Comorbid Metabolic Syndrome and Prodromal Alzheimer's Disease in a Rat Model

Nadezda Ivanova
The University of Western Ontario

Supervisor
Cechetto, David F.
The University of Western Ontario

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Abstract

Metabolic syndrome (MetS), the development of which is associated with high-caloric Western diet intake, represents a risk factor for mild cognitive impairment (MCI) and Alzheimer’s disease (AD) and appears to contribute to AD progression when MetS and AD are comorbid. The interaction between AD and MetS might be through white matter inflammation, since white matter abnormalities and inflammation are important early events in the etiopathogenesis of both diseases. In these investigations, the effect of a high-caloric diet (HCD), to induce metabolic disturbances, on white matter neuroinflammation and cognitive function was investigated in a transgenic (TG) rat model of prodromal AD and MCI (APP21 TG). Rats maintained on the HCD developed obesity, dyslipidemia, hyperinsulinemia and glucose intolerance, but not hypertension. HCD-driven metabolic perturbations significantly exacerbated white matter microglia, but not astrocyte, activation in the TG rats. There were significant deficits in spatial reference memory in the comorbid TG HCD group compared to wildtype control rats. There were no changes in hippocampal neuronal or synaptic density in the comorbid group. In addition, single-regimen chronic prophylactic treatment with a novel brain-targeted NSAID prodrug Ketoprofen-lysine was not able to decrease the activation of white matter microglia and alleviate cognitive deficits in the comorbid model. In fact, the prodrug treatment alone was associated with two deaths and widespread neuroinflammation and neurodegeneration in a subset of rats further associated with a spatial working memory decline and cognitive inflexibility. Finally, white matter microglia activation was positively associated with visceral fat deposition and with transgene presence. Dyslipidemia was related to the greater white matter inflammation in the entire rat sample, however, showed an unexpected inverse association in the comorbid model. Thus, the APP21 TG rat combined with the HCD represents a good model for future studies on prodromal AD pathology and MetS. It can be used to study related cognitive dysfunction including effects of various comorbidities on the disease presentation, as well as provide a platform for testing treatment strategies and biomarker identification. The results support an important role of white matter inflammation in the interaction between prodromal AD and MetS and as a contributor to cognitive impairment.
Keywords

Alzheimer’s Disease, Prodromal, Metabolic Syndrome, Hypercaloric Diet, Comorbidity, White Matter, Inflammation, Microglia, Preclinical Rat Model, Amyloid Precursor Protein, NSAIDs, Ketoprofen-lysine Prodrug
Summary for Lay Audience

Alzheimer’s disease (AD) is a highly prevalent and incurable brain disease in the elderly resulting in difficulty in thinking and communicating as well as memory loss. Metabolic syndrome (MetS), is another common disease in the elderly, largely due to the consumption of a hypercaloric Western diet high in fat and sugar. MetS represents a modifiable risk factor for AD. It can coexist with and likely interacts with AD, contributing to cognitive impairment. Investigating the pathological links between AD, especially at the very early stage, and MetS will advance our understanding of early disease processes, highlight therapeutic targets for early intervention and prevention, and identify markers of prodromal AD to aid early diagnosis and timely treatment. White matter abnormalities in brain are associated with cognitive dysfunction and inflammation and are important features of the initial stage of AD and MetS. Inflammation occurring in the white matter could be the basis of the interaction between AD and MetS. The studies in this thesis examined the contribution of a high-caloric (HCD)-induced MetS to the early AD pathology and changes in cognitive function using a novel genetically engineered rat carrying a human protein implicated in human AD. The combined model of AD predisposition and MetS demonstrated greater white matter inflammation which was caused by microglia (resident brain immune cells) and resulted in memory impairment. A novel brain-targeted chronic prophylactic anti-inflammatory treatment was not able to disrupt the detrimental interaction of early AD and MetS on white matter inflammation and to preserve cognitive function. However, serious neurological side effects were observed in several rats on the treatment. Accumulation of fat around the internal organs and blood lipid abnormalities were identified as potential biomarkers of increased white matter inflammation. However, abnormal blood lipids were associated with lower white matter inflammation in the combination of the early AD and MetS in the rats. In conclusion, the findings support the suggestion that white matter inflammation is a link between early AD and MetS. This rat model, closely mimicking human pathological conditions, represents a good platform for future studies on the complex relationships between AD and comorbidities, therapeutic approaches and biomarkers.
Co-Authorship Statement

The work presented in Chapter 2: Comorbidity of Prodromal Alzheimer’s Disease and Metabolic Syndrome in the APP21 Transgenic Rat is co-authored by Qingfan Liu, Cansu Agca, Yuksel Agca, Earl G Noble, Shawn N Whitehead, David F Cechetto and is under review for Journal of Neuroinflammation under the title White Matter Inflammation and Cognitive Function in a Comorbid Metabolic Syndrome and Prodromal Alzheimer’s Disease Rat Model.

The work presented in Chapter 3: Effect of the Novel Anti-inflammatory Prodrug Ketoprofen-lysine in Comorbid APP21 Transgenic Rat and Diet-induced Metabolic Syndrome is co-authored by Cansu Agca, Yuksel Agca, Earl G Noble, Markus Forsberg, Jarkko Rautio, Jukka Leppänen, Shawn N Whitehead, David F Cechetto and is not yet submitted for publication.

The work presented in Chapter 4: Negative Impact of Ketoprofen-lysine Treatment on Cerebral Pathology and Cognition in a Comorbid APP21 Transgenic Rat and Diet-induced Metabolic Syndrome is co-authored by Cansu Agca, Yuksel Agca, Earl G Noble, Markus Forsberg, Jarkko Rautio, Jukka Leppänen, Shawn N Whitehead, David F Cechetto and is not yet submitted for publication.

The work presented in Chapter 5: Relationship between the White Matter Inflammation and Metabolic and Physiological Parameters in a Comorbid Rat Model of Prodromal Alzheimer’s Disease and Metabolic Syndrome is co-authored by Cansu Agca, Yuksel Agca, Shawn N Whitehead, David F Cechetto and is not yet submitted for publication.

NI participated in design of the work, acquisition, analysis, interpretation of data and drafting the manuscript. QL participated in immunohistological staining and analysis. CA and YA developed the AD animal model. JL synthesized the KL prodrug. JR and MMF provided the prodrug and participated in the dose determination and development of administration protocol. EGN participated in design of the work related to MetS, and interpretation of the data, in particular providing the expertise in physiology. SNW participated in the design of the study, interpretation of the data. DFC had primary role in design of the study, participated in the analysis and interpretation of the data and the writing of the manuscript and overall supervision of the work.
Epigraph

“Success is not final, failure is not fatal. It is the courage to continue that counts”.

Attributed to Winston Churchill…
Dedication

To Stanislav Ivanov, my husband – You have always been my infinite support and motivator in this life. Without you there would never be this very Nadia. Thank you for seeing in me something that no one can see, even myself, and for always believing in me. With you I have also found a new family, that has truly become my own. You are my love, my friend, my everything.

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List of Abbreviations

Aβ - amyloid-β peptide

AA - Alzheimer’s Association

ACh - acetylcholine

AD - Alzheimer’s disease

APOE - apolipoprotein E

APP – amyloid precursor protein

ANOVA – analysis of variance (method of statistical analysis)

AUC – area under the curve

BBB – blood-brain barrier

BCC – body of the corpus callosum

BDNF – brain derived neurotrophic factor

BMI – body mass index

CAA – cerebral amyloid angiopathy

CD – control diet

CNS – central nervous system

COX – cyclooxygenase

CRP – c-reactive protein

CSF – cerebrospinal fluid

DAB - 3,3′-diaminobenzidine-tetrahydrochloride
DNA - deoxyribonucleic acid

DSM-5 – the Diagnostic and Statistical Manual of Mental Disorders, fifth edition

DTI – diffusion tensor imaging

EOAD – early onset Alzheimer’s disease (<65 years old)

FAD – familial form of Alzheimer’s disease with autosomal dominant inheritance

GFAP - glial fibrillary acidic protein

hAPP – human amyloid precursor protein with Swedish and Indiana mutations in the transgenic model used for this thesis

HCD – high calorie diet

HDL – high-density lipoprotein

HOMA-IR - homeostasis model assessment index of insulin resistance

IC – internal capsule

IDF - International Diabetes Federation

IpGTT – intraperitoneal glucose tolerance test

IvGTT – intravenous glucose tolerance test

KL – ketoprofen-lysine prodrug; a conjugate of a non-selective NSAID ketoprofen with an amino acid l-lysine that allows LAT1 - mediated transport through BBB

LAT-1 – large amino acid transporter 1

LOAD – late onset Alzheimer’s disease (≥65 years old)

LOX – lipoxygenase

LPA - lysophosphatidic acid
LRP1 - low-density lipoprotein receptor–related proteins 1

MCI – mild cognitive impairment

MetS – metabolic syndrome

MHC - major histocompatibility complex

MRI – magnetic resonance imaging

MWM – Morris water maze

NIA - National Institute of Aging

NOS – reactive nitrogen species

NSAID – non-steroidal anti-inflammatory drugs

NCEP ATP3 - Third Adults Treatment Panel National Cholesterol Education Program

NVU – neurovascular unit

PET – positron emission tomography

PSEN1,2 - presenilin proteins 1 and 2

PVCC – periventricular regions of the corpus callosum

RAGE - receptors for advanced glycation end products

RCT - randomized clinical trial

RNA – ribonucleic acid

ROS - reactive oxygen species

SEM – standard error of the mean

T2DM - type 2 diabetes mellitus
TG/APP21 TG – transgenic rat model of prodromal Alzheimer’s disease created on a Fischer 344 background; homozygous for pathogenic hAPP with Swedish and Indiana mutations

TNF-α – tumor necrosis factor – α

TREM-2 – triggering receptor expressed on myeloid cells 2

TSPO - 18 kDa translocator protein

WMH – white matter hyperintensities detected using brain MRI or computer tomography

WT – wildtype Fischer 344 rats
Chapter 1: Introduction

This chapter introduces the main concepts and current views on Alzheimer’s disease (AD) and metabolic syndrome (MetS) with a focus on white matter pathology and neuroinflammation in the pathogenesis of both diseases. AD and MetS often co-exist in elderly individuals and likely interact contributing to the decline in cognitive status. Investigating the underlying basis of this interaction, particularly at an early stage, and its effect on the cognitive function will advance our understanding of AD pathology and highlight potential therapeutic targets and strategies for the prevention of AD.

This section first addresses the general issues of ageing, mild cognitive impairment (MCI), dementia and metabolic vascular disorders. This section then provides a detailed description of many aspects of AD. Thirdly, there is an examination of clinical and pathological aspects of MetS. Finally, the interaction of AD and MetS is discussed.

1.1 The Problem of Ageing

Aging is a natural and integral part of a human life. Every organ system of a human body undergoes changes as we age and the brain is no exception. The aging brain changes in morphology, physiology and function. This may lead to a minor but noticeable general reduction of cognitive abilities, particularly information processing speed, attention and memory, which together constitute a so-called normal cognitive aging. Physiological changes in the aging brain including vascular and metabolic alterations, as well as imbalance in oxidant-antioxidant systems and dysfunction of the immune system make the brain highly vulnerable and sensitive to disease and injury. As a result of the overlay of multiple chronic or acute disorders, there may be an acceleration in impairment of cognitive function leading to a substantial decrease in quality of life and even loss of independence.

The aging world population has become a major concern. It is estimated that by 2050, 21.1 per cent of the world’s population will be 60 years or older and the number of older persons from this age group will increase to more than 2 billion\(^1\). Normal healthy aging is not the only course and
the number of chronic pathologies is growing and greatly affecting the quality of life of older people. Two of these chronic disorders that interfere with cognition and furthermore with a daily living are MCI and dementia. Another group of chronic diseases associated with a high mortality rate is a group of vascular disorders and related metabolic conditions.

1.1.1 Mild Cognitive Impairment

MCI or mild neurocognitive disorder (as per Diagnostic and Statistical Manual of mental disorders, fifth edition (DSM-5) classification)\(^2\), represents a “transitional” or intermediate state between normal cognition and dementia \(^3\). MCI may improve or remain stable for many years\(^4\). MCI is defined as a condition of a more severe decline in cognitive domains, usually memory (amnestic MCI) or generally across multiple domains, than that expected at a given age. Nevertheless, individuals experiencing a negative change from their usual cognitive level, remain functional and independent in the daily life tasks with a minimal aid from others.

1.1.2 Dementia

Dementia, or major neurocognitive disorder as per DSM-5 classification\(^2\), is a syndrome of severe deterioration in an individual’s mental status which includes a substantial decline in memory, attention, executive function (such as planning, organizing, problem solving and multi-tasking), inappropriate social behavior and severely compromised everyday functioning. The symptoms presented by an individual may also include language and mood disturbances, more commonly depression, apathy and anxiety.

Dementia remains one of the biggest global public health challenges. The approximate number of individuals living with this diagnosis worldwide today is estimated at 47 million and is set to grow almost twofold in the next decade \(^5\). In Canada, the numbers are expected to be nearly 1 million people in the next decade according to Alzheimer Society of Canada 2018 facts.
There are multiple diseases that are manifested as dementia syndrome, but the most commonly diagnosed form is dementia due to AD which accounts for 60-70% of all cases. The second most common cause of dementia is vascular dementia.

1.1.3 Metabolic Vascular Disorders

Metabolic vascular disorders represent a group of diseases featuring dysfunction of metabolism and pathological alterations to the vascular system, representing risk factors for cardio-, cerebro- and peripheral vascular diseases\(^6,7\). This group includes such major conditions as type 2 diabetes mellitus (T2DM) and its precursors, obesity and MetS. MetS is a cluster of several conditions including overweight status, defined by a body mass index (BMI) \(>25\) kg/m\(^2\), and obesity (BMI \(\geq 30\) kg/m\(^2\)), hyper- and dyslipidemia, glucose metabolism perturbations, insulin resistance, and hypertension.

The incidence of these chronic pathological conditions has markedly increased over the past few decades in the adult population and the prevalence has reached epidemic status particularly from a global ageing perspective\(^8\)–\(^{12}\). This growth appears to be primarily associated with unhealthy lifestyle choices, such as chronic consumption of Western diets high in fat, sugar and salt and low physical activity\(^{13}\)–\(^{18}\). The estimated prevalence of obesity is currently 650 million people worldwide\(^{19}\). People diagnosed with diabetes, 90% of whom have T2DM, account for 425 million worldwide\(^{20,21}\).

The global prevalence of MetS, which is difficult to measure, was estimated to be at about one quarter of the world population, roughly over a billion people\(^8\). In Canada, roughly 6 million people were reported to be obese between 2001-2012\(^{22}\). Canada-wide statistics indicated that in 2016-2017, 64% of adults older than 18 years were overweight and obese\(^{23}\). Obesity accounted for 27% of this statistic which is approximately 8 million people indicating a progressive increase over the past 5 years\(^{24}\). Canadian statistics in 2017 on diabetes indicated that more than 2 million people were diagnosed with diabetes and about 22% of population aged 12-79 had symptoms of MetS \(^{25}\)–\(^{27}\). The seriousness of the situation behind these numbers is that these diseases not only represent
a public health issue and increased incidence of vascular morbidities, but also impose a great risk for dementia, including AD later in life\textsuperscript{13,14,17,28–38}.

1.2 Alzheimer’s Disease

Alzheimer's disease (AD) is a neurodegenerative disease that leads to severe mental health decline and incapability to function independently in everyday life. There are two known forms of this disease. The first and most common form (about 95\% AD cases) is sporadic form of the disease that in most cases presents as senile or late-onset AD (LOAD) in late 60-80s years of life with occasional early onset AD (EOAD) cases\textsuperscript{39}. The second is a familial form (FAD) with an autosomal dominant inheritance of genetic mutations that typically has an early-onset, before 65 years, although some later in life onset cases are described\textsuperscript{40}. The progressive, irreversible and incurable character of this disease, regardless of the form, is a major societal concern given the high prevalence of this type of age-related dementing disorder in the population. Despite the extensive research conducted in the AD field, our comprehension of the causal events and pathological presentation, especially in the very early stages of the disease, is still incomplete and is an impediment to the development of therapeutic advancements. The search for biomarkers of the disease to aid in early capture has been developing intensively, and yet a timely and accurate diagnosis of AD that is essential for early intervention and better prognosis remains a great challenge.

1.2.1 Diagnosis of Alzheimer’s Disease

Accurate life-time clinical diagnostics of AD is very challenging, even with the existence of detailed criteria available to healthcare providers and clinical researchers. The diagnosis is mainly based on the patient’s complaints and their close relatives’ observations of gradually developing progressive cognitive decline from a previous cognitive level; as well as the patients’ performance on general cognitive and more sophisticated neuropsychological testing. Patient anamnensis and the family history provide additional information related to genetic, medical and life-style risk factors
which can further aid in diagnosis. Laboratory and instrumental diagnostics, including structural and functional magnetic resonance imaging (MRI), computer tomography and positron emission tomography (PET) is becoming more critical for early and differential diagnosis. However the methods are unstandardized and not widely available and therefore are not recommended for clinical use. Nevertheless, neuroimaging has become a very useful tool in clinical and pre-clinical research that has significantly advanced our understanding of pathological processes, particularly associated with the early stages of AD and MCI.

The core criteria outlined in the DSM-5, and recommended by the National Institute on Aging (NIA) and Alzheimer’s Association (AA) workgroups, allow for the identification of probable and possible AD. To qualify for the most typical amnestic type of probable AD, the patient must present with a decline in memory, and with at minimum, an impairment in one other cognitive domain. Alternatively, in non-amnestic case at least two cognitive domains from language, visuospatial and executive functioning should be impaired. The diagnosis of probable AD also requires no evidence of concomitant substantial cerebrovascular disease, major psychiatric disorder, or other neurological or systemic conditions that could explain the cognitive symptomatology. Evidence of autosomal dominant FAD increases the certainty of the probable AD case. If probable AD is not confirmed, possible AD should be diagnosed. The antemortem diagnosis is mainly verified during autopsy by the presence of amyloid plaques, neurofibrillary tangles (NFT) and cortical atrophy in the brain tissue.

1.2.2 Clinical Presentation of Alzheimer’s Disease

Cognitive presentation of AD varies, and it is recognized that variable cognitive domains may be affected. The two disease forms, sporadic and familial, are largely similar in clinical and pathological representation, though they have some differences in symptomatology and progression with a more rapid deterioration in the early-onset form.

Episodic memory impairment is considered the primary and early feature of the disease. It is characterized by difficulties in recollection of recent events and recent personal past experiences embedded in a spatiotemporal context as well as difficulty with learning and encoding new
information. Semantic or context-free memory undergoes decline during AD. In fact, semantic or context-free memory has been proposed to be affected at prodromal stages of the disease possibly preceding changes in episodic memory. This hypothesis is confirmed by pathological findings of an early NFT accumulation in the entorhinal cortex, a region primarily associated with semantic memory, as well as in the perirhinal cortex. This pathology can precede changes in the hippocampus, a key structure for context-rich spatial and episodic memory. Entorhinal cortical atrophy is suggested to be a better predictor for the conversion from MCI to AD. However, these two memory systems are in fact very closely interrelated and therefore it might be difficult to distinguish which one is solely or early impaired when interpreting the common cognitive tests.

In addition to declarative memory deficits, executive dysfunction is a recognized variant of AD manifestation and often occurs in a combination with a decline in other domains. Moreover, some clinical studies suggest it develops early in the course of the disease at a prodromal or mild AD stage. Individuals with AD show impairment on various executive function processes including working memory, response inhibition, selective attention and resistance to distraction, problem solving, set shifting and cognitive flexibility.

As the disease progresses, impairments may spread to non-cognitive functions including visuoconstrucional and perceptual abilities, social function and personality changes such as increased irritability, aggression, combativeness, and inappropriate behavior. Psychiatric symptoms such as depression and apathy may appear very early and accompany any stage of the disease. Very late in the disease sleep disorders, appetite loss, motor and gait disturbances, and deterioration of vital functions may be evident.

The early onset familial form of the disease has heterogenic clinical features with certain symptoms more attributed to the specific genetic profile. As in the sporadic variant, it also presents with episodic memory, context-free recognition, verbal memory impairment and executive dysfunctions. Various non-cognitive symptoms are characteristic of the FAD and occur more frequently than in sporadic AD. These can include behavioral, psychiatric, motor dysfunctions (e.g. extrapyramidal signs, myoclonus, spastic paraparesis), seizures, cerebral haemorrhage.
1.2.3 Mild Cognitive Impairment Preceding Alzheimer’s Disease

AD is a progressive disease and clinical symptoms develop over many years, even decades. The early phase of the disease when the symptoms start to appear and are yet not severe enough to qualify for AD diagnosis is defined as MCI. Most commonly this phase is characterized by a mild memory impairment. An additional simultaneous decline very often occurs in overall executive functioning and in executive function individual components. Typically decline is evident in working memory, response inhibition, divided attention and manipulation, task switching, cognitive flexibility and problem solving. Individuals with MCI, particularly with decline in multiple cognitive domains, are at a high risk of progression towards dementia including AD. Executive dysfunction is among the strongest predictors of the progressive decline.

There are different patterns and rates of decline in various cognitive domains, which some gradually deteriorate (e.g. immediate recall, inhibition) while others show a rapid decline just prior to AD diagnosis after a relatively long stable phase (e.g. delayed recall, episodic, spatial and working memory). From a clinical and therapeutic prospective, the MCI stage preceding AD appears to be the critical phase for early recognition, intervention and potential delay of the disease progression. However, negative results of clinical trials with potential treatment agents that were initiated at the MCI stage, point to the fact that even at this mild stage the pathological processes in the brain might already be too advanced and largely irreversible. This clinical failure is the impetus for research on the identification the earliest prodromal phases from both a pathological and possibly clinical prospective. This prodromal phase of the disease is asymptomatic and is characterized by developing pathological changes in the brain that can be detected using laboratory diagnostic measures in blood and cerebral spinal fluid (CSF) and neuroimaging. The NIA and AA group has developed recommendations for defining the earliest pre-clinical stage of AD that currently have only research application.

1.2.4 Risk factors of Alzheimer’s Disease

The biggest risk factors for AD are age and genotype, conditions that cannot be changed. Some risk factors (i.e. lifestyle) can be controlled and modulated in order to reduce the chance of
developing metabolic dysfunction and consequently decrease the risk for MCI, AD or progression of cognitive decline\textsuperscript{58,71}.

One of the most critical factors for developing the most common form of AD, sporadic LOAD, is age\textsuperscript{72,73}. With increased age our brain homeostatic systems become less functional and many defense mechanisms weaken, thus creating a favorable environment for development of pathology in response to a triggering event or injury.

AD has a strong genetic predisposition\textsuperscript{74–77}. Early-onset FAD has been causatively linked to mutations in three genes encoding amyloid precursor protein (APP) and presenilin proteins (PSEN1,2), that are directly involved in the amyloid pathway central to AD development\textsuperscript{60}. PSEN1 mutations account for the majority of AD cases\textsuperscript{60,78}. The contribution of genetics to LOAD is also substantial and heritability accounts for 60-80\% of cases\textsuperscript{79}. Sporadic and familial LOAD forms and sporadic non-familial EOAD have shown a very similar profile to FAD\textsuperscript{40,80–84}. Known genetic mutations in APP and PSEN have been found in approximately 2\% of sporadic LOAD cases, although they are unlikely to have a major role in LOAD predisposition\textsuperscript{81,85}.

The only major gene underlying susceptibility to both LOAD types is the APOE\textsubscript{ε4} gene, which encodes Apolipoprotein E (APOE). APOE\textsubscript{ε4} is present in more than half of AD cases\textsuperscript{79,86–91}. Homozygous carriers for this allele have a 10 fold increase in the risk of AD and have an earlier onset of the disease\textsuperscript{79,87,88,92}. Genome-wide studies have identified rare and more common mutations in more than 30 genome loci associated with LOAD\textsuperscript{75,76,80,81,93–96}. Most of the genes found encode proteins involved in cholesterol metabolism, the immune system, endocytosis and amyloid beta metabolism. However, these genes explain only a small part of LOAD genetic profile and impose a much smaller effect than that of APOE\textsubscript{ε4} \textsuperscript{81,97}.

There also appears to be a combinatorial effect of mutations on the risk, age of onset and course of LOAD, such as was seen in the presence of both APOE\textsubscript{ε4} and PSEN1 mutations\textsuperscript{84,98}. Thus, genetics is considered to be an important risk factor for senile AD, however, no causative mutations have been identified. This suggests there are other conditions such as lifestyle, medical conditions and environmental factors that impact the onset of the disease. The role of these other factors is confirmed by a study on twins in which not every individual developed the disease despite having the exact same genome\textsuperscript{77}. 

8
Unhealthy lifestyle choices including cigarette smoking, alcoholism, lack of physical activity and dietary preferences, that are also linked to the development of vascular and metabolic disorders, represent a group of modifiable risk factors for AD\textsuperscript{99–104}. Dietary choices are associated with both high and low risk of AD. Epidemiological and experimental studies indicate that a Western diet, characterized by high content of saturated fat, simple carbohydrates and salt, has been linked to cognitive impairment and increased risk of AD\textsuperscript{16,18,105–109}. On the contrary, adherence to the Mediterranean diet, which includes abundance of fruits, vegetables and whole grains, showed an association with reduced risk for dementia\textsuperscript{110,111}. It has been demonstrated that not only macronutrient composition of the diet (i.e. fat, protein and carbohydrate) is important, but also a content of micronutrients. Several vitamin deficiencies including vitamins D, B12, B6 and E have been linked to AD increased risk\textsuperscript{101,112–115}. The amount of the calories is as important as the nutrient source. High caloric intake exceeding energy expenditure is associated with MCI and AD\textsuperscript{15,116–118}. Animal studies have demonstrated that caloric restriction and low percentage of carbohydrates and fat in foods play a role in a reduced risk for AD\textsuperscript{119,120}.

Many medical conditions, developed throughout the lifespan and particularly during middle age, represent risk factors for AD\textsuperscript{36}. These conditions include hypercholesterolemia, hyperhomocysteinemia, atherosclerosis, cardiovascular and cerebrovascular diseases, inflammatory diseases such as arthritis, T2DM and MetS that can result in hypertension, obesity and insulin resistance\textsuperscript{33,121–137}. Most of these disorders are tightly linked to lifestyle choices including dietary preferences, are chronic conditions, and progress with aging. In addition, they often co-exist with MCI or dementia. Psychological stress, depression, infection and head injury can also have a significant contribution to the risk for AD\textsuperscript{36,138,139}.

Greater brain or cognitive reserve (largely formed by level of education), complexity of the working environment, socioeconomic status, social interaction, and involvement in leisure activities which may be related to higher mental stimulation have been positively related to a reduced risk for AD risk and may delay cognitive deterioration\textsuperscript{140–142}.

None of these risk factors alone have been proven to be necessary or sufficient for AD development. However, the more factors that accumulate can have a cumulative effect and increase the chances of manifestation of the disease pathology\textsuperscript{143}.
1.2.5 Neuropathology of Alzheimer's Disease

1.2.5.1 Current Mechanistic Hypotheses

Various pathological mechanisms are implicated in the etiopathogenesis of AD. The accumulation of amyloid plaques and formation of NFT are considered to be the hallmarks of AD\textsuperscript{74,144,145}. Other processes associated with the development of AD include glial activation, excessive neuroinflammation and oxidative stress, synaptic loss, neuronal degeneration and disruption to the white matter, as well as vascular and metabolic abnormalities, such as brain insulin resistance and abnormal glucose metabolism\textsuperscript{144,146}.

Amyloid hypothesis

Amyloid plaque accumulation in the brain tissue is a core feature of AD. The main component of these plaques is amyloid-β peptide (Aβ) produced through an altered enzymatic cleavage from (APP), endogenously expressed throughout the brain, formed by β- and γ-secretases\textsuperscript{144,147,148}. Amyloid peptide exists in multiple molecular forms starting with soluble monomers Aβ40 and Aβ42 which self-aggregate into oligomers, then into fibrils which form diffuse and later insoluble extracellular plaques. While Aβ42 composes the core of the senile cortical plaque, a more abundant Aβ40 species has a high affinity to the wall of cortical microvasculature and is associated with a cerebral amyloid angiopathy (CAA)\textsuperscript{149,150}. Oligomeric forms are considered to be the most neurotoxic and are associated with synaptic dysfunction and loss\textsuperscript{151–154}. Increased production of Aβ and its decreased clearance from the brain are both considered to be implicated in the disease pathogenesis\textsuperscript{74}. Support for the amyloid hypothesis as the core origin of AD comes from the causative role of genetic mutations of APP and amyloidogenic pathway enzymes genes PSEN1,2 in FAD\textsuperscript{60,78}. However, a major limitation of this theory arises with evidence from studies of human brain showing a lack of correlation between amyloid plaque burden and presence or severity of dementia symptoms\textsuperscript{155–157}.

Tau hypothesis

NFTs in pyramidal neurons constitute a second proteinopathy occurring in AD\textsuperscript{144,147}. Their formation is a result of an aggregation of dysfunctional insoluble hyperphosphorylated tau protein, which normally exists in unphosphorylated form and promotes microtubule stabilization and aids
axonal vesicle transport. Unlike amyloid plaque burden, tau pathology in the neocortex is highly correlated with cognitive performance\textsuperscript{158}. Nonetheless, amyloid and tau pathologies interact with each other, both are integral to AD pathology and both exist in the complex pathological network interconnected with other processes such as synaptic dysfunction, inflammation, oxidative stress.

\textit{Synaptic dysfunction}

Synaptic dysfunction is another AD pathology and is related to the disruption of communication between neurons and has been proposed as an explanation of the early signs of mental deterioration\textsuperscript{144}. Synaptic loss, particularly in the hippocampus, a structure relevant to learning and memory, and in the neocortex occurs in MCI and AD and correlates with cognitive dysfunction\textsuperscript{159,160}.

\textit{Cholinergic hypothesis}

The brain cholinergic system and its main neurotransmitter acetylcholine (ACh) plays a crucial role in learning and memory in a complex manner\textsuperscript{161}. ACh promotes neuronal plasticity, neuroprotection and neurogenesis mainly via nicotinic ACh receptors. It modulates neurotrophin expression (e.g. brain derived neurotrophic factor (BDNF), nerve and fibroblast growth factors), interacts with glia cells, including blocking excessive inflammation derived from microglia pro-inflammatory cytokine production, a pathology that can be detrimental to memory processing\textsuperscript{161}. A substantial reduction in brain ACh levels through changes in the activity of the main ACh enzymes (i.e. choline acetyltransferase, acetylcholinesterase), axonal pathology of cholinergic neurons occurring in the early stages and the loss of these cells in the later stages are evident in AD\textsuperscript{162–164}. Loss of cholinergic synapses contributes to the dysfunction in the cholinergic system. The cholinergic hypothesis of AD has led to the development of therapies for AD and are currently the only drug group approved for symptomatic treatment of the disease\textsuperscript{165}.

\textit{Mitochondrial dysfunction and oxidative stress hypothesis}

Oxidative stress is a pathological condition in which there is an elevation of toxic free radicals such as reactive oxygen (ROS) and nitrogen (NOS) species naturally produced by cells. These free radicals bind to DNA, RNA, protein and lipid molecules leading to cell damage. Mitochondria is a primary site of oxygen free radical production. Studies investigating the oxidant system in AD
patients’ brain tissue samples have concluded that mitochondrial damage and oxidative stress are common and early events in AD, occurring before the hallmark proteinopathies of AD are established and then gradually decreasing with the disease advancement. The changes include mitochondrial DNA alterations, mutations in an oxidative environment, proteasome dysfunction and low cytochrome oxidase activity. Thus, these changes have been proposed to drive amyloid accumulation and promote tau pathologies. In turn, these misfolded proteins further feed the oxidative process by inhibiting mitochondrial functioning directly and activating glia cells which contribute to the free radical pool and drive related inflammatory processes.

**Metabolic dysregulation**

Insulin and insulin growth factor 1 resistance, impaired downstream insulin signaling and altered cerebral glucose metabolism have been consistently reported in AD and accompany NFT formation, Aβ accumulation, inflammation and oxidative stress. Evidence has been provided that brain insulin, locally produced or from peripheral organs, has several effects on the brain including, positive effects on amyloid metabolism (degradation via insulin degrading enzyme action); apoptosis (via NFκB pathway) and memory and learning due to changes in various underlying mechanisms (synaptic plasticity, long-term potentiation and depression, glucose metabolism, downstream insulin signaling etc.). Studies on cognitive decline associated with AD and T2DM, with either pathology alone or in a comorbid situation, have shown improved cognitive performance by memory facilitation with treatment with insulin delivered to the central nervous system (CNS).

Cholesterol and lipid metabolism are also thought to be implicated in AD pathophysiology primarily via involvement with amyloid metabolism. However, the relationship of lipid metabolism with AD are complicated and largely debatable. There is an inconsistency in reports whether hypercholesterolemia and lipid dyshomeostasis in AD are a risk factor for AD. The cholesterol hypothesis of the disease has been questioned due to the fact that peripheral cholesterol does not cross the blood-brain barrier (BBB). Thus, the cholesterol changes outside of the brain are unlikely to directly contribute to brain homeostatic changes. Furthermore, changes in fluid cholesterol concentration of AD patients were reported to be low, high or the same as in non-diseased controls.
Animal research, using models of high-fat/high-cholesterol diet-induced peripheral metabolic disorders that develop dyslipidemia and are associated with impairments to cognition, have been supportive of a role for cholesterol in AD pathogenesis. This could happen via various mechanisms such as altered brain lipid and cholesterol profiles, increased accumulation of amyloid and phosphorylated tau proteins, oxidative stress, inflammation, and disruption to the BBB and microvasculature\textsuperscript{186–193}. The support for a role of cholesterol in AD also comes from the strong genetic link of APOE\textsubscript{e4} allele and high risk of AD development\textsuperscript{91}. APOE encoded by this gene is an important regulator of lipoprotein metabolism and cholesterol transport. APOE is also related to the insulin pathway, is involved in Aβ metabolism, including clearance of soluble Aβ and the Aβ aggregation, and is associated with inflammation and neuroplasticity\textsuperscript{93,194}.

Considerable attention has been granted to the oxysterols as the pathogenic component of AD\textsuperscript{195}. Oxysterols are products of cholesterol oxygenation, mainly by ROS, which are generated within the brain and in the periphery and are able to cross BBB\textsuperscript{180,196,197}. They show involvement in the modulation of cholesterol metabolism, neuroinflammation, apoptosis and Aβ accumulation\textsuperscript{198}. High blood and CSF oxysterol levels, particularly 24(S)-hydroxycholesterol (24S-OHC) which is excreted by the brain, have been reported in AD patients and are linked to amyloid cascade and neuronal stress\textsuperscript{198–201}. Metabolic conditions such as obesity are also associated with the increase in oxysterols and 27-OHC in particular is viewed as a candidate modulator entering the brain and interfering with cerebral homeostasis\textsuperscript{202–204}. In the highly interactive pathological environment in AD, inflammation greatly affects oxysterol levels and these substances in turn effect glial activation\textsuperscript{197,203}.

\textit{Neuroinflammation}

Neuroinflammation is one of the earliest and most critical events occurring in the brain in response to insult and plays an important and likely a key role in the pathogenesis of AD\textsuperscript{205–208}. Microglia are the key cellular component of the inflammatory processes occurring in the brain and are the first cells to become activated and proliferate in response to disturbances in cerebral homeostasis\textsuperscript{209}. Astrocytes play a major role in maintaining brain health and get readily involved in inflammatory reactions\textsuperscript{210}. A more detailed look at the role of neuroinflammation in AD is contained in a separate sub-chapter.
Neurovascular hypothesis

The sophisticated organization of the brain vascular system allows it to maintain its unique strictly regulated microenvironment. The larger caliber vessels can autoregulate cerebral blood flow via dilation-constriction mechanisms keeping the brain independent from systemic changes in the blood pressure. Capillaries are a part of the functional unit of the system called the neurovascular unit (NVU). NVU consists of anatomically and functionally closely linked endothelial, smooth muscle cells, pericytes, neurons, interneurons, astrocytes, microglia, and an extracellular compartment. Capillary endothelial cells, pericytes and astrocyte end feet contacting endothelial basal lamina comprise the crucial NVU subunit forming the BBB and is sealed by intercellular tight junctions. Each of these components crucially contributes to the proper functioning of the unit. The NVU serves as a regulator of blood supply with respect to the brain energy needs, a process called neurovascular coupling.

The BBB physically separates the CNS microenvironment from the peripheral circulation and regulates microvascular permeability for selective flux of substances to protect the brain from peripheral toxins and metabolites. Abluminal and soluble forms of low-density lipoprotein receptor–related proteins (LRP1) in conjunction with APOE regulate the efflux of brain Aβ42 and Aβ40 to the systemic circulation. Receptors for advanced glycation end products (RAGE), normally expressed in low amounts, mediate the influx of molecules from the peripheral blood, including Aβ. Glucose transporters such as GLUT1 primarily maintain the necessary high glucose concentrations, but also contribute to the BBB integrity and maintenance of cerebral blood flow.

