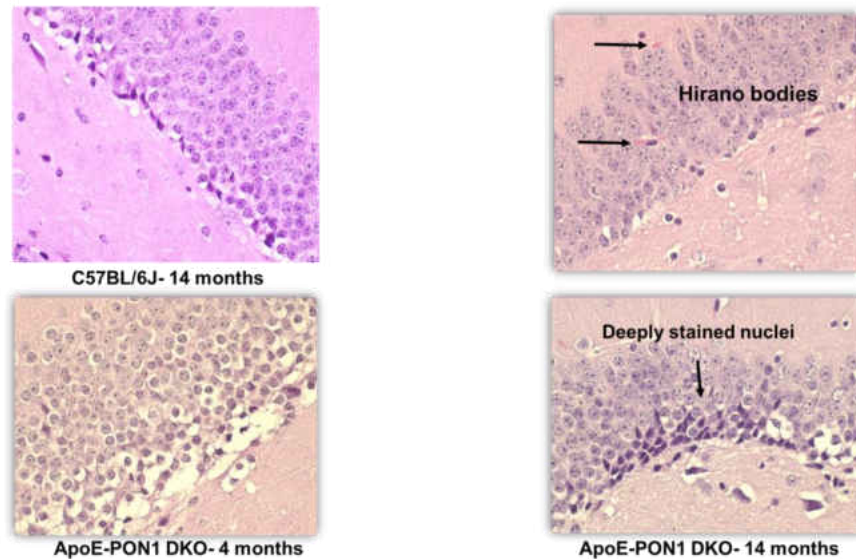
Figure 10: Hematoxylin and Eosin staining of Hirano bodies and deeply stained nuclei (40X magnification).

The images shown are from the hippocampal section of the mice stained with H and E staining. The arrows show the presence of Hirano bodies and deeply stained nuclei in the 14 months old ApoE-PON1 DKO mice.

In the 4G8 immunostained slides we see the presence of amyloid beta plaques in the hippocampal region of the 14 months old ApoE-PON1 DKO mice (Figure 11).
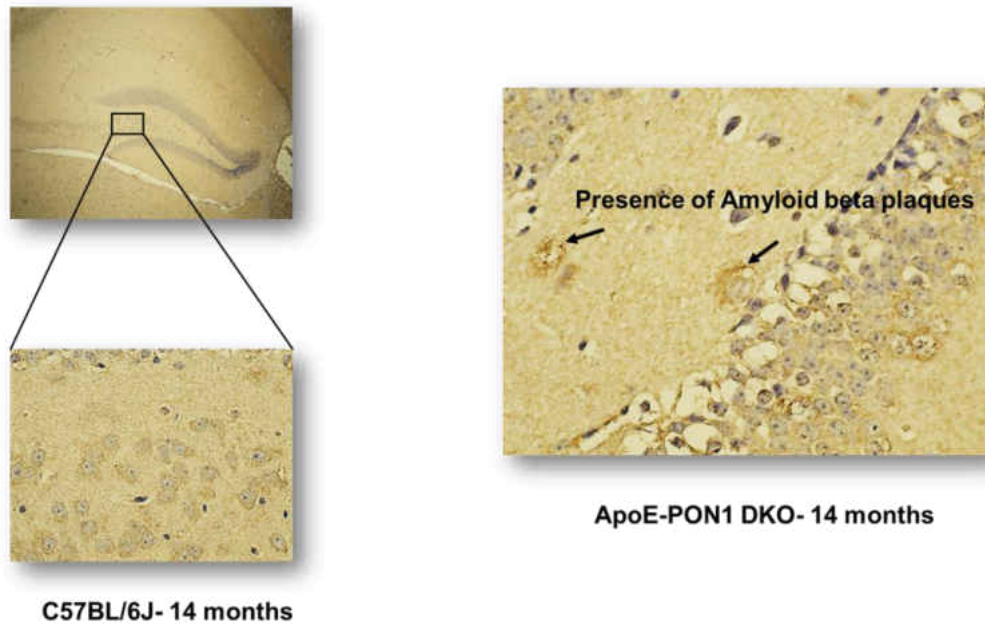
Figure 11: 4G8 Immunostaining.

The images shown are from the hippocampal section of the mice stained with 4G8 immunostain. The arrows show the presence of amyloid beta plaques in the 14 months old ApoE-PON1 DKO mice.