AD is associated with both structural and functional alterations of the cerebrovasculature. Microvascular abnormalities include decreased density, increased tortuosity, deposition of circulating lipids, and collagen, fibrinogen, amyloid and leukocyte adhesion to the vascular wall causing damage to the NVU such as endothelial inflammation and pericyte degeneration. These changes are accompanied by increased vascular reactivity and neurovascular uncoupling, vasoconstriction, hypoperfusion and ischemia creating a toxic oxidative, pro-inflammatory environment which further propagates BBB leakage. There is evidence of the loss of LRP1 and GLUT1 receptors with a parallel upregulation of RAGE. APOEe4 genotype, associated with the high risk of AD, has been linked to the BBB breakdown. Blood-
derived proteins such as fibrinogen, immunoglobulin IgG and circulating Aβ enter the brain easier when the BBB is compromised. Alternatively, clearance of cerebral Aβ is impaired which leads to its accumulation and amplification of the NVU damage, glia activation and inflammation, and ultimately axonal and neuronal damage.

The neurovascular hypothesis of AD provides an explanation for the sequential pathology seen in the disease starting with a diminished Aβ clearance and age-related alterations to the cerebrovascular system that could initiate neurovascular uncoupling, microvascular inflammation and degeneration, hypoperfusion and hypoxia. These changes which affect BBB integrity, compromise the brain biochemical microenvironment leading to synaptic and neuronal damage and loss.

The proposed two-hit concept of pathology development in AD implies that there is initial damage to the neurovascular system, such as by cerebrovascular insults, cardiovascular diseases and other risk conditions as well as age-related alterations, that leads to damage to the BBB and microvasculature. The impaired BBB and microvasculature subsequently induce oligemia or blood supply reduction, hypoxia and create a toxic environment. These vascular changes initiate early neuronal dysfunction and also promote Aβ deposition via reduced vascular clearance from the CNS and increased generation of Aβ that in turn exacerbates vascular and neuronal damage.

While it is still debatable whether cerebrovascular abnormalities are a cause or a consequence of AD, it is very likely that in fact it is both and NVU dysfunction is a significant contributor from the very initial to the end stage of the disease.

White matter changes

AD is a disease impacting both the gray and white matter of the brain. While changes to the gray matter in the pathogenesis of AD are well known and continued to be heavily investigated, the neuropathology of white matter abnormalities still remains not fully understood. White matter pathology is mainly attributed to cerebral small vessel degeneration and BBB deregulation with a contribution from inflammatory events, leading to loss of myelin and axonal fibers. White matter changes in AD are described in a separate sub-chapter.
1.2.5.2 Neuroinflammation and Role of Glia Cells in Alzheimer’s Disease

Neuroinflammation is a complex process thought to be implicated in the early stages of AD development largely contributing to its progression\textsuperscript{205,207,208,255–260}. Moreover, it interacts with all other mechanistic processes in AD pathogenesis and is a component linking these multiple pathological events. Patients with AD exhibit upregulation of genes associated with inflammation regulation (e.g. triggering receptor expressed on myeloid cells 2 (TREM2) mutations, cluster of differentiation (CD) 33, tumor necrosis factor alpha (TNF-α), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), cyclooxygenases (COX) 1 and 2) and elevation of circulating and brain tissue cytokines, particularly TNF-α and interleukin IL-6, acute phase proteins, chemokines, complement and glial activation\textsuperscript{80,206–208,255,258,260–264}. Epidemiological studies show a decreased risk of AD associated with the long-term use of non-steroidal anti-inflammatory drugs (NSAIDs) which further supports the inflammatory hypothesis of AD and suggests inflammation is a critical therapeutic target\textsuperscript{265,266}.

Brain inflammation is largely mediated by the resident innate immune cells, microglia, perivascular macrophages and immunocompetent astrocytes\textsuperscript{260}. Microglia cells conduct a constant surveillance of the brain environment and are readily activated when an aberrant process or pathogen is detected. In the AD context, the main triggers of microglia are aberrantly processed amyloid protein (e.g. Aβ monomers, oligomers, fibrils), injured neurons including those impacted by Aβ and p-tau, damaged vasculature and BBB breakdown\textsuperscript{146,257,267}. These triggers activate cells directly or through receptor binding such as RAGE, scavenger receptors, and toll-like receptors (TLRs) or through cluster of differentiation. These triggers lead to microglia phenotype changes promoting phagocytosis of apoptotic neurons, dystrophic neurite and clearance of Aβ accumulates\textsuperscript{205,268,269}.

Activated microglia further communicate with astrocytes and perivascular macrophages which help in Aβ plaque degradation via cytokines (e.g. TNF -α, IL-6, IL-1β, IL-12, IL-23), chemokines, caspases, and production and release of ROS/NOS\textsuperscript{207}. It has been proposed that glia cell numbers do not increase during AD but only undergo phenotypic change without marked proliferation\textsuperscript{270}.
Microglia appear to be activated even at the preclinical stage, when subjective cognitive decline is reported, although cognitive tests show no impairment. This activation gradually increases with the progression from MCI to eventually AD\textsuperscript{271}.

Astrocytes show a delayed involvement, becoming reactive at the MCI stage\textsuperscript{271,272}. Astrocyte activation and generation of the reactive phenotype, characterized by upregulation of glial fibrillary acidic protein (GFAP), RAGE and epidermal growth factor receptor (EGFR) are associated with Aβ plaques in AD\textsuperscript{273,274}. Reactive astrocytes aid in Aβ clearance and likely help in restriction of the focal damage though without a glial scar formation\textsuperscript{275,276}. They also interact with microglia cells and are able to modulate their activity and help down-regulate inflammatory reaction via release of anti-inflammatory factors\textsuperscript{277–279}. However, the main functions of astrocytes are metabolic support for neurons, their survival and outgrowth, synaptogenesis, myelination and BBB maintenance. During chronic inflammation, associated with triggering events, these primary functions of astrocytes are likely compromised which contributes to the propagation of the pathology\textsuperscript{280,281}. Furthermore, under pathological conditions reactive astrocytes upregulate the NF-kB pathway, overexpressing IL-1β, IL-6, mediator S100β, release pro-inflammatory cytokines and chemokines, which further activate microglial cells and propagate tissue damage including plaque formation\textsuperscript{277}.

The relationship between neuroinflammation and AD appears to be very complicated and not simply either beneficial or detrimental\textsuperscript{205,208,256,260}. While inflammation is a natural response to an injury directed toward resolving the pathological event, persistent inflammation - chronic cell overactivation by the trigger and unsuppressed pro-inflammatory cytokine release – can lead to failure of protective mechanisms, disease development and propagation. Thus, in pre-clinical studies microglial overactivation has been shown to result in decreased phagocytotic efficiency, leading to a reduced degradation of amyloid aggregates, while pro-inflammatory cytokine production was unaffected\textsuperscript{269,282–284}. However, even when microglia are overactivated, amyloid peptide toxicity, especially of oligomers, acts on glial cells to induce dystrophic state in microglia and loss of proper function\textsuperscript{146,285}.

The relationship in LOAD is further complicated by the effects of ageing on the immune system. The term inflammaging describes the age-related chronic low grade pro-inflammatory
environment in the brain which is associated with increased inflammasome generation and secretion of IL-1β and IL-18 and may contribute to age-related cognitive decline\textsuperscript{286,287}. Immunosenescence is viewed as a part of normal aging that presents as changes to cell morphology, physiology and function, and in a balance between inflammatory initiation-resolution mechanisms that might lead to cell priming and increased susceptibility to injury\textsuperscript{288–290}.

Microglia senescence is characterized by morphological changes, decreased mobility, loss of homogenous tissue distribution, altered protein expression (e.g. increased major histocompatibility class (MHCII) and reduced TREM2), increased oxidative stress and mitochondrial dysfunction\textsuperscript{291}. These changes result in less effective monitoring, defective phagocytosis and impaired communication with neurons leading to diminished response to injury, while at the same time maintaining pro-inflammatory cytokine production and release\textsuperscript{292}. Diminished amyloid peptide clearance from the brain that occurs with age further triggers activated microglia initiating a pathological inflammatory response\textsuperscript{286,293}. Experimental data indicates that senescent cell dysfunction is related to AD pathogenesis. The results show dystrophic microglial cells surround neurons with NFT pathology, suggesting this association precedes tau pathology and likely neurodegeneration in AD\textsuperscript{294,295}.

An adaptive immune system is also involved in AD and it may be recruited when resident microglia cells are not able to manage the pathological process. Circulating monocytes, attracted by microglia, can infiltrate the brain and participate in Aβ clearance\textsuperscript{267,296–299}. Furthermore, the perivascular space contains macrophages that act as antigen-presenting cells (APC) and are regularly renewed by infiltrating monocyte differentiation\textsuperscript{300}.

To support the immune system, pericytes are capable of transformation into microglia and acquire their function\textsuperscript{301}. T-lymphocytes migration into the brain has also been suggested to be involved in the AD process\textsuperscript{296}. Microglia in AD show upregulation of genes involved in APC-T-cell interaction and express MHCII, encoded by the human leukocyte antigens (HLA) genes which can modify the susceptibility to LOAD\textsuperscript{268,302}. MHCII expression is linked to antigen-presentation function and could potentially attract T-cells initiating their migration to microglia locations\textsuperscript{303,304}. Amyloid peptides and cellular debris are potential antigens presented to T-cells which show
increased reactivity to Aβ. CD8+ T-cells are thought to be involved in apoptosis, while CD4+ T-helpers can modulate microglial responses.

1.2.5.3 White Matter Changes in Alzheimer’s Disease

There is more white matter than gray matter in the brain and it consists of myelinated and unmyelinated axons, glia (including microglia, astrocytes, oligodendrocytes) and blood vessels. The white matter plays an important role in brain connectivity and cognitive function. Similar to the gray matter, it undergoes atrophy and structural changes with ageing and is not spared by neurodegenerative disorders, including AD. Eventually, white matter damage results in the loss of connectivity between gray matter regions and contributes to cognitive decline. These changes can be seen on T2 - MRI scans as hyperintensities (WMH) known as leukoaraiosis. Diffuse tensor imaging (DTI-MRI) allows an estimate of white matter spatial area and integrity. DTI-MRI can detect changes that appear normal in MRI and in histopathological examination. WMH increase with aging, are often present in MCI and AD and have been shown to highly correlate with cognitive decline. Animal models of AD have supported the clinical findings of white matter alterations in this disease. These white matter abnormalities have been viewed as an early pre-clinical stage and a core pathological event in AD, particularly in early-onset FAD, and precede classical gray matter proteinopathies and symptom onset.

WMH, or leukoraise, are thought to have a vascular origin, caused by hypoperfusion, and represent small vessel cerebrovascular disease which is commonly observed in AD and suggests an overlap between vascular disease and AD. WMH are also co-localized with amyloid pathology in AD brains and are more severe when CAA or microbleeds are present. This relationship with CAA and amyloid also emphasizes the involvement of vascular system in the pathogenesis of AD. These findings suggest the possibility that vascular abnormalities drive AD development and/or interact in an additive or synergistic way with the classical pathology as opposed to be independently co-exist.
The prefrontal, temporal and parietal lobes show the highest vulnerability to classic AD pathology and neurodegeneration. Myelination of axons from these regions continues into mid-life\textsuperscript{306,335}. These areas, the last areas to myelinate, are associated with encoding and retrieval of new memories, short-term memory formation and executive functions. These cortical areas are the first to deteriorate with normal aging and most impacted by preclinical and early AD and can explain the clinical profile observed in early AD\textsuperscript{54,65,306,336,337}. Cortical regions that are myelinated early in life (primary motor, sensory cortex) appear to be more resistant to degeneration and are affected only in the late stages of AD. These observations fall under the neuropathological retrogenesis concept which implies that aging degenerative changes begin in the last developed structures. Atrophy of the corpus callosum is evident in MCI and mild AD, begins in the later myelinating anterior subregions and propagates to posterior subregions which receive axons directly from temporo-parietal lobe regions primarily affected in the later stage of overt protein pathology in AD\textsuperscript{338,339}.

White matter degeneration and associated volume loss occurs in a spatial pattern that is depending on the AD variant and correlates with symptomatology\textsuperscript{340,341}. White matter damage and atrophy in rare disease variants was observed in the lateral temporal and parietal regions, including cingulum, posterior corpus callosum, fornix and occipital region of the brain. LOAD was associated with a less intensive regional involvement. However, there was damage and atrophy in frontal and parietal regions and the medial temporal region\textsuperscript{342}. Even when the conventional MRI scans appeared normal, the white matter of AD patients showed abnormalities with DTI in limbic pathways, cortico-cortical association tracts, interhemispheric tracts, and corticospinal tracts, which correlated with regional gray matter atrophy\textsuperscript{337,343,344}. This association between gray and white matter is not evident in MCI patients\textsuperscript{312,337,343,344}. This data suggests that in early stages of the disease, white matter damage is unlikely to be caused by anterograde Wallerian degeneration related to neuronal damage. The white matter degeneration originates from local events directly damaging myelin and axons, that leads to retrograde neuronal degeneration\textsuperscript{321}. Thus, neurodegeneration in the later stages of AD could be associated with progression in white matter damage.

The underlying pathology of WMH and abnormal white matter seen with DTI may include demyelination and axonopathy, which can be accompanied by gliosis and glial dysfunction,
pathology which is also observed in animal models of AD. Animal studies suggest that demyelination itself does not lead to axonal degeneration when normal glial function is maintained. However, these demyelinated and unmyelinated axons could be exposed to and damaged by various pathological stimuli present in AD. Vascular events including hypoperfusion and ischemia associated with BBB leakage, microvascular pathology, aberrant neuroinflammation and oxidative stress, can all contribute significantly to axonal and myelin damage.

These pathological conditions can act on vulnerable oligodendrocytes and precursor cells and promote pericyte degeneration and loss that propagates white matter damage. Local accumulation of Aβ40 and Aβ42 amyloid in white matter, due to increased production and/or impaired clearance of amyloid, can also damage glia cells and axons, aggravating axonal transport and demyelination, and promote vascular and BBB damage, including pericyte degeneration and loss, and glial overactivation. Diminished axonal APP transport, vascular clearance of APP and damage to myelin can in turn contribute to amyloid accumulation. This pathological cascade appears to be self-propagating due to a high level of interaction and influence between these events. The interaction contributes to the difficulty in identifying a causative event in the white matter disease and AD etiology as a whole.

1.2.6 Biomarkers of Alzheimer’s Disease

The use of biomarkers can increase the specificity and accuracy of the clinical diagnosis of probable and possible AD by addition of pathophysiological evidence to the observed symptomatology. There are multiple biomarkers which have long been proposed and are currently used, mainly in clinical research. However, this area is continuing to be actively explored and various new markers have been identified. The principle idea of a biomarker is to demonstrate an in-vivo pathology that can be observed at various stages of the disease including the prodromal asymptomatic phase. This would allow the biomarker to be used for diagnosis and likelihood of disease progression. The primary markers for AD are based on the key pathologies: amyloid deposition, NFT, oxidative stress, neuroinflammation, neurodegeneration, hypometabolism, BBB integrity, perfusion status, brain atrophy and WMH. These biomarkers are
grouped into factors in blood and CSF, and neuroimaging markers. Genetic screening for causative mutations associated with FAD and APOEε4 also complement diagnosis.

Evidence of cerebral amyloid deposition can be obtained from plasma or serum and CSF. These fluid measures can include Aβ1-42, Aβ1-40 and fibrinogen γ-chain levels. Low levels of fluid Aβ are suggestive of increased brain amyloid accumulation. The presence of fibrillar amyloid plaque and amyloid angiopathy can also be detected using PET with the 11C-Pittsburg component B tracer (Pi-B). Elevated concentration of total (t-tau) and phosphorylated tau (p-tau) protein measured in blood or CSF are also associated with the ongoing neurodegenerative process. It is recommended to use amyloid and tau markers in combination to increase level of information and accuracy. Thus, a combination of these proteins in a CSF Aβ1-42/t-tau ratio showed a high specificity and sensitivity as a marker. Amyloid PET scans have shown to be good at predicting progression to AD from MCI and are used in parallel with or to back up fluid amyloid level markers.

Inflammation and oxidative stress are two critical processes that are present from the very initial prodromal stages of AD, preceding protein pathologies and are promising therapeutic targets. These events can be detected using both fluid analysis and PET imaging technology. The PET imaging utilizes radiological tracers for the detection of specific proteins largely expressed by activated cells. These biomarkers can be a key to treatment management and disease assessment. PET imaging of both microglia and astrocytes, uses tracers for a highly specific microglia 18 kDa translocator protein (TSPO) and less specific astrocytic monoamine oxidase B inhibitor (MAO-B). Increased concentration of cytokines in blood and CSF, including c-reactive protein (CRP), IL-6, TNF-α, or microglia associated proteins (e.g. TREM2 receptor) have also been shown to reflect neuroinflammatory status. Specific markers of brain oxidative stress, including lipid peroxidation products (oxysterols), can be detected in blood or urine with chromatography or mass spectrometry.

Clinically, biomarkers that can be detected in blood receive a great deal of attention since they are easily obtainable using minimally invasive and potentially more widely affordable methods. A recent study examined plasma metabolites to identify novel diagnostic markers and reported a group of 24 metabolites associated with preclinical LOAD. Neurofilament light chain is another
promising marker of disease progression. It is specifically associated with degeneration of myelinated axons and can be measured in plasma or serum as well as in CSF\(^{372,377,378}\). Elevated levels and increased rates of change of neurofilament light chain in prodromal FAD were shown to be predictive of a faster symptomatic onset and highly correlated with cortical thinning\(^{377}\). AD patients also have significantly elevated levels compared to MCI and healthy individuals suggesting there is a correlation with cognitive deficits\(^{378}\). Another group of blood-derived biomarkers are autoantibodies against various neuronal proteins involved in inflammation, vascular and metabolic alterations that can be observed at various pathological stages of the disease\(^{369}\).

BBB integrity is compromised in early stages of AD and is independent of changes in amyloid or tau pathology. Permeability of BBB can be assessed with dynamic MRI with contrast agent and CSF-serum albumin ratio\(^{250}\). Microvascular damage, particularly in the hippocampal region is related to early AD and is detected in the CSF using a capillary pericyte loss marker that is a soluble platelet-derived growth factor receptor-β. Cerebral hypoperfusion is associated with the pathology of AD can be detected using single-photon emission computed tomography (SPECT) imaging. Another marker of late stage neurodegeneration is hypometabolism in temporoparietal regions and is associated with synaptic dysfunction that is detectable on PET images with a fluorodeoxyglucose ligand (18FDG). However, these markers are evidence of the ongoing advanced stage and lack specificity.

MRI provides information on structural changes of the brain. Atrophy of the hippocampal formation and medial temporal and parietal lobes seen on MRI scans is a proven marker of progression to AD dementia. However, this atrophy is usually suggestive of a more advanced stage of the disease marked by neurodegeneration\(^{379}\). MRI fluid attenuated inversion recovery (FLAIR) and T2-sequencing are able to detect WMH that are commonly present in AD and MCI and have been shown to highly correlate with cognitive decline\(^{108,316,318,380}\). Of great interest is the clinical finding that WMH, leukoaraiosis volume, specifically in the parietal and occipital lobe, tend to be present a couple of decades before symptoms of cognitive deterioration start to appear\(^{308,310,316}\) and suggest they are promising biomarkers of early disease stages for MCI and subsequent conversion to AD.
Although, often seen in AD, WMH are not specific to AD pathology. Thus, the exploration of WMH as a biomarker should be extended beyond the absence and presence, and look deeper at the underlying WMH pathology specifically associated with AD. The neuropathology of white matter abnormalities still remains not fully understood and is mainly attributed to cerebral small vessel degeneration, inflammatory events, as well as loss of myelin and axonal fibers\textsuperscript{252-254,311}. Inflammation, as an early process in AD pathogenesis, might be also developing in the white matter. Markers specific to white matter inflammation including imaging and fluid-derived approaches should be studied. A high level of peripheral inflammatory cytokine IL-8 in the serum was shown to be related to WMH in AD and this further suggests an inflammatory basis of leukaraiosis\textsuperscript{381}.

A high priority is the detection of MCI in the elderly population and in particular those individuals likely to convert to dementia. The best predictors of conversion from MCI to AD are hippocampal volume and levels of p-tau in the CSF. In addition, these measures in combination with CSF Aβ\textsubscript{1-42} or blood phospholipids levels provide the highest predictive power\textsuperscript{61,371,382-385}. Progression of an increase rate in WMH volume is a predictor of both conversion from a healthy brain to MCI and from MCI to dementia due to AD of either a vascular or mixed pathology\textsuperscript{386,387}.

Some of the biomarkers discussed above can show relatively high specificity and accuracy, although they also have certain limitations. None of them are widely accessible or have been clinically validated, standardized or approved. They are generally recommended for use only for research purposes\textsuperscript{41,42,61,367}. Most of the diagnostic biomarkers reflect an advanced pathology and likely have limited relevance to potential disease treatment. Most of prognostic biomarkers are usually predictive of a conversion in cases that are already symptomatic. Comorbid conditions including chronic metabolic and vascular diseases, age, gender and belonging to a specific ethnic group may modulate the relationship of the markers to AD or MCI pathologies or change the specificity and sensitivity of some biomarkers, suggesting that extensive investigations in this area are required.
**1.2.7 Treatment Strategies for Alzheimer’s Disease**

Currently there is no available treatment to stop AD progression and cure the disease. However, there are several approved treatment agents available for symptomatic relief. One of the drugs approved for use in mild to moderate AD cases belong to the group of reversible acetylcholinesterase inhibitors including donepezil, rivastigmine, and galantamine. The mechanism of action of these drugs is a prolongation of neurotransmitter ACh lifetime and its action at synapses, facilitating cholinergic transmission. With the progression of pathological processes into more advanced stages of neurodegeneration and loss of neuronal synapses, these drugs lose their ability to improve cognitive deficits.

Another agent proven beneficial in patients with mild to moderate disease stages is cerebrolysin, an agent that mimics properties of neurotrophic factors. Memantine, a glutaminergic N-methyl-D-aspartate (NMDA) receptor antagonist, is an approved pharmacological agent for moderate to severe AD dementia. Beneficial effects on cognition are thought to be related to its ability to reduce excessive stimulation of cortical and hippocampal neurons by glutamate via calcium cell entry reduction and diminish cell apoptosis and also increase long-term synaptic potentiation associated with memory formation. While the drugs described above show some benefit in overt AD, few or none were found to be of use in MCI. The final class of drugs that is prescribed are antidepressants used to alleviate psychiatric and behavioral symptoms of AD patients, including depression, anxiety and agitation. However, their use provides very modest beneficial effect and is associated with increased cerebrovascular complications and mortality, therefore their inclusion in clinical practice should be done with great caution.

With the increased evidence for the association between AD and metabolic disorder, including T2DM and MetS, drugs used in the treatment plan of these disorders is receiving greater attention. The peroxisome proliferator activated receptor γ (PPARγ) agonists pioglitazone and rosiglitazone, which are conventional T2DM drugs, did not demonstrate any benefit over placebo in randomized clinical trials (RCT) when used in mild to moderate AD. A pilot study on insulin sensitizing via intranasal insulin administration have shown beneficial effects in early AD. RCT on the potential for nasal insulin therapy has recently ended in 2018 and results are pending. Statins or 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (e.g. pravastatin,
simvastatin, atorvastatin) demonstrated no positive effects in preventing cognitive deterioration in the elderly. Nilvadipine, a calcium channel blocker, is widely used for the management of hypertension, showed promising results preclinically, however, there was no benefit in patients with mild-to-moderate AD.

Currently, experimental pathology-modulating treatments are being increasingly developed. The main preclinical and clinical research directions are chosen based on the key AD pathologies – amyloid and tau pathology, inflammation and oxidative stress.

Inhibition of β- (BACE) or γ-secretase and promotion of α-secretase activity are the key ways to reduce Aβ production and accumulation. Treatment agents with such mechanism of action have failed to show beneficial effects in clinical trials, although clinical trials on several new agents inhibiting BACE activity are ongoing. Vaccination with Aβ42 or passive immunization with antibodies against Aβ as well as intravenous gamma globulin which can promote clearance of amyloid from the brain, have also been studied. Although, this approach led to cognitive improvement in early trials, it did not stop further decline and, moreover, was associated with serious adverse effects. However, immunization against amyloid is being further investigated and a few antibodies, including crenezumab, gantenerumab, and aducanumab, are currently in phase II-III clinical trials involving early and mild AD stages.

Another way to interfere with the amyloid aggregation pathway is based on soluble Aβ42 binding reducing oligomerization and subsequent deposition. A small glycosaminoglycan-like molecule, Tramiprosate, with this mechanism of action failed to show clinical efficacy in a phase III clinical trial, although it unexpectedly showed benefits in APOE4 carriers.

Modulation of tau proteinopathy can be achieved by tau phosphorylation inhibitors, prevention of aggregation, microtubule stabilization or active immunization. The tau aggregation inhibitor, TRx0237, is currently in a phase III clinical trial in mild AD patients. Other trials completed with this agent produced negative results or indicated a high rate of side effects incompatible with a clinical use of these drugs. Tau immunization is also currently under clinical testing.

Another pathway of intervention is enhancement of the brain serotonergic system, which is disturbed in the AD and greatly contributes to behavioral and also cognitive symptoms of the
disease, using 5-HT₆ serotonin receptor antagonists (e.g. intepirdine) which is currently being clinically tested for mild-to-moderate AD treatment⁴¹²–⁴¹⁴.

The strategies to reduce oxidative stress associated with AD pathology throughout the entire course of the disease and particularly at the early prodromal stages, can include the use of the natural or human-made antioxidants and modulation of oxidative-inflammatory pathways. Antioxidants comprise a large group of natural or synthesized substances able to reduce cell damage by free radicals due to oxidative stress⁴¹⁵. Vegetables and fruits are enriched with these substances and when included in the diet are thought to be beneficial for mental health. Epidemiological studies suggested a positive effect of such diets with natural substances or supplements with antioxidant properties. These studies examined the impact of antioxidants on cognitive function and lowering the risk for dementia. The preclinical research demonstrated the capability of antioxidants to reduce cellular pathology and improve cognitive outcome, however, clinical research indicated a questionable benefit and even opposite effects⁴¹⁵–⁴¹⁹.

One trial with an antioxidant indicated that patients with probable AD benefitted cognitively from the N-acetylcysteine supplement⁴²⁰. Trials with resveratrol and curcumin have produced either mild positive or neutral results⁴⁰²,⁴²¹. However, a combination of antioxidants including vitamins C, E, α-lipoic acid and coenzyme Q3 in an RCT involving patients with mild-to-moderate stages of AD raised the concern that there might be a faster cognitive decline despite lower levels of selective, but not all CSF, markers of oxidative stress⁴²².

Targeting neuroinflammation appears to be one of the most promising approaches to slowing or preventing some forms of dementia, including AD, since it has been shown to be one of the earliest processes related to the disease pathogenesis. Epidemiological, neuropathological and animal studies have shown a long-term treatment with NSAIDs that inhibit cyclooxygenase (COX) activity, either selectively (COX2) or non-selectively, and that reduce the synthesis of prostaglandins, are associated with a reduced risk of AD ⁶⁵,⁴²³–⁴³⁶. However, clinical trials with NSAIDs have produced controversial results with some studies reporting positive effects and others indicating no significant cognitive improvement in MCI and AD⁴²⁵,⁴²⁷,⁴³⁷–⁴⁴⁷.

It appears that with NSAID treatment, an absence of initial cognitive symptoms and earlier intervention were the factors defining success of the study. It is likely, that initiation of NSAIDs
treatment prior to major pathology development (i.e. very early stages) of the disease and in asymptomatic phase might have a potential to prevent or slow down the progression and delay clinical onset of AD\textsuperscript{424,425,437,439,443}. Thus, NSAIDs can be viewed as a preventive measure rather than a treatment per se. However, there are clinical reports with evidence of serious adverse cardiovascular and cerebrovascular effects using this group of drugs irrespective of the selectivity of the NSAID, although the COX-2 selective drugs appeared more implicated in the adverse effects\textsuperscript{438,448–463}.

Based on these mixed results, of great importance is the question of the safety of these agents in the general population as well as those with concurrent vascular or metabolic diseases such as T2DM and MetS. This latter group are in a high-risk group for vascular complications and may already have cardiovascular diagnoses and/or cerebrovascular history\textsuperscript{448,451}. Further studies carefully investigating the exact conditions of the treatment, including the timing, dosage and duration and determining overall safety are required to understand the treatment potential of this large class of agents. Innovative drug design to enhance delivery to the brain to allow the use of lower doses and to minimize of adverse effects are needed.

Aspirin is another drug with COX enzyme inhibiting properties, was also shown to modulate oxidative and inflammatory pathology preclinically\textsuperscript{464,465}. However, no positive effects were seen in clinical trials and the treatment was also associated with increased risk of cerebrovascular events\textsuperscript{440,466}.

Experimental anti-inflammatory drug treatment is being continuously examined in preclinical research and the research yields results supportive of the anti-inflammatory hypothesis of AD risk reduction. Novel NSAIDs, as well as drug agents form various other classes have been tested\textsuperscript{467}. Some promising treatments such as CHF 5074, a modulator of microglial activation profile towards the M2 phenotype, have proceeded to the clinical trials for MCI. Unfortunately, this drug did not show statistically significant cognitive improvement\textsuperscript{468,469}.

Inhibitors of RAGE, which is a critical component of the inflammatory pathway, including PF-04494700 and Azeliragon, were proposed as therapeutic agents for AD and again failed in clinical trials\textsuperscript{470}. Triflusal, a platelet aggregation inhibitor, has shown good results in preclinical models and treatment delayed progression of MCI to AD in one clinical study\textsuperscript{465,471–473}. The drugs targeting
TNF-α such as etanercept infliximab have shown positive cognitive dynamics in mild – to – severe AD cases\textsuperscript{474–479}. Minocycline, a tetracycline antibiotic able to cross the BBB, has been increasingly reported to have good anti-inflammatory and anti-oxidant effects with positive effects on amyloid and tau-pathology in preclinical models mainly via inhibition of the NF-κB and MAPK pathways\textsuperscript{480–483}. Furthermore, this agent is associated with the white matter protection, which in light of the early stage pathology and cognitive function relationship of white matter inflammation to AD, is extremely relevant and favorable\textsuperscript{484,485}.

In conclusion, nearly all proposed treatment strategies for AD failed in clinical trials likely due to the use of agents at the advanced stage of the disease when pathology is too severe and likely irreversible. Thus, it is important to focus on the early stages of the disease, shifting the focus from advanced pathologies to that presented at the prodromal stage, a few decades before the clinical onset, and at stage correlating with cognitive deficits due to white matter disease (i.e. WMH) and its specific pathology\textsuperscript{486}.

Moreover, in many cases dementia is not the only disease present in these patients, and other chronic pathologies, such as vascular and metabolic diseases, contribute to the mixed pathology underlying cognitive decline, and consequently can influence the success in treating dementia. Many of these other chronic disorders begin in individual’s middle age and develop progressively over time eventually manifesting in a serious condition long after the initial signs. Thus, it is reasonable for prevention strategies to take into account the impact of coexisting disorders. It is important to explore the potential roles of those conditions that are risk factors for dementia and investigate mechanisms of shared pathological conditions simultaneously presenting with dementia. Reducing risk factors for the diseases including lifestyle modification and early prevention with drugs appears to be of great importance and should be pursued. Moreover, stable control over comorbid disorders should be achieved to increase the positive outcome of the treatment for dementia. A combination therapeutic approach that targets multiple links in the disease pathophysiological chain might be beneficial and should be seriously investigated.
1.3 Metabolic Vascular Disorders

1.3.1 Comorbidities of Alzheimer’s Disease: Focus on Metabolic Syndrome

Vascular metabolic conditions, such as T2DM and MetS, which represent risk factors for dementia including AD, develop in midlife and very often lead to the development of serious cardio- and cerebrovascular complications including myocardial infarction and stroke. Furthermore, these diseases accumulate with aging and are common comorbidities of AD. Increasing attention is paid to the interplay of AD with MetS due to the extreme prevalence of MetS in the elderly. MetS is also a precursor condition for the major cardiovascular diseases and T2DM and is highly modifiable in its nature.

Epidemiological and clinical studies strongly suggest the existence of an interaction between MetS and AD and a possible synergistic or additive effect on cognitive outcomes when these pathologies are simultaneously present\textsuperscript{14,35}. Individuals with MetS show a greater risk for developing MCI and AD later in life and AD patients tend to have a worse cognitive outcome and a faster progression or conversion to AD when MetS is also present\textsuperscript{28,29,129,134,138,487–497}. Experimental data from studies using rodent comorbid models of overt AD and diet-induced obesity and metabolic changes yield evidence of poor performance in cognitive tasks and increased AD-like pathology\textsuperscript{109,188,498–504}. A common gene, APOE, is implicated in the development of both AD and MetS, further suggesting a possible link between them\textsuperscript{505}.

While cerebrovascular comorbidities with AD, such as brain infarcts or strokes, are strongly and consistently associated with amplified cognitive impairment\textsuperscript{506,507}, the association between MetS and AD appears to be less consistent. The complex interaction seems to be influenced by the age of the study participants\textsuperscript{14,508}. Younger groups with age <70 years show a greater risk of AD development, whereas studies including older persons (>75-80 years) consistently report an inverse association of obesity and MCI, in which there is a slower progression and a better cognitive performance in comorbid demented subjects, suggesting a beneficial effect of the presence of metabolic changes\textsuperscript{34,509–512}.

It is possible that metabolic alterations interacting with cerebral homeostasis changes with age and with the development of the pathological processes. In addition, it might be that the age at which
vascular metabolic dysfunctions develop is a crucial factor which determines the effects on the cognitive function. The number of physiological and metabolic changes and their combination within the MetS cluster might further complicate the relationship between MetS as a whole and AD. Some changes may be individually related to AD risk in the same or opposite way, and the relationship may depend on the severity of the change. Further studies are needed to unravel the interactions between the two diseases, particularly investigating the early prodromal stages of AD where there is a greater possibility for treatment and prevention.

### 1.3.2 Diagnosis of Metabolic Syndrome

MetS is a cluster of components including central (i.e. abdominal) obesity, hypertension, insulin resistance, pre-diabetes (elevated blood glucose levels and glucose intolerance), diabetes and dyslipidemia (elevated triglycerides and reduced high-density lipoprotein (HDL) cholesterol)\(^8,10\). Central obesity is defined by measures of BMI, waist circumference or waist/hip ratio measurements. Criteria for MetS diagnosis vary slightly related to test-specific values and measurements of obesity and glucose metabolism, depending on the organization, but it always requires a combination of 3 conditions\(^8\). The most commonly used criteria from the World health organization (WHO) proposed in 1999, requires the presence of insulin resistance with glucose intolerance based on the 2h oral glucose tolerance test (OGTT) along with any two or more abnormalities (i.e. high triglycerides, low HDL cholesterol, raised blood pressure or obesity).

The Third Adults Treatment Panel National Cholesterol Education Program (NCEP) ATP3 criteria from 2005 diagnoses MetS based on the presence of any three or more components from the five identified previously. The International Diabetes Federation (IDF) 2006 guidelines include central obesity and the presence of two or more components. The last two organizations do not use OGTT, but include raised fasting blood glucose or hypoglycemic treatment and additionally diagnosed T2DM in IDF.
1.3.3 Risk Factors of Metabolic Syndrome

Modern lifestyle plays a big role in the etiology of obesity and MetS with a significant contribution from poor eating habits, particularly chronic consumption of high calorie Western diets characterized by high intake of saturated fat, simple carbohydrates (especially fructose) and salt, coupled with physical inactivity\textsuperscript{6,513–517}. While environmental factors seem to be the main drivers of the disease, genetic predisposition to intra-abdominal fat accumulation, increased BMI, obesity, insulin resistance and dyslipidemia also contribute\textsuperscript{8,513,518}. The role of parental obesity, intrauterine environment, maternal habits, diseases affecting fetal development and the postnatal environment are also recognized\textsuperscript{8}. The development of MetS also increases with increasing age\textsuperscript{519–521}.

1.3.4 Clinical Presentation and Pathophysiology of Metabolic Syndrome

Central or abdominal obesity is a core element of MetS. Obesity is a condition characterized by an excessive fat accumulation, especially around the internal organs (visceral fat), which is a highly metabolically active tissue contributing to systemic metabolic, vascular perturbations and development of chronic low-grade inflammation\textsuperscript{513,515,522–524}. However, metabolically normal obese persons do not develop the syndrome, and MetS can be diagnosed without obesity, thus emphasizing that metabolically active visceral fat rather than subcutaneous fat increase is required\textsuperscript{518}. Increased visceral adiposity is one of the major causes of insulin resistance which is characterized by the reduced sensitivity of cells to the actions of insulin such as glucose uptake and utilization, thus, leading to hyperglycemia and compensatory hyperinsulinemia (i.e. high blood levels of glucose and insulin)\textsuperscript{6,525}. The main tissues developing insulin insensitivity are adipose tissue, liver and muscle. Exposure to the high levels of abdominal fat-secreted free fatty acids is thought to play a major role in the development of insulin resistance which in turn promotes fat accumulation creating a vicious cycle. Moreover, insulin resistance plays a major role in development of the pathologies that constitute MetS and defines its clinical presentation\textsuperscript{515}.

Insulin resistance promotes lipolysis in adipocytes resulting in elevation of free fatty acid levels that further interferes with the lipid metabolism. Abnormalities in lipid metabolism, or dyslipidemia, include an increase in total cholesterol, cholesterol atherogenic fractions such as
low-density lipoprotein (LDL), very low-density lipoprotein (VLDL) particles and decrease in antiatherogenic high-density lipoproteins (HDL) and elevated triglycerides level\textsuperscript{515}. 