3.6 Gene expression of S100B and Amyloid Precursor Protein

For the detection of AD biomarkers, brains were isolated from mice, different parts of the brain were identified and separated. Biomarkers were detected in hippocampus, which is the major part of brain that undergoes damage in AD and leads to disorientation and memory loss. S100B gene expression was elevated in the hippocampus region of older (14 months old) ApoE-PON1 DKO mice compared to age matched control C57BL/6J mice and younger (4 months old) ApoE-PON1 DKO suggesting that older ApoE-PON1

DKO mice has AD pathology (Figure 12). However, the difference between younger (4 months old) and the older control and DKO mice was not significant.
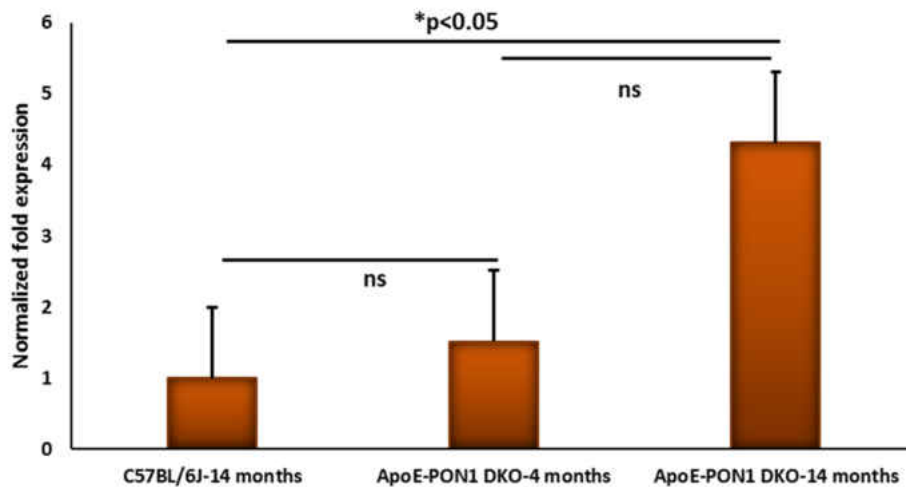
Figure 12: Gene expression of S100B in control and double knockout (DKO) mice

S100B mRNA levels in control and double knockout mice. RNA was harvested and analyzed. Fold expression was normalized by using glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The values are expressed as mean $\pm$ SD. Differences with $p<0.05$ were accepted significant.

3.7 Enzyme Linked Immunosorbent Assay (ELISA)

Significant elevated levels of S100B in older (14 months old) ApoE-PON1 DKO serum was observed compared to age matched control and younger (4 months old) ApoE-PON1 DKO mice (Figure 13).
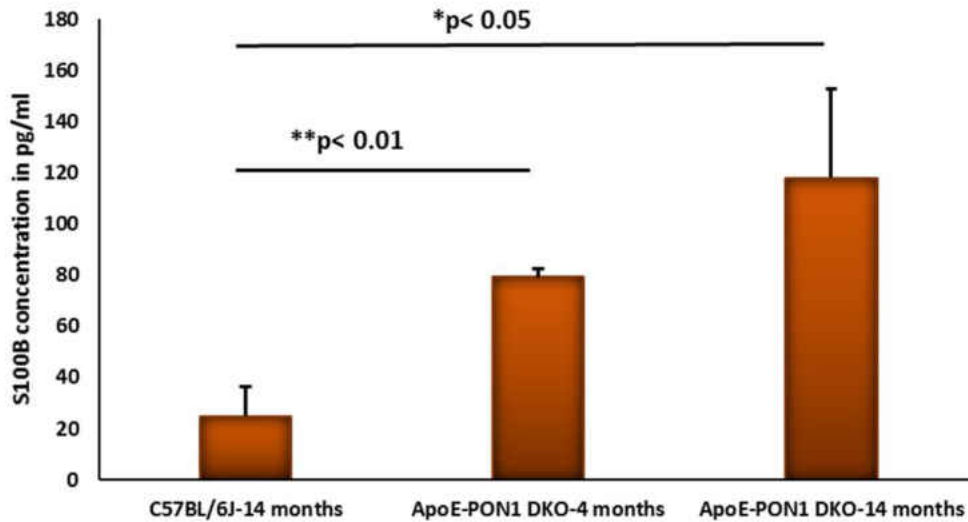
Figure 13: Serum levels of S100B in control and double knockout (DKO) mice.

The serum levels of S100B in 14 months old ApoE-PON1 DKO mice were significantly higher than age matched control and 4 months old ApoE-PON1 DKO mice. The values are expressed as mean $\pm$ SD. Differences with $p<0.05$ were accepted significant.