Insulin resistance and associated hyperinsulinemia and hyperglycemia are linked to the development of vascular insulin resistance directly associated with endothelial dysfunction, vascular reactivity change, oxidative and inflammatory damage exacerbated by elevated circulatory cytokines\textsuperscript{6}. These changes create a prothrombotic environment which together with atherosclerosis promoted by dyslipidemia and associated inflammation increases the risk for cardio- and cerebrovascular diseases. These conditions along with hyperactivation of the renin-angiotensin system and sympathetic nervous system by the insulin resistant state also leads to the development of hypertension\textsuperscript{518}.

Obesity is associated with increased NF-κB-mediated secretions of pro-inflammatory cytokines including interleukins IL-1 and IL-6, TNF-α and CRP by metabolic cells. These cytokines are triggered by excess consumption of nutrients, high caloric intake, particularly saturated free fatty acids and simple carbohydrates (e.g. fructose, glucose), leading to increased systemic inflammation that can affect multiple organs (primarily adipose tissue, liver, pancreas, muscle, gastrointestinal tract and brain)\textsuperscript{524,526-531}. Adipocytokines including leptin, adiponectin and resistin secreted by adipose tissue are important regulators of energy intake, appetite, satiety, body weight, lipid and glucose metabolism and modulators of inflammation\textsuperscript{518,532}. In morbid obesity there is leptin resistance with an increase in leptin production linked to hypertensive and pro-inflammatory actions and decreased levels of adiponectin associated with antiatherogenic and anti-inflammatory effects.

MetS has deleterious effects throughout the whole body leading to many complications including cardiovascular pathology, retinopathy, nephropathy, non-alcoholic fatty liver disease, skin conditions, problems with the reproductive system and sleep and breathing disorders\textsuperscript{515}. The CNS is not an exception and has complications, confirmed by a growing body of evidence of brain insulin resistance and its neural consequences\textsuperscript{38,178}. Chronic long-term development of these metabolic abnormalities is 5 times more likely to manifest as T2DM. The real hidden danger of MetS is that individuals with this condition are largely asymptomatic for a long period of time, but have a 2-fold increase in cardiovascular disease over the next 5 -10 years and have a 2- to 4-
increased risk of stroke, myocardial infarction and certain cancers resulting in a large contribution to mortality\textsuperscript{518,532}.

1.3.5 Cognitive Function in Metabolic Syndrome

Obesity and MetS developed during midlife are involved in the perturbation of cognitive function and are associated with global cognitive decline\textsuperscript{16,533–536}. The relationship of MetS and cognitive function are not mediated by a single individual component, but rather several components separately or in combination. Individuals with MetS show poorer scores on the mini-mental state examination (MMSE) measuring general cognitive ability, Raven’s progressive matrixes (RPM) for fluid intelligence assessment, memory assessing cognitive-affective verbal learning test (AVLT), impaired reasoning and executive functioning (e.g. on the Wisconsin Card Sorting Test, including working memory and attentional set shifting), decreased information processing speed and complex attention deficits\textsuperscript{135,491,537,538}. Executive functioning as well as informational processing speed and verbal memory were the most frequently impaired cognitive domains\textsuperscript{533}. As indicated above, it is interesting that older old adults, >85 years old, showed a better cognitive performance when metabolic components were present\textsuperscript{54}.

1.3.6 Neuropathology of Metabolic Syndrome

Obesity and MetS affect CNS homeostasis through multiple mechanism although it is mainly via neuroinflammation, insulin resistance and vascular abnormalities, with effects on both gray and white matter\textsuperscript{539–541}. The hypothalamus, especially the mediobasal region, which is a key neuroendocrine regulator of a large number of processes in the body including thermoregulation, appetite and body weight, is the most vulnerable brain structure to the vascular metabolic disturbances associated with MetS\textsuperscript{531}. However, other cerebral structures such as the hippocampus, neocortex, brainstem, amygdala, cerebellum and choroid plexus show pathological changes during the course of the disease\textsuperscript{531}. In clinical studies, obesity has been associated with frontal and temporal brain atrophy, white matter alterations, frontal and prefrontal metabolic changes and reduced blood flow in a large cortical area, resulting in decreased cognitive
performance independent from the cardio- and cerebrovascular conditions\textsuperscript{534,542,543}. MetS has also been associated with a greater incidence of silent brain infarcts that also might contribute to cognitive deficits\textsuperscript{544}.

The results of animal studies with Western high-fat, high-sugar diet induced obesity and MetS have supported the negative relation of the syndrome to learning and memory, particularly hippocampal dependent memory. These learning and memory deficits appear to be mediated by increased vascular and oxidative-inflammatory damage to the hippocampus, decreased neuronal plasticity and long-term potentiation and BDNF levels \textsuperscript{16,18,107,109,187,188,499,500,535,545–559}. Insulin resistance, hyerinsulinemia, hyperglycemia and dyslipidemia can all affect the cerebrovasculature, induce oxidative stress and inflammation and modulate tau and amyloid metabolism promoting peptide accumulation, leading to cognitive impairment\textsuperscript{135,190,504,536,539,541,555,560–563}.

\textbf{1.3.6.1 Neuroinflammation in Metabolic Syndrome}

One of the central mechanisms mediating MetS effects in the brain is neuroinflammation. The proinflammatory JNK and IKKβ/NFkB pathway appears to be upregulated in the hypothalamus and linked to energy imbalance, promoting obesity, as well as brain leptin and insulin resistance\textsuperscript{564–567}. High-fat diets in animals, commonly used to model obesity-related metabolic alterations, and increased consumption of the simple sugar fructose have been shown to increase microglial infiltration in the hypothalamic arcuate nucleus. The microglia are thought to be attracted and activated by circulating cytokines, immune cells, saturated fatty acid build up, hyperglycemia, thus, initiating local inflammatory reaction and recruiting astrocytes\textsuperscript{526,527,568–570}. It has been suggested that hypothalamic reactive gliosis, cytokine release and inflammation induce local synaptic dysfunction and neuronal damage, thus affecting outputs to the brain structures linked with the hypothalamus including the hippocampus and amygdala. These brain regions contribute to the disease progression inducing neurodegeneration and cognitive decline\textsuperscript{107,531,548,571}.
1.3.6.2 White Matter Changes in Metabolic Syndrome

MetS, and obesity in particular, are associated with reduced white matter volume and disrupted microstructural integrity with both axonal and myelin alterations, especially in the corpus callosum, cingulum, external capsule, corona radiata, internal capsule and fornix\(^{542,572–580}\). These areas contribute to the development of cognitive dysfunction and in particular executive dysfunction\(^{318,534,581,582}\). Alterations to the white matter connecting frontal and temporal, limbic structures and prefrontal regions are observed\(^{576,577}\). WMH and lacunar infarcts are also found in individuals with MetS, particularly temporoparietal regions which show an increased vulnerability\(^{108,583–588}\).

1.3.7 Treatment Strategies for Metabolic Syndrome

Lifestyle modification is a very effective preventive approach to the development of MetS. It includes changes in dietary habits, caloric restriction and regular exercise\(^{589}\). Pharmacological treatment is implemented to manage individual pathological conditions in MetS when preventive measures are not sufficient\(^{6,515,518,590}\). Weight loss medications include appetite suppression (e.g. phentermine, sibutramine) and nutrient absorption reducing (e.g. orlistat) agents. Bariatric surgery which is aimed to reduce the gastric volume is used in extreme cases of obesity in which there are additional health complications. Dyslipidemia can be controlled with statins, fibrates, cholesterol absorption inhibitors and bile acid sequestrants\(^{590}\). Angiotensin converting enzyme inhibitors and angiotensin receptor blockers are commonly used as blood pressure regulating drugs. Glycemic control can be achieved with the use of anti-diabetic drugs such as metformin, thiazolidinediones, acarbose. Some of these drugs are able to act on multiple pathological units. Administration of aspirin is commonly used to reduce risk of thrombosis and linked vascular events.
1.4 Interaction of Alzheimer’s Disease and Metabolic Syndrome

AD and MetS share multiple pathological features, including neuroinflammation, oxidative stress, alterations to the BBB, cerebrovasculature pathology, cerebral hypoperfusion, brain insulin resistance, and gray and white matter abnormalities\textsuperscript{176,562,591–597}. These processes are also tightly related to the mechanisms of amyloid and tau production and degradation\textsuperscript{598}.

Neuroinflammation is a key pathological event developing at the prodromal stage of AD and drives the progression of the disease\textsuperscript{205,207,208,257,260}. MetS is a chronic low-grade inflammatory disease which primarily originates from the visceral adipose tissue \textsuperscript{513,515,522–524} and is one of the major causes of peripheral and brain insulin resistance which, in turn drives the clinical manifestation of MetS which can include cognitive dysfunction\textsuperscript{6,525,529,591,599–604}.

Systemic inflammation, such as that occurring in obesity and MetS, can modulate CNS inflammatory system and this interaction can have a high impact on cognitive function\textsuperscript{504,605–608}. Resident microglia and macrophages respond to peripheral immune signals with increased cytokine production initiating a neuroinflammatory cascade. The peripheral immune signals such as pro-inflammatory cytokines and chemokines, can include ILs and TNF-α, and act via non-disrupted NVU and circumventricular organs that lack a BBB as well as by neural afferent pathways\textsuperscript{609}. Leukocytes, peripheral immune cells, have been shown to enter the CNS and aggravate gliosis contributing to neuroinflammation\textsuperscript{599,610}.

These observations suggest that neuroinflammation might be a potential early stage event linking these two chronic disorders which can further modulate AD-related pathology\textsuperscript{591,611–617}. Modulation of brain amyloid metabolism in MetS has been suggested to be mediated by reduced degradation and clearance and increased production of Aβ. The amyloid changes are associated with effects of inflammation (i.e. TNFα), insulin (inhibition of insulin degrading enzyme), soluble LRP and leptin resistance\textsuperscript{190,618–620}. Furthermore, APP is expressed not only in the brain, but in the peripheral tissues including adipose tissue, intestines and its expression is upregulated in obesity\textsuperscript{32,621,622}. Aβ produced on site in these peripheral tissues in increased quantities can enter the blood circulation and can enter the brain contributing to the cerebral pathology and promoting neuroinflammation\textsuperscript{623}. 
Systemic low-grade inflammation in MetS is associated with a high circulating levels of CRP, lipopolysaccharide binding protein (LBP) and α1-antichymotrypsin. This low-grade inflammation is highly correlated with cognitive impairment, particularly cognitive flexibility, working memory and attentional set shifting (i.e. executive functioning) in obese subjects and the effects appear to be independent from the major cerebrovascular diseases. These effects on cognitive performance are thought to be mediated by white matter abnormalities observed in these subjects. Cerebral white matter lesions are also common in AD and have been shown to highly correlate with cognitive decline. Thus, white matter abnormalities represent another point of interaction between these comorbidities which seems to be intimately linked to inflammatory processes.
1.5 Rational and Objectives

AD and MetS are both serious disorders affecting a large elderly population worldwide. These conditions coexist in the population and it is likely they interact contributing to the course and progression of AD-pathology and cognitive decline and influence the success of experimentally tested treatments. However, the exact underlying processes of this interaction remain unclear. Neuroinflammation, mainly driven by microglia and astrocyte activation, is a key pathological event developing at the prodromal stage of AD and a primary cause of disease progression. AD is also a white matter disease with abnormalities in major fiber tracts including glial activation. This white matter pathology is present well before symptomatic onset of AD and highlights the potential of cerebral white matter lesions as a new biomarker of cognitive impairment in MCI and AD and a possible target for prevention and early therapy.

MetS is a chronic systemic inflammatory condition and is also associated with neuroinflammation and white matter changes, which appear to contribute to cognitive impairment. Furthermore, aging which is highly associated with white matter alterations and increased risk of AD preceded by white matter lesions, also is associated with the development of metabolic disorders.

Research data suggests that aging, and particularly age-related brain neuroinflammatory dysregulation, may exacerbate existing metabolic, vascular and neuroinflammatory conditions and aggravate white matter degeneration. Thus, it is likely that an early interaction between the comorbid conditions of preclinical AD and MetS is mediated though the white matter inflammation occurring at prodromal stage of AD. This represents a critical area for research and deserves a great attention and requires extensive investigations. In order to conduct these types of investigations, there is a demand for comorbid preclinical animal models. Development of just such a model was a part of this work.

In these investigations a high calorie high-fat, high sugar diet-induced MetS was combined with an APP21 transgenic rat model of prodromal Aβ plaque-negative AD. This comorbid model was studied to test the research hypothesis. The hypothesis is that the comorbid condition of
preclinical AD and MetS will result in increased white matter inflammation and cognitive
dysfunction which can be prevented by anti-inflammatory treatment. The following objectives
were addressed:

1) To determine the impact of comorbid metabolic abnormalities (MetS) and increased pathogenic
APP environment on white matter neuroinflammation, specifically on microglia and astrocytes,
and on cognitive function; thus, defining possible targets for early therapeutic intervention
(Chapter 2).

2) To examine the therapeutic potential of a brain targeted anti-inflammatory agent by examining
the effect of the drug on the increased white matter inflammation and cognitive deficits in the
comorbid model of prodromal AD and MetS (Chapter 3).

3) To analyze the possible negative effects of a brain targeted anti-inflammatory agent tested as a
potential therapeutic strategy to target increased white matter inflammation (Chapter 4).

4) To identify potential biomarkers of white matter inflammation by characterizing the
relationship between the white matter inflammation and the observed systemic metabolic and
physiological parameters (Chapter 5).
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Chapter 2: Comorbidity of Prodromal Alzheimer’s Disease and Metabolic Syndrome in the APP21 Transgenic Rat

The aim of this study was to investigate the impact of the comorbid conditions of MetS and prodromal AD (TG for hAPP) on white matter neuroinflammation and on cognitive function (Objective 1). The manuscript for this investigation is currently under review for Journal of Neuroinflammation.

2.1 Introduction

Among age-related diseases, dementias are particular serious given their prevalence, severity and progressive and incurable characteristics. Alzheimer’s disease (AD) is the most commonly diagnosed form of dementia. The accumulation of amyloid-β peptide (Aβ), produced through an altered cleavage of amyloid precursor protein (APP), and formation of neurofibrillary tangles are considered to be the hallmarks of AD. The processes associated with the development of AD include glial activation, excessive neuroinflammation and oxidative stress, as well as vascular and metabolic abnormalities. AD is a disease impacting both the gray and white matter of the brain. While changes to the gray matter in the pathogenesis of AD are well known and continued to be heavily investigated, the neuropathology of white matter abnormalities still remains not fully understood and is mainly attributed to cerebral small vessel degeneration, inflammatory events, as well as loss of myelin and axonal fibers. However, white matter changes have been shown to develop very early, in prodromal phase and precede the onset of clinical symptoms of dementia, highlighting the importance of their further investigation.

The complex etiology and pathology of AD alone remains a focus of research, but increasing attention is paid to the interplay of AD with comorbidities such as stroke and metabolic disorders including diabetes and metabolic syndrome (MetS). MetS, which is a focus of our research work, represents a combination of conditions such as obesity, dyslipidemia, glucose intolerance, insulin resistance and hypertension. Unhealthy lifestyle choices play a big role in etiology of MetS, with chronic intake of high calorie Western diets rich in saturated fat and simple carbohydrates coupled
with a sedentary lifestyle being the most common risk factors \(^{10,11}\). MetS is a serious public health issue \(^{12}\). It begins in middle age and continues to develop over time manifesting in serious conditions such as type 2 diabetes, cardio- and cerebrovascular diseases. Moreover, it represents a risk factor for dementia, including AD \(^{13-16}\), and often coexist with it in one individual likely contributing to the course and progression of dementia \(^{17}\).

Epidemiological and clinical studies strongly suggest the existence of an interaction between MetS and dementia, including mild cognitive impairment (MCI) and AD. Individuals obese and diagnosed with MetS show a greater risk for developing cognitive decline later in life \(^{18-20}\) and AD patients tend to have a poorer prognosis when MetS is also present \(^{21}\). Experimental data from studies using rodent models of well-developed AD fed a high-fat diet yield evidence of poor performance in cognitive tasks and increased AD-like pathology including neuroinflammation \(^{22-26}\). In contrast to the earlier studies, our present study aimed to examine the early processes and interactions occurring at the prodromal phase of AD using a novel transgenic model of high cerebral amyloid levels as a predisposing environment.

Inflammation as an event associated with both dementia, including AD, and MetS has been suggested to be one of the shared mechanisms contributing to the impaired cognition and AD-like pathology \(^{27-29}\). In the current study, we examined the early effects of the comorbidity on the inflammation in the white matter which is highly susceptible to pathological changes, particularly the key cellular components of inflammatory response, microglia and astrocytes.

While there is a clear connection between metabolic diseases and AD, the exact underlying mechanisms regarding how metabolic diseases affect mental health and contribute to the existing neuropathology, especially at the very initial stages of their development, remain unclear. The gap in our understanding of this interaction appears to be a limiting factor in any success in finding effective therapeutic and preventive interventions. This highlights the importance of developing experimental models that combine prodromal phase AD-like pathology with risk factors such as MetS to investigate the potential of early intervention and prevention.

The present study was undertaken to better understand the relation between metabolic abnormalities and prodromal AD dementia, particularly studying the impact on changes in white matter inflammatory pathology and coincident cognitive deficits. The comorbidity of prodromal
AD with MetS was examined in a novel APP21 transgenic (TG) rat model of prodromal AD created on a Fischer 344 background which carries a human APP (hAPP) gene with Swedish and Indiana mutations, implicated in early-onset AD. This rat has been previously shown to express high levels of human brain APP and serum β-amyloid (Aβ1-40 and 1-42) without spontaneous Aβ plaques deposition in brain tissue with age. Thus, it allows us to study the early interaction between MetS and prodromal AD-like processes in the brain in a model with AD-predisposing conditions.

In this study we focused on the pathology of diet-induced MetS in relation to prodromal phase of AD, specifically examining the consequences of its chronic course on the white matter inflammation, one of the earliest and most critical events occurring in the brain in response to insult, particularly on its key cellular players, microglia and astrocytes. In addition, we examined the effects of diet in the prodromal AD model on behavior and cognitive function. The hypothesis is that there would be greater white matter inflammation and cognitive deficits in the combined model than in either condition alone.
2.2 Methods

2.2.1 Animals

All animal handling and experimental procedures were approved by Western University Animal Care Committee (AUP 2008-113) and were carried out in accordance with the guidelines of the Canadian Council on Animal Care and National Institute of Health Guides for the Care and Use of Laboratory Animals. A total of 24 male wildtype (WT) and 22 male APP21 TG Fischer 344 rats were involved in this study, and rats were assigned to experimental groups at random. APP21 TG rats overexpressed hAPP with Swedish and Indiana mutations driven by the ubiquitin-C promoter were created via lentiviral vector method. Rats were bred in house with original breeding pairs obtained from Drs. Yuksel Agca and Cansu Agca (University of Missouri, Colombia, MO, USA) and confirmed to be homozygous. Animals were housed in pairs under standard conditions (12:12 light/dark cycle, at 22-24°C) and maintained on a standard rat diet provided ad libitum. At the age of 8.5-9.5 months, half of the rats of each genotype were randomly assigned to a high-calorie Western type diet (HCD), while the other half continued on a standard diet (control diet, CD). Diets were provided ad libitum and rats were maintained on the diets for 12 weeks. A study timeline is shown in Figure 2-1. Body weight as well as food and drink consumption were measured twice a week throughout the experiment. Towards the end of the experiment, there were slight variations in the exact time for the physiological and metabolic measures since they would interfere with the acquisition of behavioral data. Animal numbers for each experimental dietary group were as follows: WT CD, n=12; TG CD, n=11; WT HCD, n=12; and TG HCD, n=11.

2.2.2 Diets

Rats maintained on a standard diet received chow with the following composition (in %kJ): 26 protein, 59.7 carbohydrate, and 14.3 fat with 1.52 % of saturated fatty acid (Prolab RMH 3000 5P00). The Western diet consisted of the following (in %kJ): 17 protein, 43 carbohydrate, and 40 fat with 62.4% of saturated fatty acid (D12079B, Research Diets, Inc) which included 0.21% cholesterol. The metabolizable energy from standard and Western diet (in kJ/g) was 13.31 and 19.66, respectively. The solid food was supplemented with water in the CD group and with 20%
corn syrup water solution in the HCD group as an additional source of calories (Bee Hive, ACH Food Companies, Inc, USA).

2.2.3 Intraperitoneal Glucose Tolerance Test (IpGTT) and Insulin Measurement

IpGTT was performed at two weeks prior, and 11 weeks following the change in diet (Figure 2-1). Following a 12-h overnight fast, 100-150 microliters of blood was drawn from the saphenous vein for determination of glucose and insulin baseline levels. A 60% glucose solution of D-(+)-glucose (G 8270, Sigma-Aldrich, Inc., Saint Louis, Missouri, USA) in 0.9% saline (2g/kg) was then injected intraperitoneally. Blood was collected from a tail vein repeatedly at 15, 30, 60, 90 and 120 minutes after the glucose load. Glucose levels (mmol/l) were measured using Freestyle Light Blood Glucose Monitoring System (Abbott Diabetes Care Inc, Alameda, CA). Glucose responses over time were analyzed to determine the area-under-the-curve (AUC). Fasting insulin levels were determined in serum samples using an ELISA kit (Ultra-sensitive rat insulin ELISA kit, Crystal Chem. Inc) according to the manufacturer’s instructions. Homeostasis model assessment index of insulin resistance (HOMA-IR) was calculated to estimate insulin resistance using the following formula\(^34\): HOMA-IR=(fasting glucose (mmol/l) x fasting insulin (mmol/l))/22.5.

2.2.4 Lipid Profile Analysis

Triglycerides, total cholesterol and high-density lipoprotein (HDL) cholesterol were measured in serum samples isolated from cardiac blood at time of euthanasia and analyzed at the Clinical Laboratory at University Hospital (London, ON, Canada). Non- HDL cholesterol was calculated as total cholesterol – HDL cholesterol . The cholesterol ratio (Chol:HDL ratio) was calculated by dividing total cholesterol value by HDL cholesterol number.
2.2.5 Blood Pressure Analysis

Systolic and diastolic arterial blood pressure were assessed three weeks before and at week 6 and 10 on the diet (Figure 2-1) via a non-invasive tail cuff method (CODA Blood Pressure System, Kent Scientific Corp., Connecticut, USA).

2.2.6 Open Field Activity and Anxiety-like Behavior

Locomotor activity and anxiety-like behavior were tested in a square open field arena (Med Associates Inc., St. Albans, VT, USA) over the course of 20 minutes on week 9 of the diet (Figure 2-1). Ambulatory distance and time spent in central and peripheral zones were evaluated using Activity Monitor software, Med Associates Inc.

2.2.7 Morris Water Maze

Rats first encountered the Morris water maze test (MWM) 1 week prior to the diet onset. The second testing (relearning) was performed 12 weeks after the diet following the same protocol, but with a new platform location (Figure 2-1). Rats were trained to find a hidden escape platform in a circular pool (145 cm in diameter, 58 cm in depth) filled with water, dyed with black non-toxic acrylic paint, using extra-maze cues placed on the walls around the pool. The training protocol consisted of 16 trials over 4 consecutive days (4 trials/day). The duration of one trial was 60 sec with a 30-sec inter-trial period during which time the rats remained on the platform. The platform (12cm in diameter) was placed in the middle of one of 4 virtual quadrants the pool was divided into, and this location remained unchanged during the training phase. Start positions were presented in a randomized order for every day of spatial acquisition. Learning progress was assessed using time and distance required to reach the platform (actual path length) and path efficiency (ratio of direct path length to the platform to actual path length, 1 being most efficient) in the acquisition trials. The day after the last day of training the rats were subjected to a 30-sec probe trial where the platform was removed from the pool and the rats were released from a novel start position. At the end of the training and probe prior to the dietary manipulation two
reacquisition trials in which the platform was returned to the previous position were administered to prevent memory extinction. Performance was evaluated using such parameters as time and distance travelled in the quadrant of a previous platform location (target quadrant) and swimming speed. Performance was monitored using video-tracking software (ANY-maze®, Stoelting Co., Wood Dale, IL, USA).
Figure 2-1. Project timeline. Rat’s age (in months) at the start (day 0) and the end (week 13) of the study are shown in brackets. Diets were assigned on day 0 and all testing time points are in reference to this day. Baseline measurements were completed 3 weeks prior to the start of the diet. Morris water maze spatial training was completed on week -1 (4 days, 4 trials a day) with a probe trial (Pr1) following on the day after. A second probe trial (Pr2) was completed on week 12 on a diet. Learning of a new platform location started the next day following the same protocol with a probe trial (Pr3) at the end. BP = blood pressure measurement, IpGTT = intraperitoneal glucose tolerance test, MWM = Morris Water Maze, Pr = probe trial, OF = open field test, BC = blood collection, TC = tissue collection, W = week.
2.2.8 Euthanasia and Tissue Collection

Following a 12-h fasting period the rats were weighed and euthanized by a pentobarbital overdose. Cardiac blood was collected immediately before perfusion. Epididymal fat pads were collected and weighed. Rats were then perfused transcardially with 0.01 M phosphate buffered saline (pH 7.35) followed by 4% paraformaldehyde (PFA, pH 7.35). Brains were post-fixed in PFA overnight and then transferred to a 30% sucrose solution until saturated fully submerged. Brains were sectioned coronally on a cryostat into 35 μm thick sections approximately from bregma 4.70mm to bregma -5.20mm \(^{35}\), sorted into 12 series and were stored in cryoprotectant at -20°C until used for immunohistochemistry.

2.2.9 Immunohistochemistry

Immunohistochemistry was performed on free-floating sections to visualize microglia, activated microglia, astrocytes, neurons and synapses using rabbit polyclonal antibody against the ionized calcium binding adaptor molecule-1 (anti-Iba-1; 1:1000; Wako Chemicals USA Inc., Richmond, VA, USA), mouse monoclonal antibodies directed against the MHC II receptor (OX-6; 1:1000; BD Pharmingen, Mississauga ON, Canada), glial acidic fibrillary protein (anti-GFAP; 1:2000; Sigma-Aldrich, St Louis MO, USA), neuronal nuclei (anti-NeuN; 1:1000; EMD Millipore Corp., USA) and synaptophysin, a major synaptic vesicle protein, (anti- synaptophysin; 1:1000; Sigma-Aldrich, St Louis MO, USA), respectively. Following an overnight incubation with the primary antibody at 4°C, sections were incubated with biotinylated anti-mouse or anti-rabbit secondary antibody (1:500, Vector Laboratories, Inc. Burlingame, CA, USA) followed by incubation with avidin-biotin complex (ABC kit, Vector Laboratories, Inc. Burlingame, CA, USA) reagent and then developed in 0.05% 3, 3’ diaminobenzidine tetrahydrochloride (Sigma-Aldrich, St. Louis MO, USA). Sections were then mounted on glass slides, air-dried, dehydrated, cleared in xylene and coverslipped with DePex mounting media (DePex, BDH Chemicals, Poole, UK). Detection of changes in white matter fiber myelination was done in sections pre-washed in 0.01M PBS mounted on glass slides, dried overnight and stained with Luxol fast blue following the protocol described elsewhere\(^{36}\).
2.2.10 Imaging and Quantification of Immunohistochemistry

Immunohistochemically and histochemically processed brain sections were imaged at 10x objective with a Nikon Eclipse Ni-E upright microscope with a Nikon DS Fi2 colour camera head using NIS-Elements Imaging Software Version 4.30.02 (Nikon Instruments Inc., Melville, NY). Brain sections stained for OX-6 and Luxol fast blue were scanned with Aperio digital entire-slide scanner, allowing 20x magnification (Department of Pathology, Western University, London, Ontario, Canada). Entire series of brain sections was screened for positive OX-6 signal to determine regions of interest (ROIs) for all further analysis. Analysis and quantification were carried out using 64-bit ImageJ software (Version 1.48u4, Wayne Rasband, National Institutes of Health, Bethesda, MD, USA). The investigator was blinded to the identity of rats included in the quantification analysis. Images were converted into a black-and-white 8-bit format, underwent thresholding and were calibrated prior to taking all the measurements. Based on the location of the positive OX-6 immunostaining being mainly in the white matter structures, the corpus callosum, internal capsule and fimbria of the dorsal hippocampi were chosen as ROIs. A total of six regions from three consecutive brain sections containing corpus callosum, internal capsule or fimbria were analyzed for each animal. For the assessment of activated microglia cells (OX-6 stained) in the corpus callosum and internal capsule, areas with positive signal were manually outlined using a free outline tool. Integrated density, defined as a sum of the values of the pixels in the selected area, was measured for each region and summarized into a single value per animal. To analyze changes in general microglia population (Iba-1 stained), activation of astrocytes, activated microglia in the fimbria and myelin content, white matter tracts were manually outlined and a measure of the area of coverage by positive signal (percent of the total area) was noted for each region and expressed as a weighted average. The neuronal population of the hippocampus, CA1 subregion, was visualized with NeuN immunostaining and was assessed using the NIS Elements analysis software. In the ROI sampled from two to three coronal brain sections neuronal nuclei were automatically counted in a selected field of 0.2 mm² area and an average number was generated for each animal. Synaptophysin staining was quantified in the CA1 and CA3 hippocampal subregions in a total of 8 fields per subregion, sampled from two brain sections, per animal in the ImageJ. The area of coverage by positive signal was expressed as a weighted average.
2.2.11 Data Analysis

Statistical analysis was performed using GraphPad Prism 6.0. Data were analyzed by performing t-test or One-way or Two-way analysis of variance (ANOVA), followed by Tukey’s multiple comparisons test. Data is expressed as mean ± standard error of the mean (SEM), and a *p* value of ≤ 0.05 was considered statistically significant.

2.3 Results

First, we performed an extensive physiological characterization of the model by analyzing body weights, parameters of glucose and lipid metabolism and arterial blood pressure values.

2.3.1 Body Weights, Diet, Fat Accumulation, Lipid and Glucose Metabolism

Both TG and WT rats on a HCD gained weight rapidly and weighed significantly more than CD groups as early as the first week on the diet (Figure 2-2A). Starting from week 6 on the diet, rats from the comorbid group weighed more than the WT HCD group and this weight difference remained significant until the end of the study. In addition, as shown in Figure 2-2B, epididymal fat pads mass was significantly increased with HCD consumption ($F_{(1,42)}=335.9$; *p*<0.0001), with an even greater increase in the TG rats (genotype effect $F_{(1,42)}=11.26$; *p*<0.0017, interaction $F_{(1,42)}=3.769$; *p*<0.059). Analysis of diet consumption across the 12 weeks showed a decrease in amount of food consumed (WT CD 20±0.2, TG CD 21±0.3 vs WT HCD 9±0.2, TG HCD 11±0.4 g/day), however there was a large increase in drink consumption by rats from both HCD groups (WT CD 23±0.7, TG CD 27±0.7 vs WT HCD 68±2.4, TG HCD 61±1.9 g/day). This resulted in a significantly higher total energy intake in the HCD groups during the entire period of 12 weeks (WT CD 264±3.0, TG CD 272±4.0 vs WT HCD 406±5.0, TG HCD 402±3.0 kJ/day). Based on genetic profiles, rats had different preferences for the source of calories; TG rats favored high-fat food, whereas WT rats had a stronger preference for carbohydrates from drink. Triglyceride levels were significantly elevated by HCD (Figure 2-2C).
Total cholesterol was increased in both groups on the HCD, but reached statistical significance only in the comorbid rats compared to controls. Both TG and WT rats on the HCD had an increased cholesterol content of atherogenic lipoprotein particles (non-HDL cholesterol; Figure 2-2D). HDL cholesterol levels, when analyzed separately, were not different for HCD rats in comparison to the control groups. However, the Chol:HDL ratio, a relevant clinical index, was significantly greater in both HCD groups, indicating that these rats had a decrease in HDL cholesterol and a significant shift towards the atherogenic Non-HDL fraction (Table 2-1).

Rats maintained on HCD did not show signs of hyperglycemia based on the fasting blood levels of glucose (Table 2-1). Surprisingly, a glucose intolerance pattern was observed only in WT rats maintained on a HCD, which was characterized by a greater increase in blood glucose levels at 30 minutes after a glucose injection that remained significantly increased till the end of a 2-h period (Figure 2-2E). This also translated into a significantly greater AUC for blood glucose. There appeared to be no effect of diet on glucose tolerance in the TG rats and AUC was very similar to CD group values (Table 2-1). Fasting insulin levels were significantly higher for both WT and TG rats from HCD groups (Figure 2-2F). Two-way ANOVA analysis revealed a significant effect of the diet ($F_{(1,41)}=21.20; p<0.0001$) in both genotypes. HOMA-IR index was significantly greater for both WT and TG rats from hypercaloric diet groups compared to the control groups (Table 2-1). Nevertheless, these data suggest that HCD did not lead to the development of frank diabetes, yet led to the manifestation of a pre-diabetic state. In contrast, the HCD had a robust effect on lipid metabolism.

### 2.3.2 Blood Pressure

Systolic and diastolic blood pressure values obtained at 6 and 10 weeks of diet were not different between the experimental groups, indicating that no animal group showed signs of hypertension due to dietary intervention or genotype (Figure 2-3). Additional analysis of the pressure changes with age and diet within individual groups indicated a decrease in systolic (week 6 $p=0.0025$; week 10 $p=0.0011$) and diastolic (week 10 $p=0.131$) pressure levels of TG HCD rats from the baseline (week -3) levels. HCD WT group showed an increase in diastolic pressure from baseline level at week 6 ($p=0.0065$), which then dropped to initial values at week 10 ($p=0.0004$).
Figure 2-2. Weight gain, visceral fat accumulation, lipid and glucose metabolism. (A) Body weight change over the course of the diets. (B) Post-mortem paired epididymal fat pad weight. (C) Fasting triglyceride levels measured at the end of week 12 on the diets. (D) Fasting levels of total cholesterol presented as the whole bar and its fractions: Non-HDL (upper part of a bar) and HDL cholesterol (lower part of a bar). (E) Blood glucose levels during 2-h intraperitoneal glucose tolerance test (IpGTT) after 11 weeks on the diets. Zero time point (0) represents fasting glucose value obtained immediately before glucose load. (F) Fasting insulin levels measured from a blood sample drawn at time point 0 during IpGTT. Animal numbers are as follows: WT CD (n=12), TG CD (n=11), WT HCD (n=12), TG HCD (n=11). Values are presented as mean ± SEM. Significance
is indicated by * between HCD and both CD groups (in d – for non-HDL cholesterol), † between HCD groups and ‡ between TG HCD and both CD groups for total cholesterol. RM Two-way ANOVA and One-way ANOVA, Tukey’s multiple comparisons test, p < 0.05. CD = control diet, HCD = hypercaloric diet, HDL = high density lipoprotein, TG = transgenic, WT = wildtype.
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<td>3.96±0.13</td>
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<tr>
<td><strong>Glucose, mmol/l</strong></td>
<td>3.6±0.13</td>
<td>3.63±0.14</td>
<td>3.96±0.13</td>
<td>3.91±0.12</td>
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<td>(post)</td>
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<tr>
<td><strong>AUC IpGTT</strong></td>
<td>929.98±45.91</td>
<td>786.08±53.51</td>
<td>1568.85±172.74*†</td>
<td>892.44±90.58</td>
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<tr>
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<td></td>
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<tr>
<td><strong>AUC IpGTT</strong></td>
<td>891.08±82.09</td>
<td>907.25±79.11</td>
<td>1568.85±172.74*†</td>
<td>892.44±90.58</td>
</tr>
<tr>
<td>(post)</td>
<td></td>
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<tr>
<td><strong>Insulin, pmol/ml</strong></td>
<td>243.1±28.60</td>
<td>237.9±29.16</td>
<td>243.1±28.60</td>
<td>237.9±29.16</td>
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<tr>
<td>(post)</td>
<td></td>
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<tr>
<td><strong>HOMA-IR</strong></td>
<td>0.19±0.03</td>
<td>0.17±0.03</td>
<td>0.56±0.09*</td>
<td>0.58±0.13*</td>
</tr>
<tr>
<td>(post)</td>
<td></td>
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<tr>
<td><strong>Chol:HDL, mmol/l</strong></td>
<td>1.64±0.09</td>
<td>1.49±0.06</td>
<td>2.43±0.17*</td>
<td>2.44±0.17*</td>
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</table>

Initial – data obtained prior to the diet assignment; rats used in the study were combined by genotype. Post – data collected after 11-12 weeks on the diets. Non-specified – data obtained at the end of the study. All measures (except AUC IpGTT) represent fasting state values. Values are presented as mean ± SEM. The symbols * and † indicate significance for HCD group vs both control groups and between HCD groups respectively. One-way ANOVA, Tukey’s multiple comparison test; p<0.05. Abbreviations: AUC – area-under-the-curve, CD – control diet, Chol – total cholesterol, HCD – hypercaloric diet, HDL – high density lipoprotein cholesterol, HOMA-IR – homeostasis model assessment index of insulin resistance, IpGTT – intraperitoneal glucose tolerance test, TG – transgenic, WT – wildtype.
Figure 2-3. Arterial blood pressure measured three weeks prior to and 6 and 10 weeks on the diet. A) Systolic blood pressure levels prior to the diet onset (week -3), on week 6 and 10 on the diet. B) Diastolic blood pressure levels on week -3, week 6 and 10 on the diet. Animal numbers are as follows: WT CD (n=12), TG CD (n=11), WT HCD (n=12), TG HCD (n=11). Values are presented as mean ± SEM. The symbol * indicates significance at a timepoint compared to the baseline (week -3) within the group. Two-way ANOVA, Tukey’s multiple comparisons test, p<0.05. CD = control diet, HCD = hypercaloric diet, TG = transgenic, WT = wildtype.
2.3.3 Behavioral Assessment

We monitored cognitive performance using a spatial navigation version of MWM task. First testing was done prior to the assignment of different dietary regimens to assess baseline learning abilities of rats. The testing at the end of the study evaluated effects of the HCD-induced metabolic dysregulation alone and in combination with AD predisposing conditions on learning and memory.

2.3.3.1 MWM and Spatial Learning Preceding Diet

At the end of the initial training period, one week prior to the start of the diet, all groups had learned the location of the platform to the same extent (Figure 2-4A). Distance travelled in the target quadrant during the probe trial 1 following the learning was indicative of a good memory of the platform location (Figure 2-4B).

2.3.3.2 MWM and Spatial Relearning after Diet

Following 12 weeks on the diet, the latency to platform, path length to platform and path efficiency were significantly improved in CD groups, but were not significantly improved in the HCD groups (Figure 2-5A,B,D). Comorbid rats had on average shorter path lengths compared to the WT CD and TG CD animals (p=0.0451 and p=0.0494, respectively) on day 1 of the training (Figure 2-5B). However, when performance on individual trials within day 1 was compared, there was no significant difference between the groups (Figure 2-5C). Furthermore, TG HCD rats started the day 2 at the same level as the rest of the rats and continued in a comparable manner. This suggests, that TG HCD rats encountered the platform on average faster than CD rats on the very first day of learning the new location which could contribute to the observed day1-day4 lack of difference. This could likely occur by chance, especially on the trial 1, or due to choosing a certain swim strategy such as chaining, rather than represent a true spatial learning. TG rats on the HCD showed an inconsistent learning pattern with a sudden drop in path efficiency and increase in latency and path length to platform on the second day of the task. However, by the end of the spatial acquisition phase all rats learned the task to the same extent as indicated by the absence of differences between
groups in any of these measurements at day 4 of training. Learning swim speed was comparable between the groups across days. During the probe trial (Probe 3), comorbid rats spent less time searching in the target quadrant, while the other groups had a preference for the quadrant where the platform was located during learning days. Tukey’s multiple comparisons test showed a significant decrease (p<0.01; one-way ANOVA; Figure 2-5E) in time spent in the target quadrant for the comorbid rats compared to WT control group. Swim speed did not differ between groups (Figure 2-5F). Two-way ANOVA analysis revealed a significant effect of dietary treatment (F(1,42)=7.384; p<0.01) and genotype (F(1,42)=4.462; p<0.05) for time travelled in the target quadrant with no significant interaction, but the TG HCD group was significantly different from WT CD group (p=0.0085). Altogether, these results demonstrate diet- and genotype-dependent impairment in memory consolidation with a negative outcome in the comorbid condition.

2.3.3.3 Open Field Test

Assessment of the effects of HCD alone and in conjunction with AD pathology on locomotion and anxiety level was done in the open field maze. Analysis of total ambulatory distance during a 20 min task did not result in any significant changes in the locomotor activity between groups (Figure 2-6A), however, there was a genotype-dependent decrease (F(1,43)=6.371; p=0.0154) in locomotor activity of TG rats. Time spent in the central zone of the open field arena as a measure of anxiety-like behavior was not affected by the diet. In contrast, the transgene significantly decreased (F(1,42)=10.09; p<0.01) time spent in the central zone (Figure 2-6B), suggesting that TG rats were more anxious.
Figure 2–4. Morris water maze learning and memory test performed one week prior to the diet onset. A) Latency to reach the platform in the 4-day training phase. B) Time spent in the target quadrant during the probe trial following the learning phase (Pr1) expressed as percent of total time in probe trial. Animal numbers are as follows: WT CD (n=12), TG CD (n=11), WT HCD (n=12), TG HCD (n=11). Values are presented as mean ± SEM. Significance is indicated by * between days 1 and 4 in all experimental groups. RM Two-way ANOVA, One-way ANOVA, Tukey’s multiple comparisons test, p<0.05. Cd = control diet, HCD = hypercaloric diet, TG = transgenic, WT = wildtype.
Figure 2-5. Morris water maze relearning and probe trial for memory test after 12 weeks on the diet. (A) Latency to platform in the 4-day training phase. (B) Mean path length to reach the platform in the 4-day training phase. (C) Path length to platform in individual trials of each training day. (D) Path efficiency to reach the platform during 4 days of training. (E) Time spent in the target quadrant during the probe trial (Pr3) following relearning expressed as percent of total distance in probe trial. (F) Swim speed in the Pr3. Animal numbers are as follows: WT CD (n=12), TG CD (n=11), WT HCD (n=12), TG HCD (n=11). Values are presented as mean ± SEM. Significance is indicated by ‡ between days 1 and 4 in CD groups, by * in A – between TG CD and HCD groups, in B – between CD groups and TG HCD, in E - between TG HCD and WT CD. RM Two - way ANOVA, One - way ANOVA, Tukey’s multiple comparisons test, p<0.05. CD = control diet, HCD = hypercaloric diet, TG = transgenic, WT = wildtype.
Figure 2-6. Locomotor activity and anxiety-like behavior in open field test. (A) Total ambulatory distance for 20 minutes and (B) percentage of time spent in central zone of an open field arena. Animal numbers are as follows: WT CD (n=12), TG CD (n=11), WT HCD (n=12), TG HCD (n=11). Values are presented as mean ± SEM. Significance is indicated by * for WT CD vs TG CD and TG HCD. One-way ANOVA, Tukey’s multiple comparisons test, p<0.05. CD = control group, HCD = hypercaloric diet, TG = transgenic, WT = wildtype.
2.3.4 Neuroinflammation

Neuroinflammation is one of the earliest and most critical events occurring in the brain in response to insult and plays an important role in pathogenesis of AD. Microglia are the key cellular component of the inflammatory processes occurring in the brain and are the first ones to become activated and proliferate in response to disturbances in cerebral homeostasis. Astrocytes play a major role in maintaining brain health and get readily involved in inflammatory reactions. These two types of glial cells were included in our analysis as the elements of particular interest and were visualized using immunohistochemistry technique.

2.3.4.1 Microglia Activation

We looked for signs of microglial inflammation by scanning the entire brain from all frontal to posterior levels. The pathology observed was located mainly in the white matter regions with very few activated microglia cells observed in the gray matter regions such as the cortex and hippocampus. There were no apparent differences among the groups. Microglia activation in the white matter, detected with the OX-6 immunostaining, has been shown to undergo an age-related increase in the TG rats compared to WT rats\textsuperscript{31}. The images of the OX-6 activated microglia in three white matter regions from the three-month-old TG animal demonstrate there is a low activation of microglia in the young animal (Figure 2-7A), similar to that of the WT aged rat. These images were complemented with an Iba-1 positive microglia cells from the young TG animal (Figure 2-8A).

A detailed immunohistochemical assessment of the brain sections indicated significant changes in white matter inflammation due to the combination of the diet and transgene. The comorbid condition of HCD in the TG group, resulted in a large increase in OX-6 positive activated ramified microglia in all subcortical white matter areas examined, including corpus callosum (starting as far anterior as the forceps minor), internal capsule, anterior commissure, optic tract and fimbria of the hippocampi. Representative images are shown in Figure 2-7A. TG HCD group had significant microgliosis in all white matter regions compared to all other groups (Figure 2-7B). For the TG HCD compared to the WT CD group the $p$ value was less than 0.0001 for all regions. Within the
TG groups the TG HCD was significant compared to the TG CD CD groups with $p=0.0003$ in the corpus callosum and $p=0.0002$ in the internal capsule and fimbria.

This white matter microglial activation was also genotype-dependent, with APP21TG rats showing significantly higher OX-6-positive signal in comparison to WT rats in the corpus callosum ($F_{1,40} = 17.84; p =0.0001$), internal capsule ($F_{1,40} = 49.03; p<0.0001$) and fimbria ($F_{1,12} = 53.17; p<0.0001$). In addition, two-way ANOVA analysis showed a significant effect of diet on microgliosis in the corpus callosum ($F_{1,40} =22.88; p <0.0001$), internal capsule ($F_{1,40} = 22.89; p<0.0001$) and fimbria ($F_{1,12} = 29.73; p=0.0001$). There was also a significant diet-genotype interaction on microgliosis in the internal capsule ($F_{1,40} =4.250; p=0.0458$) and fimbria ($F_{1,12}= 12.09, p=0.0046$), and almost significant interaction in the corpus callosum ($F_{1,40} = 3.809, p=0.0588$).

Comorbid impact on total microglia within the white matter tracts was also assessed (Figure 2-8). The comorbid TG HCD group had significantly more microglia than the WT CD group for both the corpus callosum ($p=0.0195$) and the internal capsule ($p=0.0013$), changes not seen in the fimbria. In the corpus callosum TG CD rats also had greater numbers of microglia than WT CD animals ($p=0.0161$).

Iba-1 stained section analysis indicated a significant transgene–dependent increase in area coverage by Iba-1 positive microglia in the corpus callosum ($F_{1,12} = 15.13; p=0.0021$), internal capsule ($F_{1,12} = 13.73; p=0.003$) and fimbria ($F_{1,12} = 7.684; p=0.0169$; Figure 2-8B). There was an additional effect of the diet on the microgliosis in the internal capsule ($F_{1,12} = 12.04; p=0.0046$).

Thus, comorbid rats demonstrated a large microglial activation in all white matter areas analyzed along with an increase in microglial proliferation in the corpus callosum and internal capsule. Additional analysis revealed transgene related effects on microglial activation and proliferation in all white matter regions. There was a diet induced activation in all regions and proliferation in the internal capsule.
2.3.4.2 Astrogliosis

Area of coverage by GFAP-immunopositive astroglia expressed as a percentage of total area of ROI was taken as a measurement of astrocyte reactivity in subcortical white matter (Figure 2-9A,B). For the comorbid TG HCD group the only observed increase in astrocytes was compared to the WT HCD group in the corpus callosum ($p=0.0095$). In the corpus callosum even the TG CD group had higher levels of astrocytes compared to the WT HCD ($p=0.0188$).

There was a transgene effect in that TG rats showed a significant increase in astrocyte density in the corpus callosum ($F_{1, 11}$=20.05, $p=0.0009$) and fimbria hippocampi ($F_{1, 11}$=8.307, $p=0.0149$), compared to WT groups.
Figure 2-7. **Activated microglia in white matter.** (A) 10x photomicrographs of representative OX-6 immunolabelled activated microglial cells in the corpus callosum, internal capsule and fimbria hippocampi from the boxed regions indicated on the whole brain section insertion, right hemisphere. Photographs of the activated microglia in the three white matter regions of the three-month-old TG rat are shown in the right column. Scale bar 200µm. (B) Integrated density as a measure of microgliosis for corpus callosum and internal capsule. Animal numbers are as follows: WT CD (n=12), TG CD (n=11), WT HCD (n=12), TG HCD (n=11). Area coverage by a positive signal (as a percentage of a total area of the region) as a measure of microgliosis for fimbria. Animal numbers are n=4 in each group. Values are presented as mean ± SEM. Significance is indicated by * between TG HCD and all other groups; by † between CD groups. One-way ANOVA and Tukey's multiple comparisons test, p<0.05. 3M = three-month-old TG rat, CD = control diet, HCD = hypercaloric diet, TG = transgenic, WT = wildtype.
**Figure 2-8. Total microglia in white matter.** (A) 10x photomicrographs of representative Iba-1 immunolabelled microglial cells in the corpus callosum, internal capsule and fimbria hippocampi from the boxed regions indicated on the whole brain section insertion, right hemisphere. Photographs of the microglia in the three white matter regions of the three-month-old TG rat are shown in the right column. Scale bar 100µm. (B) Area coverage by a positive signal (as percentage of a total area of a region) for corpus callosum, internal capsule and fimbria. Animal numbers are as follows: WT CD (n=4), TG CD (n=4), WT HCD (n=4), TG HCD (n=4). Values are presented as mean ± SEM. Significance is indicated by * for WT CD vs both TG groups in corpus callosum and WT CD vs TG HCD in internal capsule). One - way ANOVA and Tukey’s multiple comparisons test, p<0.05. 3M = three-month-old TG rat, CD = control diet, HCD = hypercaloric diet, TG = transgenic, WT = wildtype.
Figure 2-9. Reactive astrocytosis in white matter. (A) 10x photomicrographs of representative GFAP immunolabelled astrocytes in the corpus callosum, internal capsule and fimbria hippocampi. Scale bar 100µm. Magnified images of individual astrocytes are inserted at the bottom right corner of image panels in A. (B) Area coverage by a positive signal (as percentage of a total area of a region) for corpus callosum, internal capsule and fimbria. Animal numbers are as follows: WT CD (n=4), TG CD (n=4), WT HCD (n=3), TG HCD (n=4). Values are presented as mean ± SEM. Significance is indicated by * for WT HCD vs both TG groups in corpus callosum). One-way ANOVA and Tukey’s multiple comparisons test, p<0.05. CD = control group, HCD = hypercaloric diet, TG = transgenic, WT = wildtype.
2.3.5 Neuronal density

Dorsal hippocampus, particularly the CA1 region, is a crucial structure for spatial learning and memory and is very susceptible to the pathological processes in AD\textsuperscript{37–40}. We assessed whether there is a loss of neurons in the CA1 subregion of the hippocampus (Figure 2-10A). Counts of NeuN positive pyramidal neurons revealed no differences in the neuronal density between experimental groups (WT HCD vs TG HCD \( p=0.0816 \); WT CD vs TG HCD \( p=0.1844 \); Figure 2-10B).

2.3.5 Synaptic density

Synaptic density was analyzed in the CA1 and CA3 dorsal hippocampal subregions using synaptophysin immunostaining to detect synaptic vesicles (Figure 2-11A). The area of coverage by a positive signal was significantly decreased in the TG rats compared to the WT animals in both regions (CA1 \( p=0.0008, F_{(1,20)} =15.38 \); CA3 \( p=0.0001, F_{(1,20)} =22.60 \); Figure 2-11B). In the TG rats that were also on the HCD there was no additional effect of the comorbidity on the synaptic density in any of the regions (TG CD vs TG HCD in CA1 \( p>0.99 \); in CA3 \( p>0.98 \)). Rats from the Control TG and HCD TG groups showed significantly lower synaptic density compared to the HCD WT (\( p=0.0126 \) and 0.0148, respectively) in the CA1 region, and to the HCD WT (\( p=0.0153 \) and 0.0065, respectively) and Control WT (\( p=0.0342 \) and 0.0149, respectively) in the CA3 region.
Figure 2-10. Neuronal counts identified by neuronal nuclear antigen (NeuN) immunohistochemistry. (A) 20x photomicrographs of the dorsal hippocampus CA1 subregion pyramidal neurons. Scale bar 100 um. Boxed area corresponds to a field defined for cell counts. (B) NeuN positive cell counts in a field of area 0.2 mm². Animal numbers are n=4 in each group. Values are presented as mean ± SEM. Cd = control diet, HCD = hypercaloric diet, TG = transgenic, WT = wildtype.
Figure 2-11. Synaptic density in the hippocampus identified by synaptophysin (SYN) immunohistochemistry. (A) 20x photomicrographs of the pyramidal neurons in the dorsal hippocampus CA1 (top row) and CA3 (bottom row) subregions. Scale bar 50 um. Boxed area corresponds to a field defined for quantification. (B) Synaptophysin area coverage (%) in CA1 and CA3 regions of the hippocampus. Animal numbers are n=6 in each group. Values are presented as mean ± SEM. Significance is indicated by * for HCD WT vs both TG groups in CA1 and for both TG groups vs both WT groups in CA3 region. HCD = hypercaloric diet, TG = transgenic, WT = wildtype. One-way ANOVA, Tukey’s multiple comparisons test, p<0.05.
2.3.6 Myelination

Activated microglia were highly accumulated in the cerebral white matter of TG rats on HCD with some more minor transgene and diet effects. To assess if signs of demyelination of the white matter tracts were present at this level glial pathology, Luxol fast blue staining was performed (Figure 2-12A,B). We quantified the percentage of area coverage by a positive signal for both corpus callosum (Figure 2-12C) and internal capsule (Figure 2-12D). There was no statistically significant difference in myelin content between the groups and no effect of genotype or diet was detected. Thus, increased microglial activation was not accompanied by loss of myelin at this stage.
Figure 2-12. Myelination of white matter. 10x photomicrographs of representative brain sections stained with Luxol fast blue containing (A) corpus callosum and (B) internal capsule, right hemisphere. Scale bar 100µm. Area coverage by a positive signal (as percentage of a total area of a region) for (C) corpus callosum and (D) internal capsule. Animal numbers are as follows: WT CD (n=4), TG CD (n=4), WT HCD (n=4), TG HCD (n=4). Values are presented as mean ± SEM. CD = control diet, HCD = hypercaloric diet, TG = transgenic, WT = wildtype.
2.4 Discussion

The results of this investigation clearly show, for the first time, that APP21 TG predisposed to AD rats maintained on a high fat and high carbohydrate diet not only develop considerable metabolic perturbations, but they also exhibit marked widespread white matter microgliosis that was accompanied by impairment on a spatial memory task compared to the performance level of wildtype rats. However, there was no neuronal loss or decrease in synaptic density in the hippocampus of these comorbid rats. Although there were some behavioral, synaptic and inflammatory changes that could be attributed to the diet or the transgene alone, it was clear that the more significant neuroinflammation and memory and learning deficits were due to the combination of the energy-rich high fat, high carbohydrate diet and the TG condition. This is the first demonstration of the impact of hypercaloric diet on white matter in a vulnerable aging brain with increased levels of pathogenic hAPP. These TG rats have been previously characterized to have dense neuronal staining for hAPP, but no evidence of plaques\(^30,33\). This differs from previous mouse models that assessed high-fat diet induced MetS on animals with established classical AD events including amyloid plaque and tau pathology.

The hypercaloric diet approach was chosen to mimic a modern dietary pattern in the human population represented by a combination of food that is high in fat and simple sugars and carbohydrate-rich beverages\(^10,41\). This study was not designed to examine the exact effects of the specific source of fat or type of fatty acids or specific carbohydrates ingested in a large amount. The intent was to examine a combined diet with a high content of both components to deliver an excess of calories associated with induction of MetS pathology in our rat model\(^10,41,42\). We therefore cannot extrapolate on the potential effects of high fat diet or high carbohydrate diet in isolation.

Twelve weeks on the HCD were sufficient for the development of significant obesity and visceral adiposity in these rats. While rats in control groups had normal rat chow as the only source of energy, rats maintained on high-fat, high-sugar diet had an additional energy uptake from a corn syrup drink, which resulted in a reduction of food consumption in these animals, but nonetheless a greater total caloric intake per rat compared to rats on CD.
The ingestion of high fat and high carbohydrate calories had effects in the periphery and markedly altered lipid metabolism, increasing triglycerides, total cholesterol and atherogenic non-HDL fraction in rats of both genotypes. Rats TG for hAPP were more susceptible to these changes and had a greater degree of dyslipidemia. In contrast, WT rats were more prone to perturbations in glucose metabolism. Such sensitivity of lipid homeostasis to a long-term consumption of high fat diets has been shown previously and has also been reported for the Fischer 344 rat strain. However, this is the first instance where the high-fat high-sugar diet has been tested in the APP21 TG rat demonstrating a greater degree of dyslipidemia compared to the WT subjects.

Although fasting glucose levels were within a normal range for both groups on HCD, the HCD led to the increase of fasting insulin levels suggesting the development of hyperinsulinemia and insulin resistance in rats of both genotypes.

During a 2-h glucose tolerance test, WT rats had sustained high blood glucose levels indicating a decreased tolerance for glucose in this group. Interestingly, HCD did not appear to induce pronounced glucose intolerance in TG rats, at least not after the 12-week long intervention. This physiological difference in response to excessive caloric intake could implicate mutated hAPP gene inserted in the genome of rats and overexpressed in tissues other than brain (i.e. liver, kidney, lung) and its possible interaction with mechanisms of metabolism. Similar to our observation, 5xFAD mice bearing 5 human familial AD mutations including APPSwe placed on a high-fat diet for 10 weeks did not show signs of glucose intolerance in the oral version of the test compared to WT control group. The presence of carbohydrate metabolism alterations has been reported in patients with symptomatic AD, in which there are lower rates of fasting blood glucose as well as lower glucose values in the oral glucose tolerance test. However, this unique phenomenon would need further separate investigation using more sensitive methods to find out whether there is a difference in glucose metabolism and in the role of compensatory mechanisms to overcome dietary effects between the two genotypes, which could account for this diverse response to a glucose load and was not in the focus of the present study.

There was no dietary effect on the blood pressure, demonstrating that a 12-week exposure to the HCD was not long enough to develop hypertension in this rat strain. However, the non-invasive method to measure blood pressure used in this study falls short of the accuracy of invasive
techniques, and might be insensitivity to subtle early changes in blood pressure possibly present at this stage.

Behavioral analysis at the end of the diet indicated an impact of the diet-induced metabolic alterations on memory consolidation in rats with AD predisposition, however only compared to the WT CD rats. This observation clearly has implications for human populations with a high prevalence of obesity due to a hypercaloric Western style diet with advancing age and increasing levels of brain amyloid \(^{18,46}\). Studies using TG AD mouse models have shown similar effects of high-fat diet on the spatial memory domain and noted the link of these effects to the inflammatory events\(^{44,47–49}\). One study showed increased microglia activation detected \textit{in vivo} using positron emission tomography, and a greater amyloid plaque load in APP/PS1 TG mouse which received a high-fat diet and a streptozocin treatment\(^{49}\). Interestingly, a triple-transgenic AD mouse on a high-fat diet did not exhibit increase in amyloid plaque deposition or tau-pathology, rather a significantly increased number of activated microglia associated with plaques in the hippocampal region that was suggested to be the primary mediating pathology to an observed cognitive impairment\(^{47}\). Another study using a APPswe/PS1 TG mouse of AD similarly showed no effect of the Western diet on the brain parenchymal amyloid burden, however the diet resulted in decreased synaptic plasticity and blood brain barrier dysfunction which could contribute to the behavioral deficits\(^{48}\). These changes were attributed to the systemic inflammation promoted by the Western diet\(^{48}\). This is in line with studies of human brain showing a lack of correlation between amyloid plaque burden and presence or severity of dementia symptoms\(^{50,51}\). This suggests that other events contribute to manifestation and progression of cognitive decline and that neuroinflammation including white matter microgliosis and astrogliosis can be among them\(^{52}\).

As the field of AD research has started to move away from the amyloid causal hypothesis, the white matter inflammation and other white matter changes concepts have been gaining attention and recognition as important players in cognitive impairment\(^{3,7,53,54}\). White matter abnormalities visualized as hyperintensities on MRI scans are common findings among the elderly population. These signals increase with aging, are often present in mild cognitive impairment (MCI), AD and patients with metabolic disorders, and have been shown to highly correlate with cognitive decline\(^{8,55,56}\). Of great interest is the clinical finding that white matter lesions tend to be present well before symptoms of cognitive deterioration start to appear\(^{7,8,54}\). This has opened a new avenue to explore
the potential of cerebral white matter lesions as a new biomarker of cognitive impairment such as MCI and AD dementia and a possible target for prevention and therapy.

Our results clearly indicate an increased microgliosis and microglial proliferation in the white matter tracts of TG rats expressing pathogenic hAPP markedly aggravated by diet-induced metabolic dysregulations in the comorbid rats. Analysis of the brain tissue has shown a widespread inflammation of the white matter, including the corpus callosum, fimbria, internal capsule, cingulum, anterior commissure and optic tract. This finding is of considerable interest as it replicates the white matter pathology associated with advanced age, MCI, early AD and metabolic disorders in the human population. Intriguingly, the white matter inflammation appeared to be an early pathological event as there was no apparent loss of CA1 hippocampal neurons or additional to the TG background decrease in synapses in the CA1 and CA3 subregions of the hippocampus in the comorbid animals at this stage of the disease.

In the present study we also assessed myelination of two major white matter tracts, the corpus callosum and internal capsule, which appeared to be unchanged in APP21 TG rats on the HCD. Further analysis confirmed that the white matter microgliosis was not accompanied by signs of myelin loss at this stage. Nevertheless, axonal damage or perturbation to oligodendrocyte health could begin to develop and should be examined in the future studies to enhance understanding of the white matter pathological changes.

Additional brain tissue analysis should be carried out in order to identify the nature and magnitude of the inflammatory events as well as determine if these inflammatory events are precursors to or consequences of potential vascular changes and other processes that might take place at this early stage of dietary intervention and contribute to the cognitive dysfunction. However, these elements of interest were not in the focus of the present study which aimed to address the effects of HCD superimposed on the high amyloid background on the major glial cells, microglia and astrocyte, activation as an indicator of neuroinflammatory process.

Clinical data, points towards an association of cerebral white matter pathology with perturbations in executive function, processing speed and general cognition. Widespread neuroinflammatory responses to the HCD, primarily denoted by microgliosis and increased microglia cell activation, seen in the white matter of TG rats may interfere with functioning of multiple cognitive domains
leading to a general decline and may contribute to the observed impairment in the behavioral task performance. However, to establish a clear connection between the white matter inflammation and cognitive impairment, more studies including neuronal health assessment, should be performed. The spatial navigation version of the MWM used in the present study was chosen to assess learning and memory dependent on hippocampal formation that is highly vulnerable to AD pathology. However, it is not the most sensitive for specific testing of executive function components that might be affected at the prodromal stage of the disease in our TG rat model. It will be necessary to perform more sensitive tests (e.g. operant conditioning based set-shifting task) to clarify the cognitive deficits that may be related to the observed brain white matter pathology.

The sex-dependent differences in the effect of MetS on neuroinflammation and other early pathology of pre-AD and cognition were not tested in the current study using only male rats. Future projects should consider including experiments conducted on female animals to address the potential role of biological sex and endocrinological differences in the interaction of these conditions.
2.5 Conclusions

Our study using a TG APP21 rat on HCD, suggests the role of diet-induced metabolic alterations as a risk factor for white matter inflammation, which is an early brain pathology in MCI and AD, as a possible point of interaction with prodromal phase AD. Results further suggest that white matter inflammation may lead to accelerated development of cognitive symptoms, since the white matter microglial activation was accompanied by cognitive impairment in comorbidity condition compared to normal rats from WT population. The other 2 groups, TG rats on the CD and WT on the HCD did not demonstrate this significant cognitive change from the WT CD animals. Activated inflammatory cells were mainly located in the white matter which raises a number of important questions on the nature of events and mechanisms that trigger this specific response. The intense white matter inflammatory response provoked by the dietary intervention in the TG rats also suggests specific anti-inflammatory agents may be a potential treatment and preventative strategy. Several approaches could be taken in this therapeutic direction including targeting inflammatory cytokines or components of the arachidonic acid pathway that mediate the inflammation.
2.6 References


Chapter 3: Effect of the Novel Anti-inflammatory Prodrug Ketoprofen-lysine in Comorbid APP21 Transgenic Rat and Diet-induced Metabolic Syndrome

This experiments in this chapter were designed to examine the effects of a novel brain targeted NSAID prodrug Ketoprofen-lysine on the increased white matter microgliosis and cognitive deficits in the comorbid model of prodromal AD and MetS (Objective 2). This manuscript has been reviewed by all authors and is ready to be submitted for publication.

3.1 Introduction

Dementia and mild cognitive impairment (MCI), which is associated with an increased risk of converting to dementia, represent a serious public health challenge. The progressive and incurable characteristic of these age-related disorders is of considerable concern given their prevalence in the human population. Alzheimer's disease (AD), the most common form of dementia, is a neurodegenerative disease that leads to severe mental health decline. The major hallmark of the disease is an accumulation of amyloid-β peptide (Aβ), produced through cleavage of amyloid precursor protein (APP) and formation of neurofibrillary tangles. However, white matter abnormalities and neuroinflammation are thought to be implicated in the early stages of AD development and contribute to its progression. Analysis of brain and serum specimens of AD patients have shown elevation of pro-inflammatory chemokines and cytokines such as IL-6, IL-1β, TNF-α, TGF-β, increased expression of inflammatory genes and activation of signaling pathways associated with inflammation (e.g. TNF-α, NF-κB, COX1, COX2), and reactive glial activation in both grey and white matter structures.

In the human elderly population MCI and AD very often co-exist with metabolic disorders such as diabetes and metabolic syndrome (MetS). MetS can include a combination of abdominal obesity, dyslipidemia, insulin resistance and hypertension. A chronic inflammatory state is also a characteristic feature of the MetS. Inflammation appears to be a shared pathology with AD and is suggested to be a point of their interaction. This interaction between prodromal AD and
MetS was recently demonstrated in our previous study of a novel human APP21 transgenic (TG) rat model of early AD\textsuperscript{15-17} and a hypercaloric diet (HCD) designed to induce obesity and MetS. This combination of MetS in a prodromal model of AD resulted in a larger increase in neuroinflammation in the white matter of the comorbid animals compared to either condition alone.

Recognition of neuroinflammation as one of the key processes in the etiopathogenesis of the disease has opened a potential new avenue for the early treatment of dementia. Promising candidate agents for treatment belong to the class of non-steroidal anti-inflammatory drugs (NSAIDs)\textsuperscript{18-22}, that primarily block pro-inflammatory mediators production via cyclooxygenase (COX) enzyme inhibition\textsuperscript{23}. Both COX-1 and COX-2 enzymes are thought to be involved in the inflammatory processes\textsuperscript{24,25}, and it is possible that agents from this pharmacological group with various selectivity have the potential to modulate pathological changes and be considered for a treatment or prevention of AD.

Several epidemiological studies have shown an associated reduced risk of AD with a long-term treatment with NSAIDs\textsuperscript{26-30}. In addition, studies using these agents for treatment in animal models of AD have shown beneficial effects on amyloid pathology, neuronal health and cognitive function\textsuperscript{31-36}. However, clinical trials on prevention and treatment of mild cognitive impairment and AD, have produced controversial results for NSAIDs of both selective and non-selective COX inhibitor subclasses\textsuperscript{37}. Cases describing beneficial outcome of a trial were linked to the use of non-selective NSAIDs, especially with prolonged intake\textsuperscript{20,37-40}. The majority of animal studies reporting a positive effect of the treatment have also used conventional non-selective drugs\textsuperscript{40}. Although there have been many failures in clinical trials to treat or delay the progression of cognitive decline\textsuperscript{40-47}, analysis of clinical data has indicated that the timing of the treatment appears to be critical for any positive outcome. The studies suggest that the desired beneficial effect can be achieved by early intervention, before symptomatic onset and at the start of or likely before cerebral pathological changes\textsuperscript{37,48}.

Thus, there appears to be strong epidemiological support for NSAIDs in the prevention of AD, although more studies are necessary to characterize potential therapeutic effects of these agents as
well as to investigate the exact conditions and define the best regimen of treatment taking into consideration some of the lessons learned from clinical trials.

In the present study we examine therapeutic potential of a novel non-steroid anti-inflammatory prodrug, ketoprofen-lysine (KL), that was designed to specifically target the brain. This study analyzed the effect of KL prodrug on neuroinflammation and cognitive function in the comorbid APP21 TG rat model of prodromal AD combined with diet-induced MetS. The prodrug was synthetized via conjugation of a non-selective NSAID ketoprofen to an amino acid l-lysine that allows large neutral amino acid transporter (LAT1) - mediated drug transport through the blood brain barrier. This manipulation of the drug should provide a major advantage of improved drug delivery to the central nervous system49. Thus, the prodrug has a rapid uptake from the bloodstream minimizing undesired systemic side effects, good brain tissue distribution and a high cell uptake providing a targeted action.
3.2 Methods

3.2.1 Animals

All animal handling and experimental procedures were carried out in accordance with the guidelines of the Canadian Council on Animal Care and were approved by Western University Animal Use Subcommittee (AUP 2014-016).

Breeding pairs of the homozygous APP21 TG Fischer 344 rat model of AD were obtained from Drs. Cansu and Yuksel Agca (University of Missouri, Colombia, MO, USA) and were bred in house alongside wildtype Fischer 344 rats¹⁵. We have previously characterized the combination of this TG rat and HCD diet for the effects on cognitive function and neuroinflammation.

A total of 80 male wildtype (WT) and male APP21 TG rats were involved in this study. Single and paired rats were housed under standard conditions (12:12 light/dark cycle, at 22-24°C) and maintained on a standard rat diet. At the age of 10-10.5 months half of the rats of each genotype were randomly assigned to a Western type high-caloric diet (HCD), while the other half continued on a standard control diet (CD). Diets were provided *ad libitum* during the 15-week duration of the study. A subset of animals of each genotype and each dietary group was assigned to the non-steroidal anti-inflammatory KL prodrug treatment protocol. Animal numbers for each experimental group were the following:

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>TG</th>
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<tbody>
<tr>
<td>CD</td>
<td>11</td>
<td>9</td>
</tr>
<tr>
<td>CD-KL</td>
<td>10</td>
<td>8</td>
</tr>
<tr>
<td>HCD</td>
<td>11</td>
<td>10</td>
</tr>
<tr>
<td>HCD-KL</td>
<td>11</td>
<td>10</td>
</tr>
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</table>

For histological analysis, lipid and visceral fat measurements animal numbers in non-KL groups were the following: WT CD n=9, TG CD n=5, WT HCD n=6 and TG HCD n=6. One rat from TG CD-KL and one from WT CD-KL group died after 1 month of drug administration.
3.2.2 Diets

A study timeline is shown in Figure 3-1. The CD contained (in % Kcal): 26 protein, 59.7 carbohydrate, and 14.3 fat with 1.52 % of saturated fatty acid (Prolab RMH 3000 5P00). The HCD consisted of the following (in % Kcal): 17 protein, 43 carbohydrate, 40 fat with 62.4% of saturated fatty acid and 0.21% cholesterol (D12079B, Research Diets, Inc). The metabolizable energy content of CD and HCD (in kcal/g) was 3.18 and 4.7, respectively. Rats were given free access to water in CD groups and 20% corn syrup water solution in HCD groups (Bee Hive, ACH Food Companies, Inc, USA).

3.2.3 Chronic Prodrug Treatment

KL prodrug was synthesized by Dr. Jukka Leppänen (School of Pharmacy, University of Eastern Finland). Treatment was initiated 2 days after the dietary regimens were assigned.

The prodrug was dissolved in saline to a concentration of 30 mg/ml. This dilution allowed a single dose to be less than 0.1 ml to make it suitable for continuous daily subcutaneous administration for an extensive period of study (15 weeks). The treatment dose used in this study was 4.1 mg/kg body weight, which corresponds to 2.5 mg/kg of free ketoprofen. Rodent studies on NSAIDs (e.g. ibuprofen) treatment in AD models with detected plaque pathology commonly use a dose of 5 mg/kg. However, we were using a model of early stage AD and the treatment was prophylactic and initiated simultaneously with metabolic disease induction. Furthermore, ketoprofen, that is mainly used for surgical pain management in rodents, has a higher rate of adverse effects and has been shown to produce gastrointestinal damage in rats in a commonly used therapeutic dose 50. Finally, the prodrug has been engineered to easily cross the blood brain barrier. For these reasons, we chose a dose half of what has been used for other NSAIDs.
3.2.4 Physiological and Metabolic Assessment

3.2.4.1 Caloric Intake

Body weight, food and drink consumption were measured twice a week throughout the experiment and caloric intake for each category was determined.

3.2.4.2 Intravenous Glucose Tolerance Test (IvGTT)

IvGTT was performed one week before the study initiation and at week 12 of the study (Figure 3-1). Following a 10-12-h overnight fast, a needle was inserted into the saphenous vein to draw approximately 100-150 µl of blood for the determination of baseline glucose levels. A 50% D(+)glucose (G 8270, Sigma-Aldrich, Inc., Saint Louis, Missouri, USA) solution in sterile water (1g/kg body weight) was then injected in a lateral tail vein. Blood was collected from the saphenous vein repeatedly at 5, 10, 20, 30, 40 and 50 min after the glucose load. Glucose levels were immediately measured using Freestyle Light Blood Glucose Monitoring System (Abbott Diabetes Care Inc, Alameda, CA).

3.2.4.3 Lipid Profile Analysis

After euthanasia cardiac whole blood samples were collected and transported to the CORE Laboratory (University hospital, London, ON, Canada) for serum analysis of lipid metabolism that included triglycerides, total cholesterol and high-density lipoprotein cholesterol (HDL) measurements. Non- HDL was calculated as total cholesterol – HDL. The cholesterol ratio (Chol:HDL ratio) was calculated by dividing total cholesterol value by HDL cholesterol number.
3.2.4.4 Blood Pressure Analysis

Systolic and diastolic arterial blood pressure was measured using the non-invasive tail cuff method (CODA Blood Pressure System, Kent Scientific Corp., Connecticut, USA). Rats underwent an acclimation procedure followed by baseline level measurements two weeks before the experiment started. Assessment of blood pressure was repeated at week 11 of the study (Figure 3-1).