3.8 Evans Blue Dye perfusion

To evaluate the changes in vascular permeability in the brain of mice suffering from atherosclerosis, Evans Blue Dye was used to determine leakage of albumin fraction of blood into brain tissue. The intensity of the permeable color of the dye was evaluated by taking Fluorescence image using excitation wavelength 520 nm and emission of 680 nm long pass filter. Reflectance image of brain was taken with 0.1 sec exposure time. Fluorescence and Reflectance *ex-vivo* images were taken in Bruker Xtreme in-vivo machine overlayed using Bruker

MI software. The presence of EBD (red color) in the brains of older DKO mouse

(Figure 14) was observed suggesting BBB damage.

Figure 14: Evans Blue Dye Perfusion

The brain samples of C57BL/6J, 4 months and 14 months old ApoE-PON1 DKO mice (A); Fluorescence images of 14 months old C57BL/6J, 4 months and 14 months old ApoE-PON1 DKO mice (B); Overlayed images of 14 months old C57BL/6J, 4 months and 14 months old ApoE-PON1 DKO mice (C).
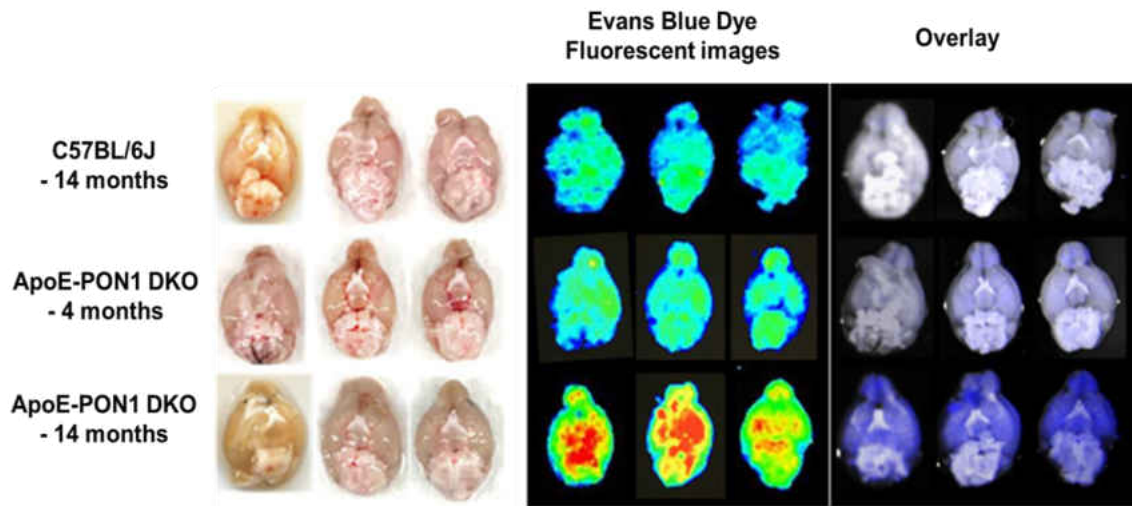
CHAPTER 4: DISCUSSION

Alzheimer's disease (AD) is one of the leading causes of death worldwide accounting for more than 35 million cases. It is the 6th leading cause of death in the US only. Alzheimer's disease is a major disease that not only affects the brain, but also other functions associated with it. At present, human Alzheimer's has no treatment, although there are potential drugs or antibodies that target the amyloid beta (Aβ) plaques, one of the most important pathological hallmarks of AD. Moreover, because the histopathological changes associated with the disease are concealed within the brain, it becomes difficult to diagnose AD. There are no or very few markers for the diagnosis of AD, so there is an increased need to know the etiology of the disease. Both genetic and environmental factors are attributed to AD. Current evidence suggests Apolipoprotein allele (ApoE) might be genetically linked to the development of Alzheimer's. ApoE gene polymorphism (ApoE4) has been implicated in late onset of Alzheimer's disease (LOAD). ApoE forms high density lipoproteins (HDL) and binds and degrades Aβ plaques. However, due to its polymorphism, ApoE is unable to degrade Aβ, thus increasing its level in the brain. Yet, the disease cannot be just genetic because younger people are not generally affected by the disease, only older people aged 65 or more are affected. Hence, other factors might be associated with the disease.

Oxidative stress has been implicated in many diseases. Oxidative stress is a very generic term and could be the result of environmental, nutritional, and pharmacological factors. There is not any single oxidative stress, and no single antioxidant enzyme that controls the body's redox potential.

During our course of study on atherosclerosis, we developed Apolipoprotein E-Paraoxonase 1 double knockout (ApoE-PON1 DKO) mice. These mice, as shown in the results section had severe atherosclerosis in the carotid and innominate arteries with 70-80% stenosis and an increase in plasma lipid levels at an older age (14 months). Based on these observations, we predicted that there will be diminished blood flow into the brain and reduced oxygen supply. Research from cell culture in a variety of systems suggest that deprivation of nutrients together with oxidative stress result in cellular death. So, in AD, based on carotid atherosclerosis, we interpreted that there would be cell death and poor utilization of blood supply. Lack of ApoE increases the levels of triglycerides and total cholesterol in the blood. This results in an increased body weight of the older DKO mice.