3.2.5 Open Field Task

Locomotor activity and anxiety-like behavior were tested in an open field arena (45.7x45.7cm) over the course of 20 min on week 12 of the experiment (Figure 3-1). Ambulatory time and distance and time spent in the central zone (27.5x27.5cm) were evaluated using video-tracking software (ANY-maze®, Stoelting Co., Wood Dale, IL, USA).

3.2.6 Morris Water Maze Task

3.2.6.1 Spatial Navigation Protocol

Rats were trained to find an escape platform submerged in a circular pool (145 cm in diameter, 58 cm in depth) using visual extra-maze cues positioned on the surrounding walls. The platform (12cm in diameter) was placed in the middle of one of 4 quadrants the pool was conceptually divided into and its location remained unchanged during the training phase. The training protocol started in week 13 (Figure 3-1) consisted of 16 trials over 4 consecutive days (4 trials/day). The duration of one trial was 60 sec with a 30-sec inter-trial period during which rats were allowed to remain on the platform. Release positions were different for each trial of the day and their order alternated for every other day of spatial acquisition.

At the end of the training, the rats were subjected to two 30-sec probe trials 7 days apart where the platform was removed from the pool and the rats were released from a novel start position. Probe 1 was introduced on day 5, the day after the training. Probe 2 was performed on day 12 after the start of the training. All sessions were monitored and recorded using video-tracking software.
Learning progress was assessed using latency and path length to reach the platform for each day of training. Probe performance was evaluated using such parameters as time and distance travelled in the quadrant where the platform had previously been located (target zone). Swim speed was monitored at all stages of the test.

### 3.2.6.2 Shifting Strategy Protocol

A cognitive flexibility (strategy shifting) protocol was adapted for use in this study\textsuperscript{51–55} to assess components of the executive function such as working memory and behavioral flexibility that were shown to be affected in early stages of AD\textsuperscript{56–61}. Immediately after Probe 2 the platform was returned to the pool to the previous location and new rules were introduced. For this paradigm rats received 8 trials grouped in 2 blocks of 4 trials each with a 30-sec inter-trial period within the block and a 1 h gap in between the blocks. Rats remained on a platform during the inter-trial period. After being familiarized with the novel protocol rats were given a 3 day shifting challenge (Figure 3-1). Platform position was fixed for each day and then changed every day, thus placing a high demand on spatial working memory.

The first trial of each day presented information about the platform position specific for each given day. The second trial was a retention trial where rats needed to recall the information learned in the first trial which is required to escape the water. The following trials were used to solidify information making the shift to a new escape location on the next day more challenging. The difference in time and path length to platform between trials 1 and 2 were analyzed as a criterion of working memory efficiency and flexibility. The difference in time and distance between trials 1 and 4 as well as trials 1 and 8 were used to evaluate learning ability in the new task setting. Old platform location preference was used for assessment of the ability to abandon previously used and now irrelevant information, i.e. persistent behaviour. For this analysis, time and distance spent searching in the platform zone (designated a 9.5 cm proximity to the platform) of the previous day during trial 1 were measured.
3.2.6.3 Cued Trials

Upon the completion of the cognitive flexibility protocol, the rats were subjected to a series of 4 cued trials in the same pool (Figure 3-1). Extra-maze cues were removed to exclude their use for spatial navigation. Instead a cue was directly mounted on top of the submerged platform that could be seen above the water surface to indicate the platform location. The platform was placed in a novel position each time and the start position was switched for every trial to interfere with the spatial learning. These trials served as a control procedure to determine if there were any visual or motivational impairments that could account for differences in learning and memory between groups. Quantification of the path length and latency to reach the platform assessed cued learning.
**Figure 3-1. Project timeline.** The age of the rats (in months) at the start (day 0) and the end of the study (week 16) are shown in brackets. Diets were assigned and began on day 0 and all testing time points are in reference to this day. Baseline measurements of blood pressure and glucose tolerance were completed in the 2 weeks before the start of the diets. Morris water maze (MWM) navigation training was completed within 4 days on week 13, with a probe trial 1 (Pr1) on the day after training. The second probe trial (Pr2) was 7 days later, on week 14 on the diet. Immediately after the Pr2, the cognitive flexibility (shifting) protocol was initiated, and was completed in 3 days. MWM cued trials were performed the next day after the shifting paradigm test. Ketoprofen-lysine prodrug was administered subcutaneously once daily at a dose of 4.1 mg/kg starting 2 days after day 0 and continuing to the end of the experiment. BP = blood pressure measurement, IvGTT = intravenous glucose tolerance test, mo = month, MWM = Morris Water Maze, Pr = probe trial, OF = open field test, BC = blood collection, TC = tissue collection, w = week.
3.2.7 Euthanasia and Tissue Collection

At the beginning of week 16 animals were weighed and then euthanized with pentobarbital overdose following a 12-h fasting period. Cardiac blood was collected immediately before perfusion. Epidydimal fat pads were collected and weighed. Rats were then perfused transcardially with 0.01M phosphate buffered saline (pH 7.35) followed by 4% paraformaldehyde (PFA, pH 7.35). The brains were post-fixed in 4% PFA overnight and then transferred to a 30% sucrose until fully submerged. The brains were sectioned coronally on a cryostat into 30μm thick sections and were stored in cryoprotecting solution at -20°C until they were used for immunohistochemical procedures.

3.2.8 Immunohistochemistry

Immunohistochemical analysis was performed on free-floating sections to visualize total microglia and activated microglia using rabbit polyclonal antibody against the ionized calcium binding adaptor molecule-1 (anti-Iba-1; 1:1000; Wako Chemicals USA Inc., Richmond, VA, USA) and mouse monoclonal antibody against the MHC II receptor (OX-6; 1:1000; BD Pharmingen, Mississauga ON, Canada) respectively. After an overnight incubation with the primary antibody at 4°C, sections were incubated with biotinylated anti-mouse secondary antibody (1:500; Vector Laboratories, Inc. Burlingame, CA, USA) followed by incubation with avidin-biotin complex (ABC kit, Vector Laboratories, Inc. Burlingame, CA, USA) and then developed in 0.05% 3, 3’ diaminobenzidine tetrahydrochloride (Sigma-Aldrich, St. Louis MO, USA). Sections were then mounted on glass slides, air-dried, dehydrated, cleared in xylene and coverslipped with DePex mounting media (DePex, BDH Chemicals, Poole, UK).

3.2.9 Imaging and Quantification of Immunohistochemistry

Immunohistochemically processed brain sections were imaged with a Nikon Eclipse Ni-E microscope using NIS-Elements Imaging Software Version 4.30.02 (Nikon Instruments Inc., Melville, NY), allowing 10x magnification. Analysis and quantification were carried out using 64-
bit ImageJ software (Version 1.48u4, Wayne Rasband, National Institutes of Health, Bethesda, MD, USA). A total of three brain sections containing anterior, mid-anterior and central-to-posterior regions of the corpus callosum and three sections from the ventral hippocampal level for the internal capsule were chosen and scanned for each animal.

Images were converted into a black-and-white 8-bit format, underwent background subtraction, thresholding and were calibrated prior to taking measurements. Based on the white matter pathology observed in our model previously, regions of interest (ROIs) included the entire body (BCC) and periventricular regions (PVCC) of the corpus callosum and internal capsule in both hemispheres and were manually outlined using a free outline tool. For the assessment of activated microglia population total area, the area covered by a positive signal (area%) was measured for each region and then expressed as a weighted average.

3.2.10 Data Analysis

Statistical analysis was performed using GraphPad Prism 6.0. The data was analyzed by performing t-test, one-way or two-way analysis of variance (ANOVA), Tukey’s multiple comparison post hoc test. Data are expressed as mean ± standard error of the mean (SEM), and a p value of ≤0.05 was considered statistically significant.
3.3 Results

We first performed a comprehensive physiological assessment.

3.3.1 Body Weight, Visceral Fat Accumulation and Diet Consumption

At the end of 15 weeks of dietary treatment, body weight gain from baseline was significantly greater in all HCD groups compared to the CD groups (Figure 3-2A) as a result of a greater total caloric intake in these animals (Figure 3-2B). Both TG and WT rats on the HCD with or without KL prodrug treatment started to gain weight rapidly and weighed significantly more than CD groups as early as the beginning of the 3rd week on the diet and remained significantly heavier to the end of study. Epididymal fat pad weights, representative of visceral fat accumulation, were significantly increased in the HCD groups compared to the control diets ($F_{(1,62)}=193.4; p<0.0001$) (Figure 3-2C). Fat accumulation also had a genotypic difference with fat deposits being larger in the TG rats (genotype effect $F_{(1,62)}=9.606; p=0.0029$).

3.3.2 Lipid and Glucose Metabolism

The HCD had a robust effect on lipid metabolism resulting in dyslipidemia (Figure 3-2D, E). Total cholesterol was significantly increased in the comorbid animals with or without the KL prodrug and also in WT HCD rats receiving the KL prodrug (diet effect $F_{(1,61)}=46.52; p<0.0001$). A similar pattern was seen for the non-HDL cholesterol (cholesterol content of atherogenic lipoprotein particles). The non-HDL cholesterol was increased in the WT HCD and comorbid rats, with and without the prodrug ($F_{(1,60)}=89.49; p<0.0001$; Figure 3-2D). However, the WT HCD on the prodrug had higher levels than the WT HCD without the drug. The Chol:HDL ratio, a relevant clinical index, was significantly elevated by HCD ($F_{(1,61)}=65.97; p<0.0001$; Table 3-1). Triglyceride levels were significantly elevated by HCD in all groups ($F_{(1,61)}=115.1; p<0.0001$; Figure 3-2E). There was no apparent effect of KL prodrug administration on lipid metabolism with an exception of effect on total and non-HDL cholesterol levels in WT HCD rats.
The IvGTT results demonstrated a glucose intolerance in WT and TG groups on the HCD (Figure 3-3). This was characterized by a significantly greater blood glucose levels at individual time points during the GTT (two-way ANOVA analysis, Tukey’s MC test). HCD groups started to deviate significantly from the control group values as early as at 5 min after the glucose injection (for WT HCD) and maintained high glucose levels up to 40 min post-load for WT rats and 20 min post-load for TG rats (Figure 3-3). Interestingly, glucose levels of TG HCD rats normalized earlier than those of the WT rats, as their glucose levels were comparable to control groups already at the 30 min time point, whereas WT HCD rats remained significantly elevated and returned to normal only by the end of the test. The glucose intolerance was also characterized by a significantly greater AUC for HCD experimental groups compared to the appropriate control diet groups (diet effect $F_{(1,76)}=103.2; p<0.0001$), except the TG HCD group which was only statistically different from WT CD, but not from TG CD rats (Figure 3-2F). TG animals had a lower AUC compared to the WT rats (genotype effect $F_{(1,76)}=12.95; p=0.0006$), suggesting a better glucose uptake from the blood stream in transgene carriers (Figure 3-2F). Rats maintained on the HCD had comparable fasting blood glucose levels compared to CD groups that remained within the normal range (Table 3-1). This data suggests that HCD led to the development of glucose intolerance in both genotypes, however, TG rats were significantly less prone to perturbations in glucose metabolism than WT animals. Prodrug administration has shown no effect on glucose metabolism.

3.3.3 Blood Pressure

Systolic and diastolic blood pressure values obtained at week 11 of diets were not significantly different between the experimental groups. Neither HCD, nor transgene presence or KL prodrug treatment had an effect on either the systolic or diastolic pressure (Table 3-1).
Figure 3-2. Physiological characteristics. A) Body weight gain expressed as a percentage of baseline value. B) Average caloric intake during the 15 weeks on the diets. Drink in the HCD groups represents energy derived from the corn syrup solution. C) Post-mortem paired epididymal fat pad weight. D) Total serum cholesterol is presented by entire bar. High density lipoprotein fraction (HDL) and the remaining combined non-high-density lipoproteins (non-HDL) are also shown in each bar. E) Blood triglyceride levels. F) Area under the curve (AUC) from the IvGTT after 12 weeks on the diets. Animal numbers for C-E are as follows: WT CD, n=9; TG CD, n=5; WT HCD, n=6; TG HCD, n=6; WT CD-KL, n=12; TG CD-KL, n=11; WT HCD-KL, n=12; TG HCD-KL, n=11. Animal numbers for A,B,F are as follows: WT CD, n=11; TG CD, n=9; WT HCD, n=11; TG HCD, n=10; WT CD-KL, n=12; TG CD-KL, n=11; WT HCD-KL, n=12; TG HCD-KL, n=11. Values are presented as mean ± SEM. * in A, B (total caloric intake), C, D (total...
cholesterol), E, F, indicates significance between HCD and CD groups. † in D, indicates significance between individual HCD and all CD groups for the Non-HDL. † in F, indicates significance between the TG HCD groups and the WT HCD group. ‡ in F, indicates significance between TG HCD group and WT CD, TG CD-KL groups. One-way ANOVA and Tukey’s multiple comparisons test p < 0.05. CD = control diet, HCD = hypercaloric diet, HDL = high density lipoprotein, IvGTT = intravenous glucose tolerance test, KL=ketoprofen-lysine prodrug, TG = transgenic, WT = wildtype.
Figure 3-3. Intravenous glucose tolerance test on week 12 on the diet. Glucose curves during the IvGTT are shown for 4 experimental groups without the Ketoprofen-lysine prodrug treatment. * indicates significance between WT HCD and both CD groups, † between TG HCD and both CD groups, ‡ between TG HCD and WT HCD. Animal numbers are as follows: WT CD, n=11; TG CD, n=9; WT HCD, n=11; TG HCD, n=10. Values are presented as mean ± SEM. Two-way ANOVA Tukey’s multiple comparisons test, p<0.05. CD = control diet, HCD = hypercaloric diet, IvGTT=intravenous glucose tolerance test, TG = transgenic, WT = wildtype.
<table>
<thead>
<tr>
<th></th>
<th>WT CD</th>
<th>WT CD-KL</th>
<th>TG CD</th>
<th>TG CD-KL</th>
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<td><strong>Chol:HDL, mmol/l</strong></td>
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<td>1.26±0.05</td>
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<td>3.14±0.22*</td>
<td>2.53±0.27*</td>
<td>2.34±0.22*</td>
</tr>
<tr>
<td><strong>Fasting glucose, mmol/l</strong></td>
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<td>4.47±0.12</td>
<td>4.27±0.15</td>
<td>4.19±0.10</td>
<td>4.56±0.15</td>
<td>4.55±0.11</td>
<td>4.47±0.13</td>
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</tr>
<tr>
<td><strong>Systolic pressure, mmHg</strong></td>
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<td>96.17±1.97</td>
<td>95.47±2.44</td>
<td>91.52±3.16</td>
<td>94.05645 ±4.18</td>
<td>95.61±2.78</td>
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</table>

Values are presented as mean ± SEM. The symbols * indicate significance for HCD groups vs appropriate CD groups, # - between WT HCD and WT HCD - KL groups. One - way ANOVA, Tukey’s multiple comparisons test, p<0.05. Abbreviations: CD – control diet, HCD – hypercaloric diet, TG – transgenic rats, WT – wild type, KL – ketoprofen-lysine prodrug recipients, Chol – total cholesterol, HDL – high density lipoprotein cholesterol.
3.3.4 Behavioral Assessment

We used two different MWM protocols to evaluate effects of comorbidity of HCD-induced MetS with genetic predisposition to early AD and the effect of the KL prodrug treatment. These were designed to assess spatial learning and reference memory, as well cognitive flexibility and working memory.

3.3.4.1 MWM Spatial Learning and Memory

During the training phase, all animals significantly improved in both latency and path length to the platform when day 1 vs day 4 performances were compared, indicating there was good learning of the spatial version of the task in all experimental groups (Figure 3-4A). In addition, neither of these measurements were significantly different between the groups at the end of acquisition phase (day 4). No significant difference was observed in the time or distance travelled in the quadrant of the previous platform location (Target zone), during both probe trials (Figure 3-4B). Swim speed was not different between the groups (Figure 3-4C). Thus, these spatial learning and memory results demonstrate an absence of diet, genotype and drug effects on the spatial navigation ability and reference memory of the rats.

3.3.4.2 MWM Cognitive Flexibility and Working Memory

Working memory efficiency and flexibility is demonstrated by a significant reduction in path length and latency to reach the platform between trials 1 and 2. This can be seen in the WT and TG CD groups. However, in comorbid rats, with or without KL prodrug treatment, this significant reduction in path length and latency from trial 1 to 2 averaged for 3 shifting days was not present (Figure 3-5A,C). The lack of reduction in path length from trial 1 to 2 on each day, suggests that the combined TG HCD rats were not using working memory as efficiently as the other groups and that the KL prodrug administration had no effect on this deficit. There was no effect of the KL prodrug treatment as no change in the pattern was seen in any of the 4 experimental group pairs.
TG CD-KL and both TG HCD groups on average had a significantly shorter swims (p=0.0011 TG HCD, p=0.0001 TG HCD-KL, p=0.0053 TG HCD-KL) and were faster (p=0.0069 TG HCD, p<0.0001 TG HCD-KL, p=0.0083 TG HCD-KL) in locating the platform on trial 1 compared to the WT CD rats (Figure 3-5A,C). However, further analysis of the trial 1 performance in each separate day showed no differences between the groups. This behavior could likely occur by chance since rats did not know the location yet or represent potential differences in swimming strategies (i.e. chaining, scanning etc) between groups and may contribute to the smaller intertrial differences in these groups.

The lack of improvement in the performance on the second trial in the comorbid groups could suggest they are using a random swim strategy instead of learning the location in the first and following trials using the spatial cues. However, when the performance in trial 1 was compared to that of trials 4 and 8, all groups without exception achieved a significant improvement in distance and latency even by trial 4 and all were comparable on the last trial of the day, trial 8 (Figure 3-5A,C). Thus, all rat groups including the comorbid rats were able to learn the location of the platform throughout the day using rules of spatial navigation and the earlier assumption of a random swimming strategy choice by comorbid groups is not accurate.

A potential reason for the lack of significance between trial 1 and 2 in the comorbid rats could be a delayed learning of new task rules. Rats from comorbid groups may have more strongly adhered to the previously learned location of the platform from the previous day and were ineffective in switching strategies that would affect progression on the novel task. In trial 1 of each day, the distance travelled in the platform zone of the previous day is an indicator of the domination of an “old rule” of spatial navigation. However, all groups exhibited no difference in swimming distance searching for the escape platform in the previous days platform zone (Figure 3-5B). Thus, comorbid rats were able to adapt to the new protocol as well as the other groups, but not as efficient or flexible in learning the new information presented on each day.
3.3.4.3 MWM Cued Trial Performance

All measurements were averaged over the 4 trials of a cued test day. There were no significant differences in latency and path length to reach the platform (Figure 3-4D) or swim speed across all experimental groups. This suggests that all groups were able to see the cue to locate the platform, were equally motivated and had the motor capability to escape the pool.
Figure 3-4. Morris water maze spatial navigation task and cued trials. A) Path length to the hidden platform during 4 days of training. B) Distance travelled in the quadrant of the previous platform location (Target Zone) as a percentage of the total distance in the probe trials 1 and 2, one week apart. C) Swim speed in probe trials. D) Average path length to the platform during 4 cued trials. Animal numbers are as follows: WT CD, n=11; TG CD, n=9; WT HCD, n=11; TG HCD, n=10; WT CD-KL, n=10; TG CD-KL, n=8; WT HCD-KL, n=11; TG HCD-KL, n=10. Values are presented as mean ± SEM. Significance is indicated by * between days 1 and 4 in all experimental groups. One-way and two-way ANOVA, Tukey’s multiple comparisons test, p<0.05. CD = control diet, HCD = hypercaloric diet, KL = ketoprofen-lysine prodrug, TG = transgenic, WT = wildtype.
Figure 3-5. Morris water maze shifting strategy task. All results are presented as an average of 3 days of testing. A) Path length to the hidden platform in trials 1, 2 and 8. B) Distance travelled in the previous day’s platform location for trial 1, expressed as a percentage of the total distance travelled in the first trial. C) Latency to find the hidden platform in trials 1, 2 and 8. Animal numbers are as follows: WT CD, n=11; TG CD, n=9; WT HCD, n=11; TG HCD, n=10; WT CD-KL, n=10; TG CD-KL, n=8; WT HCD-KL, n=11; TG HCD-KL, n=10. Values are presented as mean ± SEM. # indicates significance for trial 1 with WT CD, * indicates significance from trial 1, NS indicates not significant from trial 1. One-way ANOVA or two-way ANOVA (repeated measurement) and Tukey’s multiple comparisons test, p<0.05. CD = control diet, HCD = hypercaloric diet, KL=ketoprofen-lysine prodrug, TG = transgenic, WT = wildtype.
3.3.4.4 Open Field Exploratory Activity and Anxiety-like Behavior

Analysis of the total ambulatory distance during the 20 min arena exploration indicated that TG rats had a significantly shorter ambulatory distance than the comparable WT rats ($F_{(1,75)}=48.63$; $p<0.0001$; Figure 3-6A). A reduction in total ambulatory distance is an indication of decreased spontaneous physical and exploratory activity in these animals. Furthermore, there was an overall significant impact of the KL prodrug resulting in an increase in locomotor activity in groups receiving the prodrug ($F_{(1,75)}=8.259$; $p=0.005$; Figure 3-6B).

Time spent in the central zone of the open field arena expressed as a percentage of total testing time and decrease in the central zone is a measure of anxiety-like behavior. There was no significant group difference in the time in the arena center during the full 20 min test duration (Figure 3-6C).
Figure 3-6. Locomotor activity and anxiety-like behavior in the open field test on week 12 on the diet. Total ambulatory distance A) combined in 4 groups by genotype and diet irrespective of the prodrug treatment and B) combined by genotype and prodrug assignment. C) Percentage of time spent in the central zone during 20 min in an open field arena for all 8 experimental groups. Values are presented as mean ± SEM. Animal numbers are as follows: WT CD (n=11), TG CD (n=9), WT HCD (n=11), TG HCD (n=10), WT CD-KL (n=10), TG CD-KL (n=8), WT HCD-KL (n=11), TG HCD-KL (n=10). * in A and B, indicates significance between TG groups and WT groups. † in B, indicates significance between WT-KL and WT-ND groups. One-way ANOVA and Tukey’s multiple comparisons test or t-test, p<0.05. CD = control diet, HCD = hypercaloric diet, KL=ketoprofen-lysine prodrug, TG = transgenic, WT = wildtype.
3.3.5 Microglia Activation

We have examined and quantified OX-6 positive activated microglia cells primarily located in white matter tracts throughout the brain, including the body of the corpus callosum (BCC), the periventricular region of the corpus callosum (PVCC) and the internal capsule (Figure 3-7A). For all groups of animals, WT and TG with either CD or HCD, there was no effect of the KL prodrug on the activated microglia in any of the 3 regions measured (Figure 3-7B).

In both areas of the corpus callosum, BCC and PVCC, there was an increase in activated microglia in the TG compared to the WT rats, irrespective of diet ($F_{(1,17)}=84.87$, BCC; $F_{(1,17)}=74.13$, PVCC; $p<0.0001$; Figure 3-7C). For the internal capsule, the TG HCD rats had more activated microglia than the WT CD ($p=0.0024$) and WT HCD ($p=0.0130$) (Figure 3-7C). Furthermore, in both areas of the corpus callosum, the TG HCD had significantly more activated microglia than the TG CD, indicating a more pronounced effect of the comorbid condition ($p=0.0116$, BCC; $p=0.0118$, PVCC; Figures 3-7A,C).

An assessment of the total microglia proliferation within the white matter tracts using anti-Iba-1 immunohistochemistry indicated that there was no apparent change in the total microglia in the white matter of either the corpus callosum or the internal capsule regions (Figure 3-8).

We have also observed a marked increase in the activated microglia in multiple brain regions of a total 6 rats from all 4 experimental groups which all received a KL prodrug treatment. This indicated a potential serious negative effect of the prodrug which was studied in a more detail in these 6 cases and described in the Chapter 4 of this thesis.
Figure 3-7. Activated microglia in white matter. A) The schematics show the coronal sections at one of the levels used for analysis: Bregma 0.6 mm for the corpus callosum (on the left) and
Bregma -2.92 mm for the internal capsule (on the right). Photomicrographs (10X) of representative OX-6 immunolabelled activated microglial cells in the body of the corpus callosum (all experimental groups) and internal capsule (only HCD groups on and off the prodrug treatment) were sampled from the regions in the right hemisphere indicated by the black box on schematics. Scale bar 100µm. **B,C** Bars represent the density of the OX-6 positive microglia coverage expressed as the percentage of a total area of the region: the body of the corpus callosum, periventricular regions of the corpus callosum and internal capsule. **B** All experimental groups are shown; **C** data presented only for 4 groups not receiving the prodrug treatment. Values are presented as mean ± SEM. Animal numbers are as follows: WT CD (n=6), TG CD (n=5), WT HCD (n=5), TG HCD (n=6), WT CD-KL (n=5), TG CD-KL (n=5), WT HCD-KL (n=4), TG HCD-KL (n=6). * indicates significance between the TG and WT groups. † between TG HCD and TG CD. One - way ANOVA, Tukey’s multiple comparisons test p<0.05. CD = control diet, HCD = hypercaloric diet, KL=ketoprofen-lysine prodrug, TG = transgenic, WT = wildtype.
**Figure 3-8. Total microglia in white matter.** A) The schematics show the coronal sections at one of the levels used for analysis: Bregma 0.6 mm for the corpus callosum (on the left) and Bregma -2.92 mm for the internal capsule (on the right). 10x photomicrographs of representative Iba-1 immunolabelled microglial cells in the body of the corpus callosum and internal capsule (control WT CD, all TG groups) were sampled from the regions in the right hemisphere indicated by the black box on schematics. Scale bar 100µm. B) Area coverage by a positive signal (as percentage of a total area of a region) for corpus callosum and internal capsule. Animal numbers are as follows: WT CD (n=6), TG CD (n=5), TG HCD (n=6), WT CD-KL (n=5), TG CD-KL (n=5), TG HCD-KL (n=6). Values are presented as mean ± SEM. CD = control diet, HCD = hypercaloric diet, KL=ketoprofen-lysine prodrug, TG = transgenic, WT = wildtype.
3.4 Discussion

Targeting neuroinflammation appears to be one of the most promising approaches to slowing or preventing some forms of dementia, including AD, since it has been shown to be one of the earliest processes related to the disease pathogenesis. Furthermore, neuroinflammation is persistent over time and contributes to disease progression\textsuperscript{5,62}. A previous study using the APP21 TG rat model of prodromal AD in combination with diet-induced MetS supported the concept of the role of neuroinflammation in the early onset of the disease and mild cognitive impairment. In particular, we previously demonstrated that white matter microgliosis, in particular, may be one of the key elements in prodromal AD-related pathology and that increased white matter inflammation may be point of interaction between specific AD and metabolic disease.

A consideration of previous results along with accumulated evidence from epidemiology and supportive data from clinical and other animal research, suggests that anti-inflammatory agents may be potential treatment candidates to interrupt this neuroinflammatory interaction caused by a combination of amyloid and high fat, high sugar diet. In the present study we investigated the potential therapeutic effect of a novel non-steroidal anti-inflammatory prodrug, Ketoprofen-lysine, in the APP21 TG rat model of prodromal AD with HCD–induced MetS. As in our previous investigation, we did observe changes to metabolism characteristic of the MetS syndrome development (obesity, increased adiposity, dyslipidemia, glucose intolerance). We also observed again increased white matter microglial activation that was accompanied by working memory impairment, based on the MWM cognitive flexibility test, in the comorbid TG HCD group after 15 weeks of the HCD consumption. However, the results of this investigation did not demonstrate any obvious beneficial effect on the white matter inflammation and cognitive deficits associated with the chronic KL prodrug treatment.

The metabolic and physiological profile in the current investigation was consistent with our previous study. Chronic HCD consumption in WT and TG led to the development of obesity and markedly altered lipid metabolism. Previously, using an intraperitoneal GTT (IpGTT), we observed a genotypic difference in the response of glucose metabolism. Even with HCD exposure the TG rats were able to maintain glucose tolerance, while WT rats had greatly elevated glucose levels that did not normalize even at the end of the 2h of testing. In the present investigation, we
used an intravenous glucose challenge demonstrating that both TG HCD and WT HCD rats developed glucose intolerance.

Interestingly, the WT HCD rats again showed the highest blood glucose concentration at each time point during the test resulting in a greater AUC compared to the TG HCD rats in the present study. The lack of development of glucose intolerance in the previous study in which TG HCD rats did not show an increase in AUC compared to the control groups when using an IpGTT may, in fact, be an artefact of the different route of glucose delivery between two versions of the test. In the IpGTT, the glucose passes from the peritoneal cavity to the systemic circulation entering the liver first. In the intravenous administration it bypasses the liver allowing a direct assessment of the glucose disposal and effects of acute insulin release.

Alternatively, the mutated hAPP in our APP21 TG rats is overexpressed in several peripheral tissues including liver and this APP might interact with the mechanisms of glucose metabolism leading to the observed differences between the WT and TG animals. Similarly, the absence of glucose intolerance with the oral GTT have been reported in a transgenic 5xFAD mice also expressing a mutant APP<sub>Swe</sub>, compared to the WT group<sup>63</sup>. Clinically, both lower oral GTT and fasting glucose values were observed in an AD patient cohort<sup>64</sup>. These data suggest a potential implication of APP in the glucose metabolism regulation, findings that require additional investigation.

The KL prodrug administration did not affect any of the metabolic outcomes measured. There is evidence in the literature of beneficial effects of NSAIDs on glucose and lipid metabolism that could provide an advantage for their use in a patient population with metabolic disorders<sup>65</sup>. However, these effects are thought to be dose-dependent. In the present investigation, the treatment of the ketoprofen was administered at a relatively low dose since the ketoprofen was delivered in a prodrug form which allows faster delivery to and greater fraction entered in the brain. This low dose with a direct action in the brain would minimize the systemic exposure and effects observed by others.

We have previously shown the comorbid rats had impaired memory consolidation on the MWM re-learning task. In the present study, we aimed to assess working memory and cognitive flexibility, the processes that show an early decline in AD. The comorbid, TG HCD, rats
demonstrated preserved spatial learning ability and they did not maintain a preference for the previous days escape location during the first trial of shifting strategy version of the MWM task. However, the comorbid animals showed no improvement from trial 1 to 2 in the shifting strategy testing. These results suggest the comorbid groups may have a slower information processing speed and working memory impairment, leading to the slower switch to the changed escape location, i.e. cognitive inflexibility.

Executive behavioral deficits have been observed in these APP21 TG rats with advanced age (19 month old) compared to the WT rats when tested on a strategy shifting MWM task similar to that of the present investigation. Furthermore, another study investigated behavioral flexibility in comorbid stroke and early AD in 13 month old APP21 TG rat model and have shown impairments in strategy change efficiency. This impairment in the comorbid stroke and APP TG rats was based on an operant set-shifting task which allows for a more specific and broad assessment of the executive function. The testing in our study was done in 13.5 month old animals and we did not detect impairments in the APP21 TG rats in the control diet group using the MWM adapted protocol. This MWM protocol lacks sensitivity comparing to the operant-based set-shifting task. Nevertheless, the animals with metabolic disturbances due to the HCD in the TG animals resulted in working memory dysfunction even with the MWM protocol. Thus, our results support our previous demonstration of cognitive impairment in HCD and APP TG comorbid animals. However, as indicated, above, the KL prodrug administration did not prevent this behavioral deficit in the comorbid rats (TG on the HCD).

In the present experiments, locomotor activity tested in the open field was lower in the transgene carriers. This result is consistent with previously observed behavioral differences in this animal model and in APP transgenic mouse models of AD. Interestingly, KL prodrug treatment appeared to increase exploratory activity overall among all experimental groups, with a more pronounced effect in the WT rats. This could be due to peripheral pain-relieving action of ketoprofen which could alleviate the discomfort associated with obesity and increased load on joints in HCD groups, however, this effect was observed in all diet groups, including rats on CD. Furthermore, a rapid delivery of active ketoprofen in the prodrug form to the brain likely minimized ketoprofen systemic effects including analgesic effect. This further suggests there might be a direct central analgesic effect of the drug that is not attributable to prevention of any
increases in neuroinflammation due to the transgene or the HCD\textsuperscript{70,71}. How the ketoprofen effects the brain to produce this increase in exploratory behavior requires further exploration.

In the present study, microglial activation in the white matter indicated increased neuroinflammation in TG animals compared to WT and in comorbid animals with MetS and hAPP compared to TG animals alone. A previous investigation has shown that the APP21 TG rats demonstrate a significant increase in the OX-6 positive microglia in the corpus callosum compared to the WT from 12 and 15 months of age\textsuperscript{55}. Thus, the present investigation indicates that the HCD can markedly aggravate the age-related white matter pathology in the TG rats. These results are consistent with our previous study characterizing this combined disease model in which the comorbid group (TG HCD) had a significant increase in microglial activation in white matter tracts, including the corpus callosum and internal capsule. However, the present results indicate that the anti-inflammatory KL prodrug treatment was not able to reduce the microglial activation in the white matter in the comorbid group. Furthermore, 2 lethal cases and 6 cases of pronounced microgliosis indicated a potential serious negative prodrug effects questioning safety of this agent. One limitation to this study that needs be mentioned is that there was no vehicle-treated group to control for the effects of chronic injections as a stress factor. This study was primarily aimed to investigate the potential of the prodrug treatment to decrease white matter microglial activation, thus, only KL treatment was done. However, it is necessary to perform a follow-up saline-controlled experiments as well as dose-response studies to fully assess the treatment potential.

The hypothesis for this study was that the KL prodrug would ameliorate the white matter inflammation and the cognitive deficits in the comorbid animals. The results are very clear that there is no significant impact of the drug on these measures. Factors potentially influencing the effect of the drug in our model could include timing of the treatment initiation, duration, dosage and properties of the active compound ketoprofen \textsuperscript{72}. Potential changes in expression or function of the LAT-1 transporter in our transgenic prodromal AD model and/or HCD consumption and the establishment of metabolic dysregulation could also affect pharmacokinetics of the prodrug and its potency. Clinical trials have also indicated that NSAIDs might be ineffective when sufficient pathology already exists at the start leading eventually to disease progression and establishment of symptoms without regard to the treatment. Therefore, clinical studies suggest that an early intervention occurring prior to overt pathological changes\textsuperscript{35,37,48} or at the very early stages of the
disease is necessary for beneficial effects\(^{48,73}\). It may be that in or study, the rats by 10 months of age have already developed pathologies driving or contributing to white matter inflammation that could not be rescued or reversed by the KL prodrug administered in the current dose and timeframe for delivery. The inherent pathologies in our animals may be progressing with increasing age that by itself could contribute to the previously described phenomenon of decreased protection of drugs\(^{37}\) through dyshomeostasis in immune and vascular systems. In addition, these pathological processes can be further aggravated by transgene presence in our prodromal AD rat model and even more by the altered metabolic condition, and both of these conditions that can further sustain inflammatory process and affect treatment results. Therefore, further experimentation should be done to establish the timeframe of the development of neuropathological changes to determine the earliest point for successful intervention. Moreover, these studies should be accompanied by the safety testing of the treating agents.

Our model of prodromal AD has demonstrated inflammation primarily affecting white matter. White matter is a structure that is highly susceptible to damage and white matter pathology appears to be one of the earliest signs in diseases including AD and MetS and precedes cognitive decline\(^{2,3,6,74–76}\). Thus, it might be important to consider potential re-directing of the treatment strategy towards targeted protection of the white matter rather than focusing simply on general suppression of inflammation. This would require a detailed analysis of underlying white matter lesions and the timeline of their development. Furthermore, it might not be effective, and in fact it is likely insufficient, to target only one out of many processes of the complex pathogenesis in the progression of MCI and AD. Therefore, future research directions might also include an integration of anti-inflammatory treatment into a combined therapy that accounts for multiple key players in the disease development to increase success of the treatment and obtain the maximal beneficial effect.
3.5 References


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Chapter 4: Negative Impact of Ketoprofen-lysine Treatment on Cerebral Pathology and Cognition in a Comorbid APP21 Transgenic Rat and Diet-induced Metabolic Syndrome

This chapter presents the analysis of a subset of animals from the previous investigation that received the prodrug that developed a large amount of neuroinflammation (Objective 3). The negative effects on neuropathology and behavior associated with the long-term prophylactic prodrug treatment in a subset of rats are described. Full description of the methodology of this study is presented in Chapter 3 and the current chapter includes only highlights and additional methods used for the extended pathological examination. This manuscript has been reviewed by all authors and is ready to be submitted for publication.

4.1 Introduction

Alzheimer's disease (AD), a common cause of dementia, is a progressive incurable neurodegenerative disease that leads to a severe mental health decline. Neuroinflammatory processes occupy an important place in the pathogenesis of AD, developing at the very early stages of the disease and contributing to the progression\(^1\)–\(^5\). Obesity and metabolic syndrome (MetS), whose development is greatly associated with the intake of high-fat, -sugar hypercaloric diet (HCD), represent risk factors for dementia including AD later in life. A chronic inflammatory state is also a characteristic feature of these disorders\(^6\)–\(^8\) and has been suggested to be a point of interaction between AD and metabolic diseases\(^9\)–\(^12\).

Epidemiological studies have shown a long-term treatment with non-steroidal anti-inflammatory drugs (NSAIDs) is associated with reduced risk of AD\(^14,15,24,25,16–23\). Particularly, this association is thought to be mediated through the inhibition of cyclooxygenase (COX) enzymes COX-1 and COX-2 and blockage of downstream pro-inflammatory mediators production\(^13\), which are involved in the inflammatory processes critical to the AD development and progression\(^1,26–29\). However, clinical trials on NSAIDs have produced controversial results\(^30\), leading to the need for further studies investigating treatment potential of this class of agents.
We previously conducted a 12-week study on the effects of diet-induced metabolic disturbances on neuroinflammation and cognition in a novel human APP21 transgenic (TG) rat model of early AD\textsuperscript{48–50}. The results demonstrated an intense white matter inflammatory response accompanied by a poor cognitive performance in the comorbid condition, suggesting inflammation might be one of the key players in early AD and highlighting a potential for an anti-inflammatory treatment strategy.

We subsequently conducted another study designed to examine the therapeutic effect of a novel prodrug ketoprofen-lysine (KL)\textsuperscript{51}, a conjugate of NSAID ketoprofen and amino acid lysine, specifically targeting the brain. This study examined the impact of KL on white matter microgliosis and cognition in the comorbid model of early stage AD and diet-induced MetS. We hypothesized that chronic prophylactic treatment with a novel KL prodrug which was initiated at the prodromal AD stage simultaneously with the dietary switch for MetS induction and therefore administered at a half-treatment for this drug class dose, will prevent increased white matter inflammation and the concomitant cognitive impairment in the comorbid AD and MetS. Long-term prophylactic treatment failed to stop accumulation of white matter microglia activation induced by comorbidity.

However, there was an additional phenomenon that needs to be reported since it has considerable implications for the potential use of brain targeted NSAIDs. In all groups receiving the prodrug there was a subset of rats that demonstrated a dramatic increase in pathologic neuroinflammation in the neocortex and subcortical structures accompanied by a negative impact on spatial working memory and cognitive flexibility.
4.2 Methods

The complete study design with detailed methods section is described elsewhere.

Male Fischer 344 wildtype (WT) and TG carrying human amyloid-β precursor protein gene (hAPP) with Swedish and Indiana mutations (APP21) rats 10-10.5 months of age were maintained on either a control diet (CD; Prolab RMH 3000 SP00) or the hypercaloric diet (HCD; D12079B, Research Diets, Inc) supplemented with 20% corn syrup water solution. A total of 21 WT and 18 APP21 TG rats received the KL prodrug in once daily subcutaneous injections in a dose of 4.1 mg/kg body weight, which corresponds to 2.5mg/kg of free ketoprofen, for a full duration of the study (15 weeks).

Tissue processing and immunohistochemistry were performed to visualize total, activated microglia and astrocytes as described previously. Additional staining was done to visualize neurons using mouse monoclonal primary antibody against neuronal nuclei protein (anti-NeuN; 1:1000; Millipore Corp; USA). Systolic and diastolic arterial blood pressure was measured using the non-invasive tail cuff method (CODA Blood Pressure System, Kent Scientific Corp., Connecticut, USA) at week 11 of the study. Spatial learning abilities, working memory and cognitive flexibility were evaluated using the Morris water maze task (MWM) at weeks 13-15 on the diet as described in the original study. Data is reported as mean with the standard error of the mean (SEM). Differences in the 2-way ANOVA followed by Tukey MC test or t-test were considered statistically significant where $p \leq 0.05$. 

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4.3 Results

In total there were 39 animals receiving the KL prodrug. These animals included 10 WT CD, 8 TG CD, 11 WT HCD and 10 TG HCD. The first indication that there may be a problem with the drug treatment was the death of two rats receiving the KL prodrug, one each from the WT CD and TG CD group. These animals were found dead in their home cages after one month of drug administration. Previously, in these MetS studies alone, we have used a total 126 rats in these types of experiments that do not involve any kind of surgery or invasive treatment and we have not had any animals succumb. Unfortunately, the brains were not saved for histological analysis due to undetermined time after spontaneous death.

4.3.1 Neuroinflammation

One of the hypotheses for the overall experiment was that there would be increased white matter inflammation in the TG rats on the HCD and that the KL prodrug would ameliorate this inflammation. When we performed immunohistochemical analysis of OX-6 activated microglia, it revealed the expected white matter inflammation in the comorbid AD and MetS animals, however there was also a widespread microgliosis in 6 animals on the KL prodrug (Figures 4-1,2). Areas of OX-6 microgliosis have also shown an increase in the Iba-1 reactivity of microglial cell population (Figures 4-1,2). These animals were not localized to any one group, but were in all 4 experimental groups irrespective of the presence of the transgene or the HCD (Figures 4-1,2).

The only common element is that all of these animals with extensive neuroinflammation were exclusively from animals receiving the KL prodrug and included both genotypes and dietary regimens: WT CD, n=1; TG CD, n=1; WT HCD, n=2; TG HCD, n=2. In all animals, there were highly activated OX-6 positive microglia cells clustered together in large areas spreading across both gray and white matter. Images of the activated microglia are shown for representative animals from each group in Figures 4-1,2. This was a striking finding since this type of neuroinflammation had never been observed in any (126 rats) of the previous animals in these studies. We particularly did not expect this in those that are part of the control groups. We previously observed only localized white matter inflammation increase in comorbid animals that were TG and on the HCD.
The areas of neuroinflammation included the neocortex, thalamus, hippocampus, striatum, amygdala, septal nuclei, hypothalamus, spreading into the adjacent white matter (Figures 4-1,2). Morphologically, the microglia cells exhibited enlarged irregular soma with shortened thickened processes and an increase in the number of processes (Figure 4-1).

These brains were also stained for GFAP. Analysis of the brain sections has shown an increase in GFAP-immunoreactivity of cells in the areas of microgliosis (Figures 4-1,2). These hypertrophic GFAP+ astrocytes exhibited thickening of their processes as well as an increase in the number and length of the processes (Figure 4-1).

Staining for NeuN was done to obtain an indication of the numbers of neurons in these areas (boxed fields on Figures 4-1,2). Neuronal density expressed as NeuN positive cell automated counts, indicated there was a consistent relative decrease in the numbers of neurons in the regions of microglial activation compared to corresponding areas of a control brain from a rat not receiving the drug (Figure 4-3).
Figure 4-1. Widespread microgliosis and reactive astrocytosis in Ketoprofen-lysine prodrug recipients in groups on the control diet. 2x photomicrographs of representative brain regions from coronal sections at the levels indicated by the Bregma (mm) with immunolabelled OX-6, Iba-1 activated microglia and GFAP astroglia. Scale bar 500µm. Boxed regions refer to areas selected for neuronal cell counting. Magnified images obtained at 10x magnification show morphology of microglia and astrocytes in the core of the affected region. Scale bar 100 µm. CC = corpus callosum, CD = control diet, fmiCC = forceps minor of corpus callosum, KL = ketoprofen-lysine prodrug, LH = left hemisphere, LV = lateral ventricle, M = motor cortex, RH = right hemisphere, TG = transgenic, WT = wildtype.
Figure 4-2. Widespread microgliosis and reactive astrocytosis in Ketoprofen-lysine prodrug recipients in groups on the hypercaloric diet. 2x photomicrographs of representative brain regions from coronal sections at the levels indicated by the Bregma (mm) with immunolabelled OX-6, Iba-1 activated microglia and GFAP astroglia. Scale bar 500µm. Boxed regions refer to areas selected for neuronal cell counting. 3V = 3rd ventricle, CD = control diet, IC = internal capsule, KL = ketoprofen-lysine prodrug, LH = left hemisphere, LV = lateral ventricle, RH = right hemisphere, TG = transgenic, WT = wildtype.
Figure 4-3. Neuronal density in areas of extensive neuroinflammation in Ketoprofen-lysine prodrug recipients. Neuronal counts (NeuN+ cells) in affected brain areas of representatives from WT CD, TG CD, WT HCD and TG HCD animals in comparison to control brain of untreated WT animal on CD. Amyg = amygdala, CD = control diet, CPu = caudate putamen, HCD = hypercaloric diet, KL = ketoprofen-lysine prodrug, Prim SS Cortex = primary somatosensory cortex, SeptNu = septal nuclei, TG = transgenic, WT = wildtype.
4.3.2 Behavioral deficits

To assess the impact of KL prodrug induced neuroinflammation on cognitive performance we performed an additional analysis of behavioural data dividing rats in two groups: a group that was not receiving the prodrug (ND, n=41) and group receiving the KL prodrug that exhibited widespread neuroinflammation (DI, n=6). Both of these groups contained rats from the combination of dietary protocols and genotypes.

The analysis of spatial learning in the MWM demonstrated that both ND and DI rats had significant improvement in the time to reach the hidden platform by the end of the training (Figure 4-4A), indicating that spatial learning ability was not compromised in the DI animals. In addition, these two groups were similar in motor ability since swim speed did not differ between groups in any of the probe trials (Figure 4-4B).

In the 3-day MWM shifting strategy protocol with the platform location changing every day the average time to find the hidden platform in the first trial of the day was not different between the groups. However, the time to recall the newly learned platform location and reach the platform in trial 2 was significantly longer in DI group compared to the ND group (p=0.027; Figure 4-4C). Thus, the improvement in the time on trial 2 compared to trial 1 was only significant in ND group indicating the KL prodrug reduced cognitive flexibility due to working memory impairments.

Furthermore, analysis of the performance in trials 2-4 showed that DI rats were significantly slower in learning the platform position within each day (p=0.02; Figure 4-4D). Finally, the latency to reach the platform in trial 5, after a one-hour break, was significantly longer in DI rats (Figure 4-4E), suggesting there are difficulties in retaining information during the break. Altogether, these results demonstrate negative effects of detrimental neuroinflammatory pathology on the spatial reference and working memory and cognitive flexibility of the rats.
Figure 4-4. Behavioral performance of Ketoprofen-lysine prodrug recipients with extensive neuroinflammation. Rats divided in two groups: those not receiving prodrug (ND) and those with prodrug and with extensive gray matter inflammation (DI). Each group combines both dietary protocols and genotypes. Spatial navigation MWM. A) Latency to the hidden platform as a measure of learning progress over 4 days of training. B) Swim speed during probe trials one week apart performed right after the spatial training. Shifting strategy MWM protocol for spatial working memory and cognitive flexibility assessment. C) Average time to find the platform in trials 1 and 2 of the day as working memory efficiency criteria. D) Average latency during trials 2-4 within the day as working memory and cognitive flexibility measure. E) Latency to reach the platform in trial 5, after a one-hour break in testing day, as a measure of spatial reference memory. Trial bar in C-E) represents an average of trial time across 3 days of shifting strategy MWM task. Values are presented as mean ± SEM. Significance is indicated by * between different time points within the group and by # - between groups. t-test, 2-way ANOVA Tukey post-hoc test (in A), p≤0.05. CD = control diet, HC = hypercaloric diet, KL = ketoprofen-lysine prodrug, MWM = Morris water maze, TG = transgenic, WT = wildtype.
4.3.3 Blood pressure

Systolic and diastolic arterial blood pressure levels assessed at week 11 of diets were not different between the rats on the prodrug treatment that developed extensive neuroinflammation and non-treated rats, DI and ND experimental groups respectively (Figure 4-5).
Figure 4-5. Systolic and diastolic arterial blood pressure of Ketoprofen-lysine prodrug recipients with extensive neuroinflammation. Rats divided in two groups: those not receiving prodrug (ND) and those with prodrug and extensive inflammation (DI). Each group includes those rats both dietary protocols and genotypes. Values are presented as mean ± SEM.
4.4 Discussion

The original study aimed to investigate therapeutic effect of a novel non-steroidal anti-inflammatory KL prodrug to treat the negative effects of the combination of early AD-like pathology and a high fat, high sugar hypercaloric diet. In two separate studies this combination has been demonstrated to induce localized white matter inflammation and cognitive deficits. The investigation that included the impact of the KL prodrug on the activated microglia in the white matter and the behavior indicated no significant reduction in the white matter pathology or the cognitive deficits. However, of the 39 animals receiving the KL prodrug 2 died unexpectedly, and 6 developed widespread neuroinflammation and neuronal degeneration, irrespective of the experimental group. This widespread pathology also impacted behavioral performance when compared to untreated rats. Although this phenomenon was observed in a relatively small number or animals (total of 8 out of 39), the magnitude of the pathology and that fact this did not show up in any other animals (41 rats) not receiving the KL prodrug in this study or a previous study, such a pronounced deleterious effect that appears to be KL prodrug dependent, is important to report in light of the clinical push to use NSAIDs to treat early symptoms of AD.

The rats receiving the KL prodrug developed massive cerebral inflammation, including both microgliosis and astrogliosis that affected large regions of the cerebral cortex, basal ganglia, thalamus, hippocampus, hypothalamus and adjacent white matter. Analysis of cells positive for a neuronal marker showed neuronal loss in areas corresponding to the regions of neuroinflammation. Treatment has also appeared to lead to a lethal outcome in two cases after one month of KL prodrug treatment.

Spatial learning tested in the MWM navigation task, in which the hidden platform remained in the same position over 4 days, remained intact. However, the MWM shifting strategy, or cognitive flexibility task, adapted for use in this study to assess the executive function components, in which the platform was moved to a novel position for each of the 3 testing days, revealed significant deficits in spatial working memory and challenged learning ability under the novel task rules and changing environment indicating a cognitive inflexibility in these rats.

To our knowledge such negative effects of NSAIDs have not been reported by other animal studies in the literature to date. However, there are clinical reports with evidence of adverse cardiovascular
and cerebrovascular effects using this group of drugs\textsuperscript{57–65}. These effects include fatal and non-fatal heart failure, myocardial infarction, ischemic and hemorrhagic stroke, and transitory ischemic attack. An increased risk for serious vascular complications with the NSAIDs treatment has been registered for the general population\textsuperscript{63,66} and specifically for NSAID use in osteoarthritis\textsuperscript{65} and rheumatoid arthritis\textsuperscript{62} patient cohorts as well as individuals with a family history of AD\textsuperscript{63}. The adverse effects depended on dose and duration factors and were especially worse for long treatments at high doses.

Potential underlying mechanisms leading to the development of the adverse effects with NSAID use include vascular damage including due to oxidative stress, a shift towards a prothrombotic state due to lack of or insufficient blockage of COX-1 dependent platelet aggregation and elevation of blood pressure that might result into cerebrovascular event\textsuperscript{42,58,59,62}. There are 3 characteristics of ketoprofen that suggest the treatment agent in our study might have a prothrombotic effect and result in the adverse cardio- and cerebrovascular events. First, ketoprofen exerts a reversible inhibition of COX-1 pathway compared to other NSAIDs that have a more permanent COX-1 inhibition\textsuperscript{67}. Secondly, it has a relatively weak affinity for COX-2 expression\textsuperscript{59,64}. COX-2 is increased in the vasculature and is associated with vasoconstriction in an inflammatory environment such as that seen in metabolic disorders\textsuperscript{68}. Thirdly, ketoprofen has a half-life less than 12h\textsuperscript{69,70}. The treatment protocol of a once daily injection that we used and this dosage chosen could produce insufficient antithrombotic action in the long term.

Ketoprofen itself may have a tendency for a higher degree of adverse effects. Blood pressure levels measured via tail-cuff method were not different in the KL treated rats and does not appear to add to the pathological events observed in the present study. However, aging, AD and MetS are associated with the vascular pathology and it is, therefore, possible that cerebrovascular derangements are present in our model and the actions of the KL prodrug could intersect with or even further aggravate preexisting pathologies, resulting in cerebral insults seen in our animals. Further investigation of the vascular status in our model would be needed to make conclusions on this assumption.

In light of this, of great importance is a question of the safety of these agents in a population with concurrent chronic diseases such as diabetes and MetS as studied in our model. These are
individuals who are in a high-risk group for vascular complications and may already have cardiovascular diagnoses and/or cerebrovascular history. Presence of these conditions can potentially increase the risk of these side effects even more, leading to recurrent cardio- or cerebrovascular insults\textsuperscript{57,71}.

What is most critical about the KL prodrug we used in this investigation is the alteration in the molecular structure, adding the amino acid lysine, to enhance uptake by the brain via LAT1-mediated transport. Although this may seem like a logical approach to generate more efficacious anti-inflammatory agents to combat the earliest neuroinflammatory pathological events, it may, in fact be very counterproductive if other NSAIDs manipulated in a similar manner to ketoprofen also generate this type of brain pathology. The toxicity could result from particular NSAIDs itself, i.e. ketoprofen in our study, or could be attributed specifically to the lysine addition. Additional experimental studies need to be done to understand the clinical risks associated with the use of NSAIDs for the treatment of mild cognitive impairment and the slowing or prevention of dementia.

One of the limitations to this study is that there were no vehicle control groups in the current experimentation. Our primary goal was to assess whether the novel prodrug could reduce the white matter inflammation in our comorbid model. However, saline control experiment should be carried out to examine possible effects of chronic injection stress and their contribution to the neuropathology and behavioral performance.
4.5 References


Chapter 5: Relationship between White Matter Inflammation and Metabolic and Physiological Parameters in a Comorbid APP21 Rat Model of Prodromal Alzheimer’s Disease and Metabolic Syndrome

This chapter describes the relationships between the white matter inflammation and systemic metabolic and physiological parameters using the combined data from the two studies described in the Chapters 2 and 3 of this thesis. The relationships between behavioral performance and white matter inflammation are also described. The methods section of this chapter contains only highlights of the tests used for metabolic and behavioral measurements described in detail in the previous chapters. This aim of this study is to identify potential biomarkers of white matter inflammation (Objective 4). This manuscript has not yet been submitted for publication.

5.1 Introduction

Dementia represents one of the biggest global public health challenges these days. Alzheimer's disease (AD) is the most commonly diagnosed type of dementia and is a progressive, irreversible, and currently, incurable disease\(^1\). Extensive search for the treatment of AD has not yet yielded the desired results and research from both pre-clinical and clinical fields is continued. In addition to the search for treatment of AD, there has been an increase in studies examining biomarkers of the disease that would aid in early identification of the disease pathology\(^2\)–\(^5\). Timely and accurate diagnosis of AD is essential for early intervention and better prognosis and remains a great challenge and most likely one of the major contributors accounting for the failure of clinical trials for potential treatments. However, identified biomarkers have a limited use in the clinical examination yet and bear significance primarily for research purposes\(^6\)–\(^9\). Current biomarkers proposed for the diagnosis of AD include changes in brain inflammatory cells, changes in perfusion and metabolism on positron emission tomography, structural changes using MRI, changes in cytokine, tau and amyloid protein levels in blood and cerebrospinal fluid\(^2\)\(^,\)\(^10\)–\(^12\). Most of these parameters are not easily obtained and the techniques are not widely available in hospitals and clinics. In addition, they are expensive and require complex technical set up.
Markers of neuroinflammation, or the inflammatory process in the brain, are of special interest. Neuroinflammation, mainly regulated by microglia and astrocytes, is one of the features of pathological processes that occur in metabolic disorders in the elderly, including obesity and metabolic syndrome (MetS)\(^{13,14}\). Neuroinflammation is also a common process in neurodegenerative diseases, including AD in which it is thought to be implicated at early stages of the development and progression of the disease\(^ {15-18}\).

White matter pathology, including inflammatory changes, appears to be a promising candidate for early treatment and prevention. White matter abnormalities are often visualized as hyperintensities (WMH) on MRI scans in individuals from the elderly population and are thought to be characterized by excessive neuroinflammation and glial activation as well as by loss of myelin and axonal fibers, however, the particular pathology and chronological events might differ with the specific disease\(^ {19-21}\). These abnormalities increase with aging, are often present in the prodromal phase of AD\(^ {22,23}\), mild cognitive impairment (MCI)\(^ {24}\) and in patients with MetS\(^ {25}\), and have been shown to highly correlate with cognitive decline and, moreover, occur well before symptoms of cognitive deterioration become evident\(^ {22,26}\).

Potential biomarkers of these WMH include increased blood insulin levels and hypertension, which represent individual components of MetS\(^ {27}\). Blood IL-8 levels were found to be associated with the WMH particularly in AD pointing to a potential inflammatory basis of this pathology\(^ {28}\).

The presence of WMH, however, might indicate more advanced changes to the white matter, thus, investigation into the early pathological events, such as white matter inflammation, and their markers is needed.

Neuroinflammation, as a feature of both AD and MetS, and represents a risk factor for MCI and AD, and also appears to be a shared pathology between these disorders. AD and MetS are often coexisting in human population and neuroinflammation is suggested to be a point of their interaction\(^ {29,30}\). This interaction between pre-AD and MetS was recently demonstrated in our study of a novel human amyloid precursor protein (hAPP) transgenic (TG) rat model of early AD (APP21) and a hypercaloric diet (HCD) induced obesity and MetS. The combination of prodromal AD and MetS resulted in a larger increase in neuroinflammation, specifically in the white matter throughout the entire brain primarily driven by OX-6+ activated microglia of the comorbid animals.
compared to either condition alone. This increase in white matter neuroinflammation was accompanied by spatial reference memory impairment in the comorbid group compared to the control animals and occurred in the absence of neuronal loss in the hippocampus suggesting that white matter neuroinflammation in particular could be one of the early brain pathologies contributing to cognitive deficits observed in MCI and dementia, including AD cases.

The results summarized above suggest the importance of finding potential biomarkers of the neuroinflammatory process, including those specific to the white matter, to identify a population at risk for dementia and to be able to intervene and prevent further development of cognitive decline. Thus, an examination of the relationship between changes in metabolism and/or physiological markers associated with MetS and early signs of white matter inflammation in a prodromal AD model could be an important first step in developing an understanding of potential biomarkers for AD pathology at specific stages.

In this study, we performed an analysis of the relationships between systemic blood based metabolic changes and physiological parameters with activation markers of the key brain inflammatory cells, microglia and astrocytes, in the white matter. This would enable the identification of potential measurable indicators, biomarkers or precursors to brain pathogenic white matter inflammatory processes. Using the measures collected from our pre-clinical model, we focused on those which are easy to obtain in the clinical setting. We also analyzed the relationship between behavioral performance and white matter inflammation.
5.2 Methods

5.2.1 Animals and Diets

All animal handling and experimental procedures were approved by Western University Animal Care Committee (AUP 2008-113, 2014-016) and were carried out in accordance with the guidelines of the Canadian Council on Animal Care and National Institute of Health Guides for the Care and Use of Laboratory Animals.

These results were drawn from 2 cohorts of animals. The first comprised a total of 24 male wildtype (WT) and 22 APP21 TG Fischer 344 rats 8.5-9 months old at the start of experiment involved in a study of the characterization of the comorbid model of pre-AD and MetS. Additional animals were included in the analysis from a second study investigating the therapeutic effect of the anti-inflammatory prodrug using the same model of Mets and pre-AD and involved a total of 15 male WT and 11 TG rats 10-10.5 months of age. Rats were bred in house with original breeding pairs obtained from Drs. Yuksel Agca and Cansu Agca (University of Missouri, Colombia, MO, USA) and confirmed to be homozygous \(^{31,32}\). Animals were housed in pairs under standard conditions (12:12 light/dark cycle, at 22-24°C). A subset of rats of each genotype was randomly assigned to a high-calorie Western type diet (D12079B, Research Diets, Inc) supplemented with 20% corn syrup drink (HCD), while the other half continued on a standard diet (control diet, CD; Prolab RMH 3000 5P00). Diets were provided \textit{ad libitum} and rats were maintained on the diets for 12 - 15 weeks.

For this correlational analysis, rats from both studies were combined for the same parameter with an exception of glucose and insulin metabolism measures with the following numbers for each experimental group: WT CD n=21, TG CD n=16, WT HCD n=18, TG HCD n=17.

5.2.2 Physiological and Metabolic Measurements

Detailed methodology can be found in the original studies. Briefly, the body weight at the end of the experiment was recorded and epididymal fat pads were collected and weighed at the time of euthanasia. The latter was used as a measure of visceral adiposity. Glucose metabolism was
assessed using the glucose tolerance test (GTT) at 11-12 week following the change in diets, with either the intraperitoneal (Ip; IpGTT) or intravenous (Iv; IvGTT) version of the test. Fasting serum insulin levels were determined and the homeostasis model assessment index (HOMA-IR) of insulin resistance was calculated for a subset of rats. Fasting serum lipid profile analysis included measurements of triglycerides, total cholesterol, calculations of non-high density lipoprotein (non-HDL) cholesterol and cholesterol ratio (Chol:HDL ratio; total cholesterol/ HDL cholesterol) which is an “atherogenic” index used clinically to assess cardiovascular risks.

5.2.3 Behavioral Testing

The Morris Water Maze test (MWM), in which rats were trained over 4 days to find a hidden escape platform in a circular pool using extra-maze cues and then tested in a probe trial with no platform, was used to assess spatial navigation and reference memory. In the first study, rats were exposed to initial training before the dietary assignment, then underwent re-learning of the novel platform location at the end of experiment with a probe trial following a day after. In the second study rats received training only at the end of dietary treatment which was again followed by a probe trial. Spatial reference memory was evaluated during the probe trial using time and distance spent searching for a platform in the target zone where it used to be located expressed as a percentage of the total probe swim. Rats from the second study were then challenged with a modified 3-day shifting strategy task in which platform location was changed every other day and spatial working memory (cognitive flexibility) was assessed using differences in time and distance (i.e. path length) to reach the platform in trials 1 and 2 of the task.

5.2.4 Immunohistochemistry

Following a 12-h fasting period all the rats were euthanized by a pentobarbital overdose and sequentially perfused with phosphate buffered saline and 4% paraformaldehyde (PFA). Coronal sections of 30-35 μm thickness were immunohistochemically processed to visualize activated microglia and astrocytes using monoclonal antibodies directed against the MHC II receptor (OX-6; 1:1000; BD Pharmingen, Mississauga ON, Canada) and glial acidic fibrillary protein (anti-
GFAP; 1:2000; Sigma-Aldrich, St Louis MO, USA), respectively. Areas of interest included the entire body (BCC) and periventricular regions (PVCC) of the corpus callosum as well as the internal capsule (IC) in both hemispheres. Area coverage by a positive signal (area%) was measured for each region in the Image J software and expressed as a weighted average. Detailed protocols are described elsewhere.

5.2.5 Data Analysis

Statistical analysis was performed using IBM SPSS software. Statistical analysis was done on the entire set of data including all rats, on the data sets separated based on either genotype or diet and on individual experimental groups. Data for each measured parameter was first screened for outliers using 2SD±mean rule. All measures were checked for normality using the Shapiro-Wilk test. For the interregional white matter inflammation correlation, a partial correlation analysis was performed on the entire dataset controlling for diet and genotype. Statistical significance was set at a p value of ≤ 0.05.

Firstly, correlations were computed for each behavioral outcome and white matter inflammatory marker to assess the relationship between the cognitive performance and cerebral pathology. Bivariate correlation matrix was created next to define potential predictors (independent variables) of the white matter inflammatory cellular pathology (dependent variable) from the measured peripheral outcomes to be included in the regression model. Independent variables that were making significant contributions to the explanation of the variance of the dependent variable were selected based on the correlation coefficient significance. Individual linear regressions were performed with each independent variable to model its relationship with dependent variable, the white matter inflammatory parameter. Single input models were compared between each other to identify a better model. Multiple linear regression analysis was next performed with low or non-correlated independent variables to establish whether any model including more than one independent variable explains the variance of the dependent variable better and improves over models with a single entered component. Assumptions for linear regression were checked for each model: linearity of relationship between independent variable and dependent variable using scatterplots; independence, normality of distribution, linearity and homoscedasticity of residuals
using normal probability plot, standardized predicted values over standardized residuals scatterplot, Durbin-Watson test; multicollinearity using VIF, tolerance values, coefficients from bivariate correlation matrix. Models were compared by number of parameters including F statistics of overall significance of the model (p value), standard error estimate, t value, standardized coefficients (β) and adjusted R².
5.3 Results

5.3.1 White matter inflammation relationships

Microglia activation throughout the brain of individual rats assessed using OX-6 immunomarker was highly positively correlated across examined white matter regions: BCC and PVCC Spearman’s rho=0.982, p<0.0001; BCC and IC rho=0.813, p<0.0001; IC and PVCC rho=0.810, p<0.0001. This relationship held true while controlling for genotype and diet (Table 5-1). In the individual groups, the BCC region activated microglia coverage was highly correlated with PVCC (r>0.9, p<0.0001) and IC (r>0.78) for all groups except WT CD, that did not show any significant relationship with the IC region inflammation. All groups with an exception of control rats on the CD had a PVCC-IC significant positive correlation (r>0.65, p<0.009).

Astrocyte reactivity in the BCC measured by GFAP(+) signal area coverage (%) of the region positively correlated with the OX-6 activated microglia coverage in the BCC in the entire dataset (rho=0.36, p=0.004). When genotype and diet were entered as controlling factors, the correlation lost significance. Thus, this relationship was due to both inflammation makers correlated with the particular genotype and diet. Individual experimental group analysis revealed that the correlation between these two inflammatory markers in the BCC region was significant only in the comorbid, TG HCD, group (r=0.517, p=0.04).
Table 5-1. Correlation of microglia activation in the cerebral white matter

<table>
<thead>
<tr>
<th></th>
<th>BCC</th>
<th>PVCC</th>
<th>Internal capsule</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCC</td>
<td>-</td>
<td>0.974</td>
<td>0.835</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&lt;1.2e-38</td>
<td>3.067e-018</td>
</tr>
<tr>
<td>PVCC</td>
<td></td>
<td>-</td>
<td>0.819</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.064e-016</td>
<td></td>
</tr>
</tbody>
</table>

Strong positive correlation suggests that activation of white matter microglia increased globally throughout individual rat brains for both wildtype and transgenic rats on either control or hypercaloric diet. Pearson’s coefficient (r) and corresponding p values are shown for correlations of OX-6+ microglia (area%) between ROIs. Partial correlation was performed controlling for genotype and diet; n=66. BCC – corpus callosum body, PVCC – corpus callosum periventricular sub-regions, ROI – region of interest.
5.3.2 Behavioral performance and white matter inflammation relationships

Linear regression analysis was performed to test whether the spatial reference memory probed in the MWM at the end of study in each of the protocols was related to the white matter microglial activation level. The relationships with the target zone swim parameters were analyzed. The combined dataset showed a negative relationship between OX-6 microglia coverage in IC and the distance travelled in the target zone out of the total probe swim path (\( r=-0.368, \ p=0.015, \ F_{(1,42)}=6.427, \ R^2=0.114 \)) in the re-learning paradigm. This suggests a better behavioral performance (i.e. better spatial memory), as represented by the greater distance travelled searching in the target zone of the pool, is associated with a lower white matter microglial activation in the IC region. This relationship was not observed within either individual genotype subsets or individual experimental groups, however it appeared in the HCD subset and maintained the directionality and strength of significance (\( r=-0.53, \ F_{(1,19)}=7.427, \ p=0.013, \ R^2=0.281 \)). Astrocyte reactivity in the white matter was not associated with any parameter of the cognitive function.

Analysis of the groups also indicated specific relationships between behavior and neuroinflammation. Time spent searching in the target zone, expressed as a percentage of the total probe trial time, was strongly inversely correlated with microglial activation in the BCC for TG HCD. This suggests there is a poorer cognitive performance on the test with increase in microglial activation in the BCC (Table 5-2). This was observed in the second study subgroup (\( n=6 \)) of rats for which this was a first ever encounter with the maze. In this rat group, a significant negative linear relationship was also found between OX6-positive microglia area coverage in the BCC and shifting strategy task performance. Thus, comorbid rats tended to have a smaller intertrial difference in the swim path, a smaller improvement, that suggested they were less cognitively flexible, when the activation level of the microglia in the BCC was higher (Table 5-2). In contrast to these results, TG CD rats (\( n=4 \)) showed a very strong positive correlation (\( r=0.96, \ F_{(1,3)}=35.193, \ p=0.01, \ R^2=0.895 \)), suggesting a greater performance associated with the greater inflammation in the white matter of the corpus callosum.
Table 5-2. Relationship of the behavioral performance in the MWM of TG HCD rats with OX-6 microglial activation in the BCC

<table>
<thead>
<tr>
<th></th>
<th>Pearson’s r</th>
<th>Adjusted R²</th>
<th>p</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>MWM shifting Δ distance*</td>
<td>-0.836</td>
<td>0.624</td>
<td>0.038</td>
<td>9.315</td>
</tr>
<tr>
<td>MWM target zone time (%.s)*</td>
<td>-0.897</td>
<td>0.756</td>
<td>0.015</td>
<td>16.467</td>
</tr>
</tbody>
</table>

MWM – Morris water maze; TG HCD – transgenic rats on the hypercaloric diet (comorbid group); * results of the second study only (n=6); shifting Δ distance – 3- day average difference in distance (path length) to reach the hidden platform between trials 2 and 1 with a greater value indicating a better cognitive performance.
5.3.3 Metabolic, physiological parameters and white matter microgliosis relationships

Correlations and linearity of the relationship of OX-6 positive activated microglia coverage in each of the three different white matter regions (BCC, PVCC, IC) were done individually for each measurement and included an examination of the whole dataset as well as genotype, diet and specific group analyses (Table 5-3).
### Table 5-3. Metabolic measures and white matter microgliosis correlations

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Diet</th>
<th>Body weight</th>
<th>Visceral Fat</th>
<th>Total Cholesterol</th>
<th>Triglycerides</th>
<th>Non-HDL Cholesterol</th>
<th>Chol:HDL</th>
<th>AUC GTT</th>
<th>Fasting Insulin; HOMA-IR</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Entire Dataset</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>BCC</strong></td>
<td>( \beta = 0.513 ) &amp; ( F_{(1,64)} = 22.839 ) &amp; ( R^2 = 0.263 ) &amp; ( \beta = 0.341 ) &amp; ( F_{(1,64)} = 5.711 ) &amp; ( p &lt; 0.001 ) &amp; ( \beta = 0.376 ) &amp; ( F_{(1,64)} = 10.558 ) &amp; ( p = 0.005 ) &amp; ( \beta = 0.494 ) &amp; ( F_{(1,64)} = 22.701 ) &amp; ( p &lt; 0.0001 ) &amp; ( \beta = 0.293 ) &amp; ( F_{(1,64)} = 5.824 ) &amp; ( p = 0.019 )</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>PVCC</strong></td>
<td>( \beta = 0.543 ) &amp; ( F_{(1,64)} = 26.695 ) &amp; ( R^2 = 0.294 ) &amp; ( \beta = 0.363 ) &amp; ( F_{(1,64)} = 9.708 ) &amp; ( p &lt; 0.001 ) &amp; ( \beta = 0.398 ) &amp; ( F_{(1,64)} = 12.045 ) &amp; ( p = 0.003 ) &amp; ( \beta = 0.516 ) &amp; ( F_{(1,64)} = 23.184 ) &amp; ( p &lt; 0.0001 ) &amp; ( \beta = 0.334 ) &amp; ( F_{(1,64)} = 7.803 ) &amp; ( p = 0.007 ) &amp; ( \beta = 0.31 )</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>IC</strong></td>
<td>( \beta = 0.352 ) &amp; ( F_{(1,63)} = 34.227 ) &amp; ( R^2 = 0.352 ) &amp; ( \beta = 0.04 ) &amp; ( F_{(1,63)} = 8.38 ) &amp; ( p &lt; 0.001 ) &amp; ( \beta = 0.436 ) &amp; ( F_{(1,63)} = 14.78 ) &amp; ( p = 0.005 ) &amp; ( \beta = 0.282 ) &amp; ( F_{(1,63)} = 7.803 ) &amp; ( p = 0.007 ) &amp; ( \beta = 0.256 )</td>
<td></td>
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</tr>
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</table>

| **Presence of Transgene (TG rat dataset)** | | | | | | | | | |
| **BCC** | \( \beta = 0.396 \) & \( F_{(1,30)} = 5.58 \) & \( R^2 = 0.129 \) & \( \beta = 0.482 \) & \( F_{(1,30)} = 9.072 \) & \( p = 0.025 \) & \( \beta = 0.513 \) & \( F_{(1,30)} = 11.937 \) & \( p = 0.008 \) & \( \beta = 0.431 \) & \( F_{(1,30)} = 9.072 \) | |
| **PVCC** | \( \beta = 0.463 \) & \( F_{(1,30)} = 8.166 \) & \( R^2 = 0.188 \) & \( \beta = 0.534 \) & \( F_{(1,30)} = 11.937 \) & \( p = 0.008 \) & \( \beta = 0.534 \) & \( F_{(1,30)} = 11.937 \) & \( p = 0.002 \) & \( \beta = 0.431 \) & \( F_{(1,30)} = 9.072 \) | |
| **IC** | | | | \( \beta = 0.37 \) & \( F_{(1,29)} = 4.59 \) & \( p = 0.041 \) & \( \beta = 0.37 \) & \( F_{(1,29)} = 4.59 \) & \( p = 0.041 \) & \( \beta = 0.37 \) & \( F_{(1,29)} = 4.59 \) | |