The lack of ApoE cautioned us that the ApoE-PON1 DKO mice might have specific abnormalities related to AD. The histopathology of mice Alzheimer's can be only compared to humans on the factors that are reminiscent of Alzheimer's pathology. Our results show aggregation of Aβ plaques in older mice as also seen in human Alzheimer patients. In contrast, the younger ApoE-PON1 DKO mice did not have any plaques. Our histology results also show loss of pyramidal

and granular cells in the hippocampal region of the brain, as well as presence of Hirano bodies and deeply stained nuclei, all of which are histological characteristics of AD.

At present, S100B, tau proteins, C-reactive proteins, Amyloid Precursor Protein, Presinilin are known to be specific markers for AD. Our results show an increase in S100B gene expression in older ApoE-PON1 DKO mice compared to age matched control and younger ApoE-PON1 DKO mice. In general, S100B protein designates inflammatory changes. This suggests that Alzheimer's or pathology seen in our animals also reflect highly elevated inflammatory response. This raises an important question, whether chronic inflammation contributes to Alzheimer's disease. If so, whether those who are predisposed to Alzheimer's for example, those who have defective ApoE allele, would require pharmacological agents and supplements that would reduce Alzheimer's and reflect in delayed onset of Alzheimer's or a progressive change from abnormal pathology to normal pathology could be only speculated.

Disarticulation of the blood brain barrier (BBB) increases permeability of neurotoxic proteins and other proteins in the serum resulting in vascular dysfunction and cell death. Vascular permeability of the BBB can be assessed by the extravasation of Evans Blue Dye (EBD) in the brain parenchyma. As noted in our results, there is an increased distribution of EBD across the brain tissues of older ApoE-PON1 DKO mice, suggesting gross differences in permeability. This would suggest that nutrients or neurostimulators, and neurotoxins are going to be

distributed in the brain causing neuronal death and affecting the function of the brain.

Phosphorylation and dephosphorylation reactions are very common in biology. They reflect the ongoing state of metabolic changes in our system. Whether these changes could be reversed has not yet been established. Also, if atherosclerosis of arteries is significant in inducing apoptotic changes in addition of phosphorylated proteins, then one can devise strategies to control atherosclerosis.

Already nutritional and pharmacological means are available as regulators of atherosclerotic process. At present, they relate to two kinds of drugs, Statins, that specifically control cholesterol synthesis and PCSK9, which on the other hand decrease overall cellular metabolism of cholesterol. While statins and AD have been studied and there are confusing line of evidence whether statins could affect Alzheimer's, the potential of PCSK9 antibodies to control AD have not been established. Future research as well as long term epidemiological changes are required on this account.

Cholesterol, phospholipids, unsaturated fatty acids are all important components of the brain, particularly myelin sheath. These are all lipids that the brain utilizes for its structural integrity as well as functions. However, the contribution of dyslipidemia and cholesterol to AD appears to be questionable, as recent studies point out that very little Low Density Lipoprotein (LDL) is taken up

by neuronal tissues. This raises the question as to whether prevention of atherosclerosis or lowering plasma LDL would have a significant effect on AD. This will give us an opportunity to develop modulations in atherosclerosis that might lead to beneficial changes in neurological diseases. Also, it remains to be seen if the neurological symptoms could be reversed. Increase in cholesterol and triglyceride levels result in hypercholesterolemia, which induces $A\beta$ secretion and also leads to atherosclerosis.

If atherosclerosis or differences in Apo E lead to AD, there might be simpler ways of correcting it. So, one of the future questions of the study is to ask the question whether AD could be prevented by preventing atherosclerosis from early childhood.

In this study, we demonstrated that animals that are deficient in apolipoprotein E with severe carotid stenosis demonstrate Alzheimer phenotype. However, the experiments do not delineate specific role of ApoE versus coronary occlusion. So, further experiments are needed using LDLR knockout mice, which is also an important model to study atherosclerosis to suggest specific role of ApoE or occlusion as opposed to nutrient deprivation.

Paraoxonase 1 is synthesized in the liver and it circulates in the blood. The ability of PON1 to cross the blood brain barrier has not yet been determined. It is possible that localized factors are more important in causing oxidative stress in the brain. PON2, however is synthesized by brain tissues. Future experiments

involving PON2 knockout mice are needed to suggest the role of PON2 in

oxidative stress in ApoE deficient atherosclerotic mice.

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