<p>| <strong>HCD Dataset</strong> | | | | | | | | | |
| <strong>BCC</strong> | ( \beta = 0.547 ) &amp; ( F_{(1,31)} = 13.255 ) &amp; ( R^2 = 0.188 ) &amp; ( \beta = 0.109 ) &amp; ( F_{(1,31)} = 6.038 ) &amp; ( \beta = 0.109 ) &amp; ( F_{(1,31)} = 6.038 ) &amp; ( \beta = 0.109 ) &amp; ( F_{(1,31)} = 6.038 ) | ( (Iv) \beta = 0.802 ) &amp; ( F_{(1,19)} = 16.192 ) |</p>
<table>
<thead>
<tr>
<th>Genotype</th>
<th>Diet</th>
<th>Body weight</th>
<th>Visceral Fat</th>
<th>Total Cholesterol</th>
<th>Triglycerides</th>
<th>Non-HDL Cholesterol</th>
<th>Chol:HDL</th>
<th>AUC GTT</th>
<th>Fasting Insulin; HOMA-IR</th>
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</thead>
<tbody>
<tr>
<td>PVCC</td>
<td>β=0.611</td>
<td>β=0.506</td>
<td>β=0.506</td>
<td>β=0.64</td>
<td>β=0.64</td>
<td>β=0.64</td>
<td>β=0.64</td>
<td>(Ip) β= -0.442</td>
<td></td>
</tr>
<tr>
<td></td>
<td>F(1,32)=19.06</td>
<td>F(1,32)=11.038</td>
<td>F(1,32)=11.038</td>
<td>F(1,32)=9.015</td>
<td>F(1,32)=9.015</td>
<td>F(1,32)=9.015</td>
<td>F(1,32)=9.015</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>p=0.008</td>
<td>p=0.002</td>
<td>p=0.002</td>
<td>p=0.01</td>
<td>p=0.01</td>
<td>p=0.008</td>
<td>p=0.008</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>R²=0.373</td>
<td>R²=0.233</td>
<td>R²=0.233</td>
<td>R²=0.364</td>
<td>R²=0.364</td>
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**Co-morbid TG HCD Dataset**

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<th>Total Cholesterol</th>
<th>Triglycerides</th>
<th>Non-HDL Cholesterol</th>
<th>Chol:HDL</th>
<th>AUC GTT</th>
<th>Fasting Insulin; HOMA-IR</th>
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191
### TG CD Dataset

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<th>Triglycerides</th>
<th>Non-HDL Cholesterol</th>
<th>Chol:HDL</th>
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<td>(Iv) $\beta = -0.619$</td>
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<td>$R^2=0.306$</td>
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|          |      |             |              |                   |               |                      |          |        | $\beta = -0.814$ |
|          |      |             |              |                   |               |                      |          |        | $F_{(1,3)}=15.722$ |
|          |      |             |              |                   |               |                      |          |        | $p=0.004$                     |
|          |      |             |              |                   |               |                      |          |        | $R^2 = 0.621$               |

### WT rat Dataset

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<th>Visceral Fat</th>
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<th>AUC GTT</th>
<th>Fasting Insulin; HOMA-IR</th>
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<tr>
<td>BCC</td>
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<td>$\beta=0.516$</td>
<td>$\beta=0.561$</td>
<td>$\beta=0.425$</td>
<td>$\beta=0.439$</td>
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<td>$R^2=0.292$</td>
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<td>$R^2=0.284$</td>
<td>$R^2=0.244$</td>
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</table>

| PVCC     | $\beta=0.517$ | $\beta=0.516$ | $\beta=0.561$ | $\beta=0.425$ | $\beta=0.439$ | $\beta=0.544$ |                |
|          | $F_{(1,30)}=10.922$ | $F_{(1,30)}=8.955$ | $F_{(1,30)}=11.279$ | $F_{(1,30)}=5.548$ | $F_{(1,30)}=6.816$ | $F_{(1,30)}=12.587$ |                |
|          | $p=0.002$ | $p=0.005$ | $p=0.002$ | $p=0.025$ | $p=0.014$ | $p=0.001$ |                |
|          | $R^2=0.242$ | $R^2=0.204$ | $R^2=0.249$ | $R^2=0.128$ | $R^2=0.158$ | $R^2=0.272$ |                |

| IC       | $\beta=0.585$ | $\beta=0.493$ | $\beta=0.502$ | $\beta=0.439$ | $\beta=0.377$ | $\beta=0.382$ |                |
|          | $F_{(1,30)}=15.648$ | $F_{(1,30)}=9.612$ | $F_{(1,30)}=10.104$ | $F_{(1,30)}=7.147$ | $F_{(1,30)}=4.974$ | $F_{(1,30)}=5.122$ |                |
|          | $p<0.0001$ | $p=0.004$ | $p=0.003$ | $p=0.012$ | $p=0.033$ | $p=0.031$ |                |
|          | $R^2=0.321$ | $R^2=0.243$ | $R^2=0.227$ | $R^2=0.165$ | $R^2=0.114$ | $R^2=0.117$ |                |

Only significant correlations at a significance level of <0.05 are shown for OX-6+ activated microglia (area% coverage of the white matter region) in the corpus callosum (BCC, PVCC) and IC. Transgene in this context is human amyloid precursor protein with Swedish and Indiana mutations. $\beta$ – standardized coefficient (Pearson’s R), $R^2$ – adjusted $R^2$. BCC – corpus callosum body, CD – control diet, PVCC – corpus callosum periventricular sub-regions, HCD – hypercaloric diet, HOMA-IR – homeostasis model assessment index of insulin resistance, IC – internal capsule, Ip – intraperitoneal glucose tolerance test, Iv – intravenous glucose tolerance test, TG – transgenic rat, WT – wildtype.
5.3.3.1 Corpus callosum

When the entire dataset was used, there were several factors that were significant predictors of the BCC microglial activation. These included genotype (β=0.513, $R^2=0.263$); visceral fat accumulation (β=0.494, $R^2=0.128$; 5-1A); and diet (β=0.341, $R^2=0.102$). Final body weight was a less strong predictor (β=0.376, $R^2=0.244$). Total cholesterol levels had a weak, yet significant positive association and the lowest prediction power (β=0.293, $R^2=0.071$). When visceral fat mass and genotype were considered together, both genotype and visceral fat were significant predictors of microglial activation in the BCC (β=0.401 fat and β=0.425 genotype) and together they strengthened the regression model ($F_{(2,62)}=22.452$, $p<0.0001$, $R^2=0.398$).

The data set was then split in two groups based the presence of transgene (WT or TG) for one set and based on the diet (CD and HCD) for another analysis. The regression analysis indicated visceral fat accumulation was the strongest significant predictor in both genotypes (Figure 5-1B) and correlated in a positive manner in the HCD rat subset (Figure 5-1C). Genotype remained a significant predictor of BCC neuroinflammation in both dietary datasets. Additionally, weaker correlations that were also significant were detected for body weight, non-HDL cholesterol, triglycerides and for the GTT, including area under the curve (AUC) for glucose levels using the IpGTT in the WT subset and AUC using the IvGTT method in the HCD group.

Thus, genotype was strongly associated with the microglia activation in the BCC in every dataset. Among the measured parameters, visceral fat mass was associated with a greater BCC microglia activation irrespective of the genotypic characteristics, however was predictive of it only in the HCD environment.

Analysis of the entire dataset indicated significant predictors of the PVCC microglial activation included genotype (β=0.543, $R^2=0.294$); visceral fat mass (β=0.516, $R^2=0.266$; Figure 5-1D); body weight (β=0.398, $R^2=0.158$); and diet (β=0.363, $R^2=0.132$). Weaker, but significant, relationships were also seen with total cholesterol and non-HDL cholesterol levels. When visceral fat mass and genotype were both included in the regression model, it improved and both genotype and fat were still significant individual predictors (β=0.417 fat and β=0.451 genotype, $F_{(2,62)}=26.787$, $p<0.0001$, $R^2=0.442$).
In the WT data sample, cholesterol ratio, visceral fat (Figure 5-1E), body weight, diet as well as non-HDL cholesterol and triglycerides each had a significant contribution to the variance of the microglial neuroinflammation. The strongest association with microglial activation was with the Chol:HDL ratio, which had a better prediction capacity ($\beta=0.544$, $R^2=0.272$) than body fat ($\beta=0.523$, $R^2=0.249$). The strongest relationship with the OX-6 microglial activation in the PVCC region in the TG rat sample was found with the body fat mass ($\beta=0.534$, $R^2=0.261$; Figure 5-1E). In both HCD and CD environments genotype was the strongest factor significantly related to the inflammation in the PVCC. In addition, the amount of visceral fat was the next strong predictor after the genotype in the HCD subset mass ($\beta=0.506$, $R^2=0.233$; Figure 5-1F), followed by the AUC in the IpGTT.

5.3.3.2 Internal capsule

Microglia activation in the IC region was associated with the genotype as the strongest factor for the entire rat dataset ($\beta=0.352$, $R^2=0.352$) as well as both dietary subgroups (CD $\beta=0.681$, $F_{(1,31)}=24.433$, $p<0.0001$, $R^2=0.463$; HCD $\beta=0.588$, $R^2=0.325$). For metabolic measures visceral fat showed the strongest relationship with neuroinflammation in the entire dataset ($\beta=0.436$, $R^2=0.19$; Figure 5-1G) and HCD subset ($\beta=0.105$, $R^2=0.233$; Figure 5-1I). Body weight and diet showed much weaker, yet significant correlation in the whole sample regression analysis. Total cholesterol and non-HDL fraction were also positively but weakly correlated to the inflammation.

Multiple regression model which included both genotype and fat mass showed an increased precision with both variables contributing significantly to the variation in the inflammation ($R^2=0.45$; $\beta=0.321$ fat and 0.523 genotype, $F_{(2,62)}=25.383$, $p<0.0001$). Visceral fat was significantly correlated with the IC activated microglial coverage in both genotypes with a stronger relationship of the fat amount in WT rats (Figure 5-1F). Additional correlations indicated that diet was the strongest predictor ($\beta=0.585$, $R^2=0.32$), while Chol:HDL ratio, body weight, non-HDL cholesterol and triglycerides which were the weakest and poorly explained the variance of inflammatory outcome in the WT rat subset. In the HCD subset the measure of AUC in IvGTT or AUC in IpGTT were identified as having a negative correlation with IC microgliosis (AUC IvGTT, $\beta=-0.726$, $R^2=0.475$; AUC IpGTT, $\beta=-0.464$, $R^2=0.176$).
Thus, transgene presence and HCD was associated with the greater microglia activation in the corpus callosum, both in the body and periventricular regions, and the IC. Greater visceral fat mass was associated with a greater microglia activation in all three white matter regions analysed irrespective of the genotypic characteristics, however was predictive of it only in the HCD environment.

5.3.3.3 Relationships in individual experimental groups

Group-wise linear regressions on the BCC activated microglia coverage identified candidate parameters only in the comorbid (TG HCD) group.

Figure 5-2 indicates there was a strong negative correlation with final body weight ($\beta=-0.522$, $p=0.033$); fasting total cholesterol levels ($\beta=-0.623$, $p=0.013$); triglycerides ($\beta=-0.637$, $p=0.011$); non-HDL cholesterol ($\beta=-0.64$, $p=0.01$); Chol:HDL ($\beta=-0.639$, $p=0.008$) and fasting insulin levels ($\beta=-0.671$, $p=0.048$).

Linear regression analysis indicated that a lower Chol:HDL ratio was the best predictor of increases in the BCC microglial activation level in the comorbid rats ($\beta=-0.639$, $R^2=0.367$). This was also true for the PVCC brain region for which Chol:HDL ratio model produced a strong negative relationship ($\beta=-0.658$, $R^2=0.396$).

For inflammation in the IC, group-wise linear regressions indicated a relationship with fasting insulin ($\beta=-0.814$, $R^2=0.621$); HOMA-IR ($\beta=-0.76$, $R^2=0.524$); and AUC IvGTT ($\beta=-0.619$, $R^2=0.306$) in the TG CD group.
Figure 5-1. Relationships between visceral fat accumulation and white matter activated microglia. Visceral fat pads mass indicated as fat pad weight and OX-6+ activated microglia coverage expressed as percent of the total area of the white matter structure in A-C) BCC; D-F) PVCC; G-I) IC regions. Significant linear relationships were found for the datasets: A) entire set ($R^2=0.128$, $p=0.002$); B) TG ($R^2=0.207$, $p=0.005$) and WT ($R^2=0.292$, $p=0.001$); C) HCD ($R^2=0.136$, $p=0.02$); D) entire set ($R^2=0.266$, $p<0.0001$); E) TG ($R^2=0.261$, $p=0.002$) and WT ($R^2=0.249$, $p=0.002$); F) HCD ($R^2=0.233$, $p=0.002$); G) entire set ($R^2=0.19$, $p<0.0001$); H) TG ($R^2=0.107$, $p=0.041$) and WT ($R^2=0.227$, $p=0.003$); I) HCD ($R^2=0.233$, $p=0.002$). Black and colored solid lines on each graph show linear regression line. Dotted lines indicate 95% confidence interval of linear regression. BCC = body of the corpus callosum, PVCC = periventricular regions of the corpus callosum, IC = internal capsule regions.
Figure 5-2. Relationships in the comorbid group between metabolic measures and microglia activation in the corpus callosum. Significant relationships between BCC OX-6 activated microglia coverage expressed as percent of the total region’s area and A) end of experiment body weight (R²=0.252, p=0.033), B) serum fasting total cholesterol (R²=0.34, p=0.013), C) non-HDL cholesterol (R²=0.364, p=0.01), D) Chol:HDL ratio (total cholesterol/HDL cholesterol); (R²=0.367, p=0.008), E) triglycerides (R²=0.361, p=0.011), F) fasting insulin levels (R²=0.371, p=0.048). The results are from the co-morbid model of transgenic rats on the hypercaloric diet (TG HCD). BCC = body of the corpus callosum, Chol = cholesterol, HDL = high density lipoprotein cholesterol. R² = adjusted R².
5.3.4 Metabolic, physiological measures and white matter astrocyte reactivity relationships

Correlations and linearity of relationship of the GFAP activated astrocytes in the BCC and IC regions were done individually for each measurement and included whole dataset, genotype, diet and individual group analyses.

Astrocyte reactivity in the BCC was related to the genotype when analyzing the entire dataset ($\beta=0.292$, $F_{(1,60)}=5.591$, $p=0.021$, $R^2=0.085$). Astrocytosis in the corpus callosum was inversely related only to the glucose metabolism, AUC IvGTT, in the data subsets from CD ($\beta=-0.743$, $F_{(1,9)}=11.101$, $p=0.009$, $R^2=0.503$) and TG ($\beta=-0.754$, $F_{(1,9)}=11.852$, $p=0.007$, $R^2=0.520$) rat groups (Figure 5-3A).

Reactive astrocytosis in the IC was inversely associated with the glucose metabolism, AUC Ip GTT, in the entire group of all rats ($\beta=-0.625$, $F_{(1,13)}=8.351$, $p=0.013$, $R^2=0.391$), WT ($\beta=-0.813$, $F_{(1,5)}=9.750$, $p=0.026$, $R^2=0.593$) and HCD subsets ($\beta=-0.792$, $F_{(1,5)}=8.343$, $p=0.034$, $R^2=0.628$); (Figure 5-3B). The WT rat subset had negative significant, but weak relationships with body weight, visceral fat and Chol:HDL ratio.

The TG CD group showed a strong inverse association between the AUC IvGTT and GFAP BCC coverage ($\beta=-0.976$, $F_{(1,2)}=40.682$, $p=0.024$, $R^2=0.93$).
Figure 5-3. Relationships between glucose tolerance and reactive astrocytes in the body of the corpus callosum and internal capsule. Glucose tolerance presented as glucose AUC in the GTT with greater AUC numbers corresponding to low glucose tolerance. GFAP+ reactive astrocyte coverage expressed as percent of the total area of A-C) body of the corpus callosum and D-F) internal capsule regions. Significant relationships between glucose intolerance and astrocyte reactivity are shown for the datasets: A) TG CD individual group (β = -0.976, p=0.024, R²=0.93); B) TG combined set (β = -0.754, p=0.007, R²=0.520); C) CD combined set (β = -0.743, p=0.009, R²=0.503); D) entire set (β = -0.625, p=0.013, R²=0.391); E) WT combined set (β = -0.813, p=0.026, R²=0.593); F) HCD combined set (β = -0.792, p=0.034, R²=0.628). AUC = area under the curve, CD = control diet, GTT = glucose tolerance test using intravenous (IvGTT) or intraperitoneal (IpGTT) method, HCD = hypercaloric diet, TG = transgenic, WT = wildtype. Solid lines on each graph represent linear regression lines. Dotted lines indicate 95% confidence interval of linear regression. R² = adjusted R².
5.4 Discussion

This investigation was directed toward identifying potential markers of the cerebral white matter inflammation in prodromal AD by studying associations between metabolic parameters and cerebral white matter activated microglia and astrocytosis in APP21 TG and MetS rat models, alone or in combination.

First it was determined whether there is any association between the neuroinflammatory markers and outcomes of behavioral tests utilized to assess spatial reference and working memory. White matter activated microglia, but not astrocyte reactivity, showed correlations with the cognitive performance. The general trend was indicative of an inverse correlation, i.e. a better cognitive task performance was associated with a lower white matter microglial activation. Interestingly, specific group analysis revealed differences in directionality of the relationship. The comorbid rats, TG on the HCD, followed the general trend. Unlike the general trend of an inverse correlation, the TG CD group had a very strong positive correlation, suggesting a greater performance associated with the greater neuroinflammation. These observations, however, should be treated with caution as the analysis was limited by the low sample size since the behavioral protocols were different for the two cohorts of animals.

Linear regression examined the relationship of metabolic measures with microgliosis and astrocytosis in the corpus callosum and internal capsule. Microgliosis was strongly associated with the amount of visceral fat and lipid metabolism, whereas astrocytes reactivity was correlated with glucose metabolism. In addition, genotype appeared to be the leading predictor in the degree of neuroinflammation in every region, in this case the transgene was the hAPP with Swedish and Indiana mutations associated with early familial AD (APP21). These animals have high APP and amyloid beta proteins in the brain and develop increased white matter microglial activation with aging and with the HCD in our studies.

The most profound metabolic predictor of an increase in microglia activation in both white matter regions was greater visceral fat mass. The association between visceral adiposity and WMH have also been observed in humans. This seems to be in line with the notion of obesity as a systemic low-grade inflammatory condition and increased visceral fat accumulation as an active pro-inflammatory secreting tissue influencing inflammatory status in the brain. The amount of
visceral fat together with the transgene demonstrated the greatest contribution to the white matter microglial activation.

Correlations with lipid profile parameters, including total cholesterol, atherogenic fraction and triglycerides levels indicated these parameters were also possible contributors to the amount of microgliosis, although not to the same extent as visceral fat, body weight or genotype. In the combined datasets, hyperlipidemia was significantly associated with higher levels of microglia activation in the white matter, a phenomenon which is associated with AD pathogenesis. This data fits one of the existing patterns observed in epidemiological studies which related high levels of total cholesterol, LDL-cholesterol and triglycerides, low HDL levels in mid-life with the greater risk of AD development\(^{43-45}\).

There appeared to be different associations when the individual set of comorbid rats was compared to the combined dataset. This relationship was inversed in the TG HCD group, suggesting a negative association between blood lipids and white matter microgliosis in comorbidity condition. Such negative relationship between lipid profile and WMH load, although not significant, was noted in a sample of cognitively normal middle-aged subjects with a family history of AD, the majority of whom also had MetS\(^{27}\).

The strongest predictor of the OX-6 activated microglia accumulation in the corpus callosum for the comorbid rat sample was Chol:HDL ratio, which represents the ratio of two main cholesterol fractions, HDL and atherogenic non-HDL. A higher lipid ratio, which is observed in metabolic disorders, and is generally related to a greater risk for cardiovascular disease and dementia, was associated with lower levels of white matter microgliosis in comorbid group. Analysis of the lipid profile of our comorbid rats have indicated that these rats had blood levels of the HDL cholesterol, similar to that of CD rats. However, total cholesterol and the Chol:HDL ratio was significantly greater compared to controls, which suggests that the increase in the ratio was due to the greater non-HDL fraction.

The literature provides evidence of the cholesterol paradox in the AD relationship\(^{46-50}\). The inconsistency of results regarding cholesterol association with AD points to a differential association with respect to the age of subjects and possibly even the stage of the disease. High blood total cholesterol and non-HDL cholesterol fractions are associated with decreased risk of
AD, particularly in the subjects of a greater age, whereas the opposite relationship is seen in middle age\textsuperscript{51–53}. This could be due to observed declines in cholesterol along with higher energy demands of the brain with increased age\textsuperscript{54}. Moreover, lower serum cholesterol levels were observed in AD patients a decade prior to the clinical onset, suggesting lower cholesterol levels are associated with the prodromal stage of the disease\textsuperscript{55}.

With respect to the white matter, axonal myelin sheaths are highly enriched by lipids and loss of membrane cholesterol and gangliosides along with myelin degradation and low biosynthesis all have been associated with aging and AD pathology\textsuperscript{56–59}. It is possible, that increased cholesterol levels might counterbalance these effects. Thus, the analysis of the association between MRI-detected white matter lesions and cardiovascular risk factors in the elderly participants of the Cardiovascular Health Study, showed a significant progression of the white matter lesions over time in participants with high HDL and low LDL blood cholesterol levels as well as with the use of statins\textsuperscript{60}. In another study dyslipidemia (high triglycerides and low HDL cholesterol levels) were found to be positively associated with healthy white matter microstructure in generally healthy adults\textsuperscript{61}.

Furthermore, animal studies have shown the dual role of high dietary cholesterol pointing to anti-inflammatory effects, including promotion of M2 “anti-inflammatory” microglia phenotype and higher IL-4 and IL-6 levels, of higher lipid levels in the aging brain\textsuperscript{62}. This could relate the higher total cholesterol and non-HDL levels with the lower white matter microglial activation in the prodromal AD model in our study.

The genetic background of our TG rats, specifically hAPP with Swedish and Indiana mutations, could also contribute to the observed relationship with lipid metabolites in our model since APP plays a role in cholesterol metabolism\textsuperscript{63,64}. One study have shown that human astrocytes homozygous for APP Swedish mutation lead to disrupted cholesterol metabolism in the brain, in particular low intracellular cholesterol levels, impaired lipoprotein endocytosis and LDL receptor function were seen\textsuperscript{65}. These changes might contribute to AD pathology such as white matter degeneration. APP is widely expressed as in the brain so in peripheral tissues. It is possible that mutated forms might also have effects on peripheral lipid homeostasis, which are yet to be investigated.
The effects of the mid-life hypercholesterolemia, dyslipidemia and associated increased levels of oxidized cholesterol forms (oxysterols) might be particularly detrimental to the white matter health, including myelin synthesis and breakdown regulated by glia, and thus predispose to AD pathology later in life. Oxysterols which are able to cross the blood-brain barrier were determined as a potential links between diet and obesity-related dyslipidemia and AD pathogenesis including via their contribution to neuroinflammatory and amyloidogenic events.66–78. Treatments that lower cholesterol, i.e. statins, may help protect vulnerable white matter during the mid-life period, but can be detrimental in advanced aging and fully developed AD and thus have no effect on the slowing down the progression of the disease in the clinical trials.54

Interestingly, lower astrocyte reactivity, corresponding to a lower inflammation, was observed with a greater glucose AUC in the GTT, i.e. glucose intolerance likely related to insulin resistance. This is somewhat contradictory to what is usually expected since a greater insulin resistance is associated with poorer brain health. However, in our study the TG rats did not develop pronounced glucose intolerance compared to WT rats on the HCD. Similarly, patients with AD have been shown to have lower rates of fasting blood glucose as well as lower glucose values in the oral glucose tolerance test.79

There are some limitations to be considered for this investigation. Firstly, the data samples obtained from the two animal studies were not large. This factor could decrease the precision and power some of the relationships, such as related to the glucose and insulin metabolism parameters and in specific subsets. Secondly, we have considered only a limited number of factors that could contribute to the brain inflammatory pathology. There might be other measures that better explain the variation in the white matter inflammation and which could greatly improve the prediction of the defined models. Thirdly, there were only two cellular markers of the neuroinflammation analysed in the current study based on the data derived from our initial experiments. However, other white matter pathological processes, including for example oligodendrocyte health, axonal degeneration, myelination level and vascular status that could occur at the prodromal stage of the disease, could have a better correlation with systemic metabolic parameters and provide a better explanation of the cognitive performance. Finally, microglia and astrocyte immunohistochemical detection was limited to examination of a single marker of each type of cell. Other markers of activation of these cells should be considered.
In summary this study highlights the potential of visceral fat accumulation and lipid metabolism components such as cholesterol ratio, to be markers of activation of microglia in the white matter in a prodromal AD model. Astrocyte reactivity, another inflammatory factor, was associated with the glucose metabolism and insulin resistance. More studies investigating the association between metabolic measures found in the blood and specific brain pathology associated with the prodromal stage of AD and MCI should be pursued to clarify potential relationships and their usefulness as minimally invasive biomarkers in various age groups. Comorbid conditions including chronic metabolic and vascular diseases, gender, genetic variations and attributes of certain ethnic groups may modulate these relationships, thus requiring even more extensive investigations in this area.
5.5 References


64. Grimm, M. O. W. *et al.* Regulation of cholesterol and sphingomyelin metabolism by amyloid-β and presenilin.


Chapter 6: Discussion

6.1 Summary of Results

The studies constituting this thesis were undertaken to enhance our understanding of the interaction between metabolic vascular condition and pathogenic environment of the early prodromal stage of AD. Specifically, the studies aimed to investigate the effects of such interaction on the white matter inflammation and the impact on the cognitive outcome, and to determine the potential of manipulating inflammation with a novel brain-targeted NSAID to prevent pathological and cognitive changes. A high calorie high-fat, high-sugar diet-induced MetS in the APP21 TG rat model of prodromal AD was used to test the research hypothesis that the comorbid condition will result in increased white matter inflammation and cognitive dysfunction which can be prevented by anti-inflammatory treatment. The following objectives were addressed:

1) To determine the impact of comorbid MetS and increased pathogenic APP environment on white matter inflammation, specifically on microglia and astrocytes, and the effect on cognitive function; thus, defining possible targets for early therapeutic intervention (Chapter 2).

2) To examine the therapeutic potential of a novel anti-inflammatory agent by analyzing its effect on white matter inflammation and cognition in the comorbid model of prodromal AD and MetS (Chapter 3).

3) To analyze the possible negative effects of a brain targeted anti-inflammatory agent tested as a potential therapeutic strategy to target increased white matter inflammation (Chapter 4).

4) To identify potential biomarkers of white matter inflammation by characterizing the relationship between white matter inflammation and systemic metabolic and physiological parameters (Chapter 5).

In support of the hypothesis, the comorbid rats demonstrated increased white matter microglia, but not astrocyte, activation, which was accompanied by spatial reference and working memory deficits and cognitive inflexibility (Chapters 2 and 3). TG rats on the HCD were the only group demonstrating spatial reference memory consolidation deficit compared to control group of the wild type rats suggesting that intense white matter inflammatory response to the presence of
metabolic alterations could be promoting and accelerating the development of this type of
cognitive decline (Chapter 2). These results highlighted that white matter inflammation could be
a potential treatment target.

Experiments to demonstrate that an anti-inflammatory agent is a potential therapeutic approach,
did not find support in our studies. The chronic treatment with a novel NSAID KL prodrug did not
prevent the potentiating effect of the MetS on the white matter inflammation and cognitive
dysfunction at the prodromal AD phase (Chapter 3). Moreover, the use of this particular prodrug
was associated with detrimental effects in several animals on cognition and cerebral homeostasis
expressed as substantial inflammatory pathology involving both microgliosis and reactive
astrocytosis in large regions of gray and white matter of the cerebral cortex and subcortical
structures (Chapter 4). Additional analysis indicated that white matter inflammation, specifically
white matter activated microglia, is found to negatively correlate with cognitive performance. A
general trend of a better cognitive task performance was associated with a lower white matter
microglial activation (Chapter 5).

The data overall supports the need for further studies to clarify the potential of an anti-
inflammatory treatment. However, this research should carefully choose the types of agents and
consider timing as well as determine the best therapeutic window. In the meantime, identifying
biomarkers for white matter inflammation is important as it could have implications for diagnosis,
treatment and prevention.

The attempt to find potential biomarkers of this pathology indicated that the presence of the
mutated form of APP and amount of fat accumulated around internal organs is highly positively
correlated with white matter microgliosis. Blood lipid metabolism components including total
cholesterol, atherogenic fraction, triglycerides levels and Chol:HDL ratio were also contributors
to the amount of microgliosis. In particular, the comorbid condition has shown a negative
association of white matter inflammation with the lipid profile, while the entire rat sample and WT
dataset had a positive relation. These results indicate there are complex relationships between
blood and physiological parameters and white matter microglial activation, which are complicated
by the coincidence of metabolic and early AD pathologies. Thus, biomarker research should
consider the presence of comorbidities that might affect the relationship between metabolic
measures and cerebral pathologies and therefore have an effect on interpretation and subsequent clinical application of the biomarker.

6.2 Comorbid Model of Prodromal Alzheimer’s Disease and Metabolic Syndrome

MetS is a chronic disorder highly prevalent among the elderly which is associated with increased risk for and is a common comorbidity of MCI and AD\textsuperscript{1-6}. It is thought that these diseases interact with each other and might have synergistic effect on cerebral homeostasis and cognitive function\textsuperscript{7,8}. The exact basis of such interaction has not been conclusively defined yet. Analysis of pathogenetic commonalities indicates that inflammation and white matter lesions might be the events linking the two conditions, specifically at the early prodromal stages of AD.

To investigate this idea we developed a preclinical model of this comorbidity in the TG rat overexpressing pathogenic hAPP, implicated in human FAD, which shows increased production of APP and Aβ levels and yet no plaque and NFT pathology\textsuperscript{9,10}. Thus, these TG rats model the early stage of disease pathology.

Obesity and MetS in rodents are often induced via dietary manipulation and include high-fat or high-carbohydrate hypercaloric diets with a various composition and macronutrient percent content\textsuperscript{11-13}. In our studies we combined high fat, rich in saturated fat and cholesterol, food with a corn syrup drink, rich in simple carbohydrates, that together resulted in increased caloric value to closely mimic the genesis of obesity in human populations with unhealthy dietary habits\textsuperscript{11,14}. Twelve to fifteen weeks on this HCD led to successful development of MetS in our rats. In particular, the following components developed: obesity, hypertriglyceridemia, increase in levels of total and atherogenic non-HDL cholesterol, shift towards greater Chol:HD ratio, insulin resistance, hyperinsulinemia and glucose intolerance\textsuperscript{15}. Comorbid rats showed greater dyslipidemia and only modest glucose intolerance compared to the WT rats. This smaller reduction in glucose tolerance compared to the WT rats is similar to observed in AD patients and could be related to peripheral pathogenic hAPP effects on regulation of glucose metabolism\textsuperscript{16,17}. Rats of both genotype on the HCD also had increased visceral adiposity which was greater in TG rats, suggesting establishment of pro-inflammatory environment in these animals\textsuperscript{18-22}. In our animals, HCD did not induce hypertension, a parameter that is important component of the syndrome.

Behavioral analysis using MWM variations showed modest cognitive changes in spatial reference memory consolidation, spatial working memory and ability to rapidly adjust to the environmental
changes (i.e. cognitive flexibility) driven exclusively by HCD consumption in the TG rats with AD predisposition. This suggests there is a potentiating effect of comorbidity on cognitive deficits. This cognitive profile demonstrated by MWM closely resembles executive dysfunction, processing speed and general cognition deficits seen in humans with white matter lesions who are obese, have MetS or are in the initial phase of AD\textsuperscript{23–34}.

Other investigations have shown that the APP21 TG rats used in our studies develop executive dysfunction with increased age compared to WT animals supporting the clinical relevance of this model\textsuperscript{35,36}. In our studies, a non-specific and less rigorous MWM protocol to test executive domain was used and the TG rats alone did not develop executive dysfunction. However, executive dysfunction was shown in the comorbid animals there is a potentiating effect on behavioral deficits.

Brain analysis identified predominant widespread white matter microgliosis and reactive astrocytosis in TG rats, which was not accompanied by evident demyelination. MetS combined with the prodromal AD environment significantly increased the white matter microgliosis compared to TG condition alone, although there was no additional increase in astrocyte reactivity or change to myelination level. In addition, the numbers of neurons in the hippocampus were not found to be affected in TG rats compared to WTs\textsuperscript{35}. There was also no apparent hippocampal neuronal loss in the comorbid animals. While there was a decrease in hippocampal synaptic density in TG rats compared to WTs, it was not further aggravated by the presence of metabolic syndrome in the comorbid rats. Thus, the HCD was efficient in modeling MetS traits similar to that observed in humans and indicated possible effects of comorbidity on physiology and metabolism which might be relevant and important for clinical consideration. Moreover, the combined model indicated synergistic effects of comorbidity on neuroinflammation, specifically white matter inflammation demonstrated by increased microglia activation, and behavioral deficits suggesting a potential link between the AD and MetS and white matter inflammation might be underlying mechanism potentiating the effect of comorbidity on cognitive deficits.
6.3 White Matter Inflammation

The Fischer 344 rat strain used in these studies have shown to be especially prone to white matter alterations, specifically development and progression of widespread inflammation, with increased aging, similar to what is seen in humans as they age. APP21 TG rats created on the Fischer 344 background have previously been shown to develop accelerated microglia activation in white matter compared to the WT rats of this strain. This phenomenon was also observed in our animals as described in this thesis.

The regions of microglial activation included the corpus callosum, particularly the anterior part starting at the forceps minor and more found in the periventricular areas, fimbria, internal capsule, cingulum, anterior commissure, hippocampal commissure and optic tract. The predominance of white matter changes in the anterior region of the corpus callosum is similar to the pattern seen in human aging and the early phase of AD, changes that support the retrogenesis hypothesis of white matter disruption due to local events prior to neurodegenerative changes.

Significantly more activated microglial cells were observed in the corpus callosum, internal capsule and fimbria hippocampi of TG rats compared to WT rats in our studies. HCD-induced MetS collectively was also associated with significant increase of white matter microgliosis. However, it was the comorbid rats of prodromal AD and MetS that demonstrated the most marked increase in white matter microglial activation in all three regions compared to all other groups, indicating a synergistic response of the two diseases.

Although the TG animals demonstrated an increase in astrocyte reactivity, there was not an additional increase induced by the comorbid condition with the HCD-induced MetS. This could be due to microgliosis in the white matter being a very early pathological change preceding reactive astrocytosis or alternatively a specific form of response to this comorbidity with obesity and metabolic changes.

Rats of both genotype on the HCD also had increased visceral adiposity which was even greater in TG rats on the diet, suggesting the establishment of pro-inflammatory environment in these animals. The enhanced white matter inflammation in the comorbid rats which also had the highest amount of visceral fat supports the idea of systemic inflammation in MetS contributing to and
promoting the brain inflammatory processes and in this case, specifically the white matter microglial response\textsuperscript{21}. Furthermore, as only the comorbid rats showed cognitive impairments this supports the association of high systemic inflammatory status due to increased visceral adiposity with cognitive dysfunction in obese individuals\textsuperscript{18–22,49–52}.

There was no apparent hippocampal neuronal loss in the comorbid rats suggesting that white matter pathology, specifically inflammation, is associated with the behavioral deficits and explain the observed profile in this model. These findings of changes in white matter inflammation and cognitive impairment, are also supported by the analyses that demonstrated an inverse correlation between white matter activated microglia and cognitive performance in total rat sample and especially in the comorbid animals. A better cognitive task performance is associated with lower white matter microglial activation.
6.4 Anti-inflammatory Treatment

Consistent evidence from the literature and the present results, of the involvement and critical role of neuroinflammation in MCI and AD, from prodrome to end stage, strongly suggests that modulation of inflammation is a promising approach to disease prevention or slowing\textsuperscript{53,54}. Epidemiological studies also support this idea, particularly the use of NSAIDs for blocking inflammation via COX enzyme inhibition associated with reduced prostaglandin, thromboxane and prostacyclin production\textsuperscript{55}. Some clinical trials appear to support the efficacy of anti-inflammatories in MCI and AD, whereas others show no benefit of NSAID use in control of disease progression\textsuperscript{56}.

These drugs are largely effective at the systemic level and have limited ability to cross BBB. The transport and availability of active free drug in the CNS site of action is very limited, particularly due to their extensive binding to plasma proteins in systemic circulation\textsuperscript{57,58}. Modification of drug structure can lead to favorable changes in its properties leading to enhanced transport into the CNS and increased concentration of biologically active compound in the brain.

A novel engineered non-steroidal anti-inflammatory prodrug, KL, showed the ability to cross the BBB via LAT-1 transporter, achieve a good brain tissue distribution and drug release in the brain\textsuperscript{59}. We have tested this brain targeted prodrug in the comorbid rat model and found that the long-term prophylactic treatment initiated at the time of dietary intervention at a half-therapeutic dose for this class of drug, did not reduce the enhanced white matter microglial activation and did not have an effect on the behavioral deficits. However, the chronic prodrug administration was associated with two lethal cases and development of widespread neuroinflammation and neuronal degeneration accompanied by cognitive deficits in several rats irrespective of the experimental group.

Literature analysis showed that some studies reported an increased risk of adverse cardio- and also cerebrovascular events in patients taking NSAIDs that may have been happening in our animals on the prodrug\textsuperscript{60–68}. The lack of positive effects of the drug on the pathology and behavioral deficits, and the presence of possible direct negative pathological effects could be related to the treatment protocol, such as the dose, time of initiation and also the prodrug properties used in our study. The pathological APP environment in the TG rats combined with metabolic abnormalities
could alter transporter-mediated influx of the prodrug in the CNS. Clinical data suggest the timing of drug application is the most critical condition for the success of anti-inflammatory treatment\textsuperscript{56,69–71}.

White matter inflammation, similar to what was developed in our model, is thought to be the earliest pathology seen in AD. It is possible that white matter damage due to inflammation or other events leading to or contributing to increased microgliosis in our TG model has been present for some time even at the start of pharmacological intervention, even though there is no classical plaque or NFT pathology. Thus, the prodrug was unable to prevent or reduce the enhanced pathology in the comorbid animals using the drug administration protocol in these studies.

Prolonged exposure to the high concentration in the brain of free ketoprofen could lead to brain damage seen in our rats. This could be linked to insufficient inhibition of negative COX effects (e.g. pro-thrombotic, vasoconstrictive) in the current protocol\textsuperscript{61,62,65,72,73}. Alternatively, these effects might be related to the inhibition of COX enzyme activity in general which aside from the pro-inflammatory and neurodegenerative action are involved in neuroprotection mediated by certain prostaglandins, docosanoids, resolvins and neuroprotectins. Beneficial effects of COX include regulation of synaptic activity and plasticity, hippocampal long-term potentiation, neurovascular coupling and cerebral blood flow, neuronal survival, anti-thrombotic and even anti-inflammatory properties (e.g. modulation of leukocyte trafficking, downregulation of cytokines expression in glia)\textsuperscript{74–80}.

Under pathological conditions the equilibrium maintained by COX is disrupted, and effects are initially shifted towards the inflammatory which could eventually turn into neurodegeneration. Nevertheless, it is possible that there are still some protective effects in place to counteract compromised homeostasis and diminishing them pharmacologically needs to be approached cautiously.

The data overall supports the need in further studies to clarify the potential of anti-inflammatory treatment in preventing and slowing down white matter inflammation associated with AD and cognitive deficits. Initiation of treatment as well as the therapeutic window are critical conditions for the success of drug therapy and should be established by future experiments. These parameters could vary with the presence of concurrent chronic conditions, such as metabolic vascular
disorders, that might show an accelerated rate of development of brain pathology, be prone to complications associated with the NSAID treatment and also impact pharmacokinetics and dynamics of drugs\textsuperscript{60,81}.

Caution should be exercised with the drug synthesis of brain-targeted NSAIDs in the race for a better CNS penetration. This could in fact potentiate adverse effects, rather than increase the benefits of the treatment. It might also be that some systemic effects of NSAIDs would be beneficial such as lowering systemic inflammation present in obesity and MetS, which could decrease pro-inflammatory signaling to the brain and therefore CNS immunity activation, i.e. microgliosis. It is also possible to consider various NSAIDs and alternative drug agents with a different mechanism of action that will not diminish COX-mediated beneficial effects.
6.5 Biomarkers

Success of the treatment appears to depend on the pathological stage it is initiated at. Early intervention might grant beneficial effects by acting on the prodromal pathology such as white matter inflammation. It is important to investigate potential biomarkers of the cerebral white matter inflammation that may have relevance for disease treatment. Currently, white matter lesions that has been linked to cognitive dysfunction can be detected on conventional MRI as hyperintensities or atrophy. However, these lesions might already be too advanced and not quite useful from a therapeutic intervention point of view\textsuperscript{82–84}. Early inflammatory pathology might already be present even when the white matter appears normal on MRI. The DTI MRI method is more sensitive and could provide an insight into structural white matter changes associated with pathological changes to myelin and axons\textsuperscript{85–89}. PET imaging can provide information specific to microglial activation\textsuperscript{90–95}. More easily obtainable and affordable markers such as blood derived substances and physiological measures present a more favorable approach and should be studied.

The experiments described in this thesis examined associations between blood metabolic and physiological parameters and cerebral white matter activated microglia and astrocytosis using a preclinical rat model that included a model of comorbid hAPP TG and MetS. The amount of visceral fat was a very strong predictor of microgliosis and better explained its variance than body weight. Lipid metabolism components showed more complicated relations. While entire heterogenous rat sample including both diets and genotypes showed a weak positive association, comorbid rats demonstrated a negative relationship with every component. Chol:HDL ratio appeared to be the strongest contributor to the variations in microglia activity.

Epidemiological data does not provide a single directional relationship between blood lipids and AD, suggesting potential effects at different disease stages. The epidemiology data actually suggests a beneficial role of increased cholesterol levels for the brain under pathological conditions\textsuperscript{96–98}.

There was a strong genotypic relationship to the amount of activated white matter microglia. The TG APP variant with Swedish and Indiana mutations associated with early FAD correlated with greater microglial activation. Thus, comorbid conditions with AD, such as metabolic vascular diseases including MetS and T2DM, and potentially the stage of the disease appear to complicate
and can significantly modulate the associations between the markers and specific pathology. Age, gender, ethnicity and genetics may further contribute to the relationship of certain markers with AD or MCI pathologies. Thus, biomarker research should consider the presence of comorbidities that might have a direct effect on the interpretation and subsequent clinical application of the biomarker.
6.6 Limitations

The TG rat model of AD bears the hAPP gene mutations which are causally linked to the early onset autosomal dominant FAD\(^9\). However, genome studies revealed that variants of these gene are implicated in the sporadic LOAD as well\(^1\)\(^0\)\(^0\),\(^1\)\(^0\)\(^1\). The clinical profile of these two types of AD are very alike. Although there might be different mechanisms driving the disease development, these forms are very similar pathologically. Particularly, white matter lesions commonly seen in LOAD are also attributed to the onset of FAD type\(^8\)\(^2\),\(^1\)\(^0\)\(^2\),\(^1\)\(^0\)\(^3\). Thus, this rat model offers valuable opportunities for the investigation of the roles of white matter pathology and white matter microglia activation in a common form of AD pathogenesis and cognitive decline. This model is also relevant to age-related pathology and vascular dementia.

The dietary approach of modeling obesity and MetS provides a great advantage since it closely mimics human habits. These models create a complex \textit{in vivo} environment to study diseases and their interaction in more realistic conditions. However, dietary composition for experimentation is not standardized and varies greatly in fat, sugar and protein amounts which introduces a certain variability to the systemic and brain pathology that might introduce variable responses in different species and strains\(^1\)\(^0\)\(^4\),\(^1\)\(^0\)\(^5\). Similarly, the duration of feeding might impact the development of pathologies.

In these studies, the diet was provided for 12-15 weeks and was enough to model obesity and related dyslipidemia, insulin resistance and glucose intolerance characteristic of a pre-diabetic condition. However, the animals did not develop frank T2DM with fasting hyperglycemia. Blood pressure elevation was not evident in the HCD rats compared to control animals. Blood pressure was measured with a non-invasive technique which does not absolutely exclude a possibility of subtle early changes that might be developing at this stage, that could have been detected using sensitive invasive methods. Nevertheless, our model demonstrated characteristic features of MetS in a particular combination that appear relatively early in the course of the disease, and this provided us the opportunity to study early interaction with AD predisposition.

Behavioral testing done in our model utilized MWM task to assess spatial learning and memory, as well as working memory and behavioral flexibility, which are all related to the health of the white matter. However, MWM is not the most sensitive or specific task for assessment of executive
function. Operant conditioning based set-shifting would be a much more appropriate test for this purpose\textsuperscript{36}. However, this test requires a considerable food restriction period for motivational purposes prior to training and uses sugar pellets as a reward during the testing and the testing period might be a several weeks long. This significantly complicates the integration of the task in its original form into our studies when our animals are on the diet-induced pathology regimen. In fact, there is evidence of pathology reduction and reversibility with caloric restriction and diet modulation\textsuperscript{11,106–109}.

Analysis of the cerebral pathology was focused on the white matter changes, particularly glia-mediated inflammation. Microglia and astrocyte activation, which indicates a pro-inflammatory environment, were detected by immunohistochemistry using a single antibody for each type of cell, OX-6 for MHC II (M1 pro-inflammatory phenotype) and anti-GFAP, respectively\textsuperscript{110–112}. Although these markers may have some limitations, they have a good selectivity, are associated with increased activation of cells in a pro-inflammatory condition, including aging, and are commonly used\textsuperscript{40,113–119}.

Quantification methods included integrated density defined as a sum of pixels in the area and area coverage by antigen positive cells, which were analyzed on converted to black and white images, and allowed assessment of the cell activation level by the number of antigen-expressing cells, rather than the antigen expression level in cells. Additionally, the number of Iba-1 stained microglia cells was used to assess the changes in the total microglia numbers. These complementary microglia staining analysis indicated that comorbidity was specifically associated with an increase in activation of cells and not due to an increase in their number. GFAP staining does allow a conclusive answer to whether increased transgene-dependent astrocytic response was due to an increase in individual cell reactivity or increased number of cells.

We did not detect neuronal loss or additional to transgene-related synaptic density decrease in the hippocampal region by immunohistochemical staining suggesting depletion of neurons or synapses in the hippocampus is not responsible for the cognitive profile of the comorbid rats. It is likely that white matter microglial activation and/or other white matter pathology underlies these disturbances. However, this does not exclude the possibility that some functional and morphological neuronal changes could begin to develop at a later time. It is more likely that
synaptic dysfunction associated with the microglia hyperactivation and white matter damage could contribute to the cognitive impairments.\textsuperscript{48,120}

The conclusions of the NSAID prodrug treatment ineffectiveness were based on a single treatment protocol. This protocol used a half-dose of a common therapeutic dose. This was considered sufficient since it was initiated at the time of dietary change and was viewed as a prophylactic measure. It is possible that this dose, the frequency of injections, the duration and the time of initiation at that particular age could be insufficient to modify the observed pathological microglia activation. Earlier intervention in younger rats could potentially provide some benefit.

Additional studies with brain targeted anti-inflammatory drugs need to be pursued and should rely on a determination of the white matter inflammation time course. However, a large increase in brain inflammation observed in several prodrug-recipients suggests there may be a safety concern for the brain-targeted agent even with the moderate dose delivered daily and a higher therapeutic dose may not be feasible. However, a vehicle-controlled study should be performed to rule out the possible effects of the chronic stress due to injections on the brain and behavior and determine the unique negative effect of the drug treatment.

Correlational studies utilized a limited data sample which likely affected precision and power of associations, particularly between behavior and glucose metabolism and microgliosis. A limited number of measures were tested for relationship with white matter pathology. Nevertheless, observations of differential patterns seen in the whole rat sample and the comorbid group showed significant correlations and may be relevant to the humans.

Finally, these studies used only male rats 8.5-10.5 months of age at the start of experiments. There could be sex differences in the response to the diet and in the interaction with AD early pathology, which are important and should be investigated.
6.7 Future directions

The studies presented in this dissertation represent the basis for future experiments. The pathological and cognitive presentation of the MetS may slightly vary reflecting the diversity of components comprising the syndrome in a particular case seen among both humans and animals. Each of the metabolic and physiological changes in MetS may contribute to CNS pathology and AD-related changes either individually or as a combination. Thus, it might be important to study various phenotypes to observe both short-term and long-term progression of pathological events to unravel the dynamics of changes in the CNS, including white matter glia activation and function. Also, in the light of importance of understanding the early pathology, studies on the time course of white matter microglia activation could be carried out with initiation of the experiment at a younger age.

The use of more specific and sensitive behavioral methods is required to adequately characterize the changes in cognitive function in the comorbid condition and the effects of the treatment. These behavioral methods need to be first adapted for use in diet-induced metabolic disease models.

The detection of astrocytes and microglia was done using a single antigen expressed by activated cells in a pro-inflammatory condition. Greater specificity and more information could be achieved by using other markers of activated cells that may indirectly identify potential triggering events for changes in amyloid, APP or myelin debris. Such molecules of interest related to microglia function during inflammation to be considered are TREM-2, LRP, complement system receptors, galectin-3, Mac-1,2, TSPO\textsuperscript{121–125}.

While post-mortem tissue analysis is highly detailed and informative, PET and DTI MRI techniques can provide an advantage of indicating in vivo pathology detection and to trace the changes and progression over time. Glial activation, a marker of neuroinflammation, can also be detected using PET and might significantly advance our understanding of pathology when incorporated in preclinical studies\textsuperscript{126–129}. Cerebral pro-inflammatory status analysis could further be complemented by CSF or blood cytokine level determination.

Brain tissue should be further analyzed in a greater detail using available immunohistochemical and imaging methods in order to identify the nature and magnitude of the inflammatory events as
well as to determine if the white matter microglia activation is a precursor to or a consequence of potential vascular changes and other processes. These other processes include myelin and/or axonal damage and loss, oligodendrocyte dysfunction and death, are potential events that might take place at this early stage of dietary intervention and contribute to the cognitive dysfunction. The white matter damage or excessive microglia activation appearing at this early stage could affect synaptic function and number. The comorbid model which addresses the early AD disease pathology demonstrated subtle cognitive changes. Synaptic pathology might be associated with these cognitive deficits and could be investigated using various methods that include immunohistochemistry, electron microscopy, PET imaging and electrophysiology.

These processes associated with microglia activation such as vascular, axonal, myelin, synaptic pathologies could be better correlated with cognitive decline and have a stronger association with the blood and physiological parameters associated with MetS which could further be used as peripheral biomarkers. Moreover, these studies on other associated pathologies and processes could define other potential therapeutic targets and strategies directed towards white matter protection.

NSAIDs are an intensively studied class of drugs for MCI and AD treatment. They inhibit COX activity and downstream production of eicosanoids and docosanoids modulating both pro-inflammatory and innate neuroprotective mechanisms. Additionally, blockage of COX pathways might increase arachidonic acid which is also a substrate for lipoxygenase (LOX) enzymes, such as 5-LOX and 12/15-LOX. This could result in an elevated release of leukotrienes contributing to inflammation, oxidative stress and neuronal injury and modulation of tau and amyloid pathways.

Obesity, particularly when induced by hypercaloric high-fat/sugar diets, might change the brain lipid profile and membrane composition such as increase arachidonic acid production. Another derivative of membrane phospholipids metabolism is lysophosphatidic acid (LPA) involved in promotion of insulin resistance and AD-specific pathology via enhancement of tau phosphorylation and APP processing by β-secretase into Aβ peptide, and glia activation. LPA production is increased in obesity and AD. AD is also associated with the increased
ATX gene expression, coding enzyme autotoxin involved in synthesis of LPA\textsuperscript{155,156}. Comorbid conditions may aggravate these pathways.

Thus, inhibitors of LOX and production or action of leukotrienes and LPA could be an alternative to NSAIDs drug choice with beneficial effects for its use in comorbid patients and should be investigated\textsuperscript{157–160}. One of the drugs that inhibits 5-LOX leukotriene production and alleviates inflammation is minocycline, a tetracycline antibiotic used for treatment of infection \textsuperscript{161,162}. Research data suggests a strong anti-inflammatory potential of this drug mediated by a direct action on microglia and interference with microglia T-cell communication\textsuperscript{48,163–166}. This might be a favourable strategy for AD and especially comorbidity with MetS as it specifically targets microglia, which appear to have a major role in the disease neuropathology and white matter inflammation in particular. Minocycline and derivatives might block excessive glia activation by the systemic inflammatory signalling\textsuperscript{48,111,166–168}.

Blocking or suppressing pro-inflammatory cytokine (e.g. TNF-\(\alpha\)) action via receptor binding modulation is also a promising strategy\textsuperscript{79,169–174}. Etanercept (Enbrel) which is a recombinant receptor for TNF-\(\alpha\) cytokine, one of the major key players in AD primarily secreted by microglia cells, showed benefit in preclinical and clinical cases\textsuperscript{175–178}. Monoclonal antibodies to TNF-\(\alpha\), MAbs, is a second group of treatment candidates\textsuperscript{175,176,179–181}. Minocycline which was discussed earlier has also been shown to have an anti-TNF-\(\alpha\) effect\textsuperscript{172,174}. Nevertheless, TNF-\(\alpha\) also has physiological functions which could be disrupted with drugs targeting this cytokine\textsuperscript{172,173}. Direct inhibition of TNF-\(\alpha\) receptors might be beneficial or detrimental depending on a cell type, receptor type and disease stage, thus more careful studies and more targeted and selective therapy should be developed\textsuperscript{182–184}.

In addition to pharmacological treatments, lifestyle changes are a readily available way of interfering with the disease development. Reduction and reversibility of the neuropathology has been observed with caloric restriction and diet modulation\textsuperscript{11,106–109,185}. The efficacy of this approach on decreasing white matter microglia activity, including in the comorbid model, should also be studied either independently or in combination with the drug method. Moreover, poor diet and lack of exercise are risk factors for metabolic conditions, MCI and dementia. Thus, modification of lifestyle at an early age could reduce the incidence of metabolic vascular disorders
and possibly prevent cognitive decline with aging. It is likely that a therapeutic plan combining pharmacological methods targeting several key pathological events and non-pharmacological measures might provide successful prevention or control over the MetS, diabetes and dementia.
6.9 Conclusions

The studies constituting this thesis support the initial hypothesis that comorbidity of pathogenic hAPP TG model of prodromal AD with MetS increases white matter inflammation, as demonstrated by microglia activation, and this comorbidity leads to cognitive deficits. TG CD rats developed some increased white matter activation compared to that in the WT group but did not show cognitive changes. Pathogenic hAPP also increased the total number of microglia cells. Comorbidity further exacerbated microglia activation, but not proliferation in TG HCD rats compared to TG normal rats and resulted in significant behavioral deficits. White matter microgliosis was further associated with the visceral fat accumulation which supports the idea of contribution of systemic inflammation to the cerebral glia activation.

While there was a genotype-dependent increase in the astrocyte reactivity in TG rats, the comorbid condition did not have an impact on this pathology as there was no additional increase in activation of these cells. Thus, it appears that comorbidity with diet-induced MetS exacerbated exclusively white matter microgliosis which suggests this is the key element of prodromal AD-related pathology and it may be the point of interaction between these diseases and related to the cognitive health. This pathological linking event might be an extremely important target for intervention allowing early modulation of the disease pathogenesis and disruption of detrimental synergistic effects of concurrent diseases.

While the use of a particular brain-targeted NSAID prodrug in a single protocol did not provide support for the anti-inflammatory intervention, it highlighted several important points for consideration including the importance of when treatment is started, potential serious adverse effects and the need for investigations of alternative drug agents. Thus, this APP21 TG rat model combined with the diet-provoked metabolic alterations is an important and clinically relevant model for studying early pathology in AD, early biomarkers and relationship with the metabolic vascular diseases. Furthermore, it is an important arena for testing pharmacological and nonpharmacological treatments and preventive measures that target the pathological link between these diseases, particularly white matter microgliosis, with the goal of preservation of cognitive function.
6.10 References


166. Giuliani, F., Hader, W. & Yong, V. W. Minocycline attenuates T cell and microglia activity to impair cytokine production in T cell-microglia interaction diseases of the central nervous system, such as we have demonstrated previously that the interac- substantial increase in tumor necrosis fa. J. Leukoc. Biol. 78, 135–143 (2005).


Appendices

Appendix A: Ethics Approval

A1 Animal Use Protocol #1

From: eSiriusWebServer [mailto:esiriusadmin@uwo.ca]
Sent: September 23, 2014 12:29 PM
To: [Redacted]
Cc: auspc@uwo.ca
Subject: eSirius Notification - New Protocol Modification Has Been APPROVED2008-113::6

Western

AUP Number: 2008-113
PI Name: Cechetto, David
AUP Title: Mechanisms of Vascular Cognitive Impairment and Prevention of Stroke and Its consequences

Official Notification of AUS Approval: A MODIFICATION to Animal Use Protocol 2008-113 has been approved.

The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

Submitted by: Kinchlea, Will D
on behalf of the Animal Use Subcommittee
A2 Animal Use Protocol #2

From: eSiriusWebServer
To: esiriusadmin@uwo.ca
Date: 04/06/2016 9:07 AM
Subject: eSirius Notification - New Protocol Modification Has Been APPROVED
Cc: auspc@uwo.ca

AUP Number: 2014-016
PI Name: Whitehead, Shawn N
AUP Title: Role of vascular risk factors in cognitive decline

Official Notification of AUS Approval: A MODIFICATION to Animal Use Protocol 2014-016 has been approved.

The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

Submitted by: Kinchlea, Will D
on behalf of the Animal Use Subcommittee
Appendix B: Diet Composition

B1 Hypercaloric Diet (HCD)

The content of the pelleted rodent Western diet which was used to induce metabolic syndrome MetS in these studies (HCD groups) and its detailed fatty acid composition are provided below.
**Product Data - D12079B**

### Description
RD Western Diet

### Used in Research
- Obesity
- Diabetes
- Osteoporosis
- Hypertension
- Atherosclerosis
- Metabolic Syndrome

### Packaging
Product is packed in 12.5 kg box. Each box is identified with the product name, description, lot number and expiration date.

### Lead Time
5-7 business days.

### Gamma-Irradiation
Yes. Add 10 days to delivery time.

### Form
- Pellet, Powder, Liquid

### Shelf Life
Most diets require storage in a cool dry environment. Stored correctly they should last 6 months.

### Control Diets
Custom diets available on request.

---

**Formula**

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<th>kcal%</th>
</tr>
</thead>
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<tr>
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<td>17</td>
</tr>
<tr>
<td>Carbohydrate</td>
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<td>43</td>
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<tr>
<td>Fat</td>
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<tr>
<td><strong>kcal/gm</strong></td>
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### Ingredient

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<th>kcal</th>
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</thead>
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<td>780</td>
</tr>
<tr>
<td>DL-Methionine</td>
<td>3</td>
<td>12</td>
</tr>
<tr>
<td>Corn Starch</td>
<td>50</td>
<td>200</td>
</tr>
<tr>
<td>Malto-dextrin 10</td>
<td>100</td>
<td>400</td>
</tr>
<tr>
<td>Sucrose</td>
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<td>1364</td>
</tr>
<tr>
<td>Cellulose</td>
<td>50</td>
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</tr>
<tr>
<td>Milk Fat, Anhydrous*</td>
<td>200</td>
<td>1800</td>
</tr>
<tr>
<td>Corn Oil</td>
<td>10</td>
<td>90</td>
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<td>Mineral Mix S10001</td>
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</tr>
<tr>
<td>Vitamin Mix V10001</td>
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</tr>
<tr>
<td>Choline Bitartrate</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Cholesterol, USP*</td>
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<td>0</td>
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<td>Ethoxyquin</td>
<td>0.04</td>
<td>0</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>1001.54</td>
<td>4686</td>
</tr>
</tbody>
</table>

*Anhydrous milk fat typically contains approximately 0.3% cholesterol.

On this basis, D12079B contains approximately 0.21% cholesterol.


Diet formulated to match Teklad Western Diet #TD88137, except that 1% Corn Oil replaces 1% Butter Fat.
# Fatty Acid Profile

**Typical Fatty Acid Composition of D12079B**

<table>
<thead>
<tr>
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</tr>
</thead>
<tbody>
<tr>
<td>Butter, Anhydrous</td>
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<tr>
<td>Corn Oil</td>
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</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
</tr>
<tr>
<td>C2, Acetic</td>
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</tr>
<tr>
<td>C4, Butyric</td>
<td>6.4</td>
</tr>
<tr>
<td>C6, Caproic</td>
<td>3.8</td>
</tr>
<tr>
<td>C8, Caprylic</td>
<td>2.2</td>
</tr>
<tr>
<td>C10, Capric</td>
<td>5.0</td>
</tr>
<tr>
<td>C12, Lauric</td>
<td>5.6</td>
</tr>
<tr>
<td>C14, Myristic</td>
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<tr>
<td>C14:1, Myristoleic</td>
<td>3.0</td>
</tr>
<tr>
<td>C16, Palmitic</td>
<td>53.5</td>
</tr>
<tr>
<td>C16:1, Palmitoleic</td>
<td>4.6</td>
</tr>
<tr>
<td>C18, Stearic</td>
<td>24.4</td>
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<tr>
<td>C18:1, Oleic</td>
<td>52.7</td>
</tr>
<tr>
<td>C18:2, Linoleic</td>
<td>10.6</td>
</tr>
<tr>
<td>C18:3, Linolenic</td>
<td>2.9</td>
</tr>
<tr>
<td>C18:4</td>
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</tr>
<tr>
<td>C20, Arachidic</td>
<td>1.9</td>
</tr>
<tr>
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</tr>
<tr>
<td>C20:4, Arachidonic</td>
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<tr>
<td>C22, Behenic</td>
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<td>C22:1, Erucic</td>
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<td>C22:4, Clupanodonic</td>
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<tr>
<td>C22:6</td>
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<tr>
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<tr>
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<td>Polyunsaturated (g)</td>
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<tr>
<td>Saturated (%)</td>
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</tr>
<tr>
<td>Monounsaturated (%)</td>
<td>30.7</td>
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<tr>
<td>Polyunsaturated (%)</td>
<td>6.9</td>
</tr>
<tr>
<td>Omega-6 Fatty Acid (gm)</td>
<td>10.61</td>
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<tr>
<td>Omega-3 Fatty Acid (gm)</td>
<td>2.9</td>
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<tr>
<td>Omega6:Omega 3 ratio</td>
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</tr>
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</table>

D12079B FA content

Formula: Copyright © Research Diets, Inc.
**Nutrition Facts**

**Valeur nutritive**

Per 2 tbsp (30 mL)  
par 2 c. à table (30 mL)

<table>
<thead>
<tr>
<th>Amount / Teneur</th>
<th>% Daily Value</th>
<th>% valeur quotidienne</th>
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<tbody>
<tr>
<td>Calories / Calories</td>
<td>130</td>
<td></td>
</tr>
<tr>
<td>Fat / Lipides</td>
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<td>0 %</td>
</tr>
<tr>
<td>Sodium / Sodium</td>
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<tr>
<td>Carbohydrate / Glucides</td>
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<td>11 %</td>
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<tr>
<td>Sugars / Sucres</td>
<td>12 g</td>
<td></td>
</tr>
<tr>
<td>Protein / Protéines</td>
<td>0 g</td>
<td></td>
</tr>
</tbody>
</table>

Not a significant source of saturated fat, trans fat, cholesterol, fibre, vitamin A, vitamin C, calcium or iron.

Source négligeable de lipides saturés, lipides trans, cholestérol, fibres, vitamine A, vitamine C, calcium et fer.

**INGREDIENTS**: GLUCOSE, GLUCOSE-FRUCTOSE, WATER, REFINERS’ SYRUP, SALT.
**B3 Control Diet**

This rat chow from Prolab is used as a standard rodent food in the animal facility in which the rats included in these studies were housed. This diet continued to be used in the rats from both control diet groups (CD) for the entire duration of studies.
DESCRIPTION
Prolab® Rat/Mouse/Hamster 3000 is formulated primarily for growth and reproduction in Lab Rats. This diet is formulated using managed formulation, delivering Constant Nutrition®. This is paired with the selection of highest quality ingredients to assure minimal inherent biological variation in long-term studies.

Features and Benefits
• High quality animal protein added to create a superior balance of amino acids for optimum performance
• Supports optimum growth and efficient reproduction performance of rats, hamsters and mice
• Formulated to feed rats, hamsters and many mouse strains

Product Forms Available
• Pellet form, 12 mm x 12 mm x 25 mm length (3/8" x 3/8" x 1"

Other Versions Available
• SPF/SPF Prolab® IsoPro® RMH 3000

GUARANTEED ANALYSIS
Crude protein not less than ......................... 22.0%
Crude fat not less than ......................... 5.0%
Crude fiber not more than ......................... 5.0%
Ash not more than ......................... 6.0%

INGREDIENTS
Ground wheat, dehulled soybean meal, wheat middlings, ground corn, fish meal, porcine animal fat preserved with BHA, dehydrated alfalfa meal, calcium carbonate, brewers dry yeast, soybean oil, dicobalt phosphate, salt, DL-methionine, L-lysine, choline chloride, monodentate dimethylylaminobisulfate, magnesiu oxide, ferrous sulfate, pyridoxine hydrochloride, cholecalciferol, vitamin A acid, cholecalciferol, vitamin B6, supplement, riboflavin, thiamine mononitrate, zinc oxide, folic acid, calcium pantothenate, nicotinic acid, manganese oxide, ferrous carbonate, copper sulfate, zinc sulfate, calcium carbonate, cobalt carbonate, sodium selenite.

FEEDING DIRECTIONS
Prolab® RMH 3000 is especially designed for growth and reproduction of rodents. It contains all the nutrients that are required for growth, lactation, and reproduction. This diet should be fed free choice in a self-feeder. Keep a constant supply of fresh water available.

Rats—All rats will eat varying amounts of feed depending on their genetic origin. Larger strains will eat up to 30 grams per day. Smaller strains will eat up to 15 grams per day. Feeders to rat cages should be designed to hold two to three days supply of feed at one time.

Mice—Adult mice will eat up to 5 grams of pelleted ration daily. Some of the larger strains may eat as much as 8 grams per day per animal. Feed should be available on a free choice basis in wire feeders above the floor of the cage.

Hamsters—Adults will eat up to 14 grams per day.

Important: A feeding program is only as effective as the management practices followed.

CAUTION: Store in a dry, well-ventilated area, free of pests and insects. Do not use molybdenum or insect-infested feed.

For information regarding shelf life please visit www.labdiet.com.

CHEMICAL COMPOSITION

Nutrients

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<th>%</th>
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</thead>
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<tr>
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<tr>
<td>Lysine</td>
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<tr>
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<tr>
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<td>Fat (acid hydrolisis), %</td>
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<td>Neutral Detergent Fiber*, %</td>
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<tr>
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Minerals

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<td>Calcium</td>
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<tr>
<td>Potassium</td>
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</tr>
<tr>
<td>Magnesium</td>
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Sulfur, % | 0.31
Sodium, % | 0.23
Chloride, % | 0.40
Fluorine, ppm | 17
Iron, ppm | 380
Zinc, ppm | 120
Manganese, ppm | 100
Copper, ppm | 15
Cobalt, ppm | 0.42
Iodine, ppm | 0.98
Chromium (added), ppm | 0.01
Selenium, ppm | 0.41

Vitamins

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<tr>
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</tr>
<tr>
<td>Riboflavin, ppm</td>
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<tr>
<td>Niacin, ppm</td>
<td>60</td>
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<tr>
<td>Pantothenic Acid, ppm</td>
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<tr>
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<tr>
<td>Ascorbic Acid, mg/gm</td>
<td>—</td>
</tr>
</tbody>
</table>

Calories provided by:

<table>
<thead>
<tr>
<th>Source</th>
<th>Calories</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein, %</td>
<td>25.999</td>
</tr>
<tr>
<td>Fat (ether extract), %</td>
<td>14.276</td>
</tr>
<tr>
<td>Carbohydrates, %</td>
<td>59.725</td>
</tr>
</tbody>
</table>

*Product Code

1. Formulation based on calculated values from the latest ingredient analysis information. Since nutrient composition of natural ingredients varies and some nutrient loss will occur due to manufacturing processes, analysis will differ accordingly.

2. Nutrients expressed as percent of ration except where otherwise indicated. Moisture content is assumed to be 10.0% for the purpose of calculations.

3. NDF = approximately cellulose, hemicellulose and lignin.

4. ADF = approximately cellulose and lignin.

5. Physiological Fuel Value (kcal/gm) = Sum of decimal fractions of protein, fat and carbohydrate (use Nitrogen Free Extract) x 4.9, 4 kcal/gm respectively.
Appendix C: Rat Tail Cuff Blood Pressure Measurement

Blood pressure measurements were done using the CODA High Throughput Non-Invasive Blood Pressure System (Kent Scientific; CODA-HT6). Complete system user guide can be found online at https://www.kentscientific.com/Customer-Content/www/products/Files/CODA-HTManual.pdf.

Briefly, rats are placed in the holders, plastic tubes enclosing animal’s body and exposing the tail for blood pressure measurements. Two cuffs are placed on the tail, occlusion cuff which is close to the base of the tail and distally located volume change detecting cuff. Animals are placed on the warming platforms to increase the blood flow. The system allows simultaneous measurement of 6 animal. The holders are available in two sizes and have an adjustable position of the nose cone to accommodate rats of a different size. Prior to experiment acclimatize animals to the tube, gradually increasing the duration of restraint, to heating, presence of cuffs and allow a few acclimation days of test blood pressure measurements (5 days on average).

Habituate rats in the room before the start of experiment for a minimum of 30min. In the meantime, test the cuffs for malfunctions. Preheat platforms with empty holders placed on them at 39°C (L4). Place rats in the holders, cover with blankets and heat for 10-15min at reduced to 38-35°C (L3-2) temperature. Maintain 32°C (L1) heat for the duration of experiment, monitoring the tail blood volume and rat behavior. Place the cuffs on the tail and begin measurements. One session (one day experiment) includes minimum of 25 and on average 50 cycles. Monitor position of the cuffs for the accurate measurement, as rats tend to move and throw off the distal cuff. Clean tubes and platforms from feces and urine, smell and porphyrin traces with detergent (Sparkleen) between rats and at the end of experiment. Repeat the session over multiple days (3-5) choosing the same time of a day.

At the end of the session export measurements to excel file and set up the table to calculate systolic and diastolic pressure mean of multiple cycles within one day to account for the blood pressure variations and then across multiple days. Apply manual filtering of the accepted by the software cycles. Remove readings that have a tail blood volume of 15ml or less. Use the readings with in-day standard deviations less than 30, with the number of good cycles greater than 15 and mean blood volume be more than 50ml.
Appendix D: Ketoprofen – lysine Prodrug

Structural formula of the Ketoprofen-lysine prodrug was designed by Jukka Leppänen at the University of Kuopio, Finland. The prodrug has a molecular weight of 418.92 g/mol. The structural formula and kinetics of the rat brain uptake of the prodrug were demonstrated in the original work publication (Gynther, M. et al. Brain uptake of ketoprofen-lysine prodrug in rats. (2010) Int. J. Pharm. 399, 121–128). Figures inserted in this chapter were adapted from the original paper and have not been modified.

Fig. 1. Structures and selected properties of ketoprofen and ketoprofen-lysine amide (1). Lysine pro moiety is indicated with red color. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Fig. 3. Kinetics of 1 rat brain uptake. Relationship between the concentration of 1 in the perfusion medium and the brain uptake of 1. K_{inj} and V_{max} are 231.6 ± 60.4 μM and 1.50 ± 0.20 pmol/mg/min, respectively. The possible fraction passive diffusion of the brain uptake was not determined. The data is presented as mean ± SD (n = 3).
Appendix E: Tissue Staining Protocols

E1 Solutions

**Phosphate Buffered Saline (PBS) 0.1M pH 7.35 1L (10X) – STOCK**

Na$_2$HPO$_4$ (anhydrous) 10.9g  
NaH$_2$PO$_4$ (anhydrous) 3.2g or monohydrate (NaH$_2$PO$_4$$\times$H$_2$O) 3.7 g  
NaCl 90g  
Distilled water (dH$_2$O) 1000ml (~980ml)  
Mix to dissolve and adjust pH to 7.35 using HCl and NaOH dropper. Store at room temperature.

**PBS 0.01M pH 7.35 1L (1X) – WORKING SOLUTION**

Dilute 1:10: 1 part PBS 0.1M (10X) stock solution with 9 parts of distilled water:  
PBS 0.1M 100ml  
dH$_2$O 900ml  
Mix to dissolve and adjust pH to 7.35. Store at room temperature.

**Phosphate Buffer Saline with 0.2% TritonX detergent (0.2% PBST) 0.01M**

PBS 0.01M ~500ml  
Triton X-100 1ml  
Use 1ml syringe (without a needle) to draw 1ml of triton. Stir to dissolve completely.

**Parafromaldehyde (PFA) 4% pH 7.35 1L stable for 2 weeks at 4°C**

PFA 40g  
ddH$_2$O 500ml (stir and heat to 30 - max 50°C, then cool down)  
NaOH 10N ~15 drops to help dissolve  
PBS 0.1M 100ml  
ddH$_2$O 390ml (to from 1L solution). Filter before use.

**Phosphate buffer (PB) 0.1M 1L pH7.2-7.3**

Na$_2$HPO$_4$ (anhydrous) 10.9g  
NaH$_2$PO$_4$ (anhydrous) 3.2g or monohydrate (NaH$_2$PO$_4$$\times$H$_2$O) 10.7g  
dH$_2$O 1000ml (~980ml)
Cryoprotectant 1L

- 0.1M PB 500ml
- Sucrose 300g
- Ethylene glycol 300ml
- PVP-40 10g – optional and was not used in these studies
- ddH$_2$O up to 1000ml

Pour a sample of solution in an Eppendorf and put in a freezer overnight to check that cryoprotectant does not freeze.

30% Sucrose 1L

- Sucrose 300g
- ddH$_2$O 1000ml

0.3% Gelatin for mounting brain sections on glass slides

Dissolve 1.5g gelatin powder in 500ml ddH$_2$O, heat (not boil) and stir for better dissolving, cool down to room temperature before use. Keep refrigerated. Good to use within a week. Reheat to room temperature before each use.
E2 Immunohistochemistry Protocol

Primary antibodies:
- Anti-Iba-1, rabbit polyclonal (1:1000; #019-19741, Wako Chemicals USA Inc., Richmond, VA, USA)
- OX-6, mouse monoclonal (1:1000; #554926, BD Pharmingen, Mississauga ON, Canada)
- Anti-GFAP, mouse monoclonal (1:2000; #G3893 Sigma-Aldrich, St Louis MO, USA)
- Anti-NeuN, mouse monoclonal (1:1000; #MAB377 EMD Millipore Corp., USA)
- Anti-synaptophysin, mouse monoclonal (1:1000; #S5768 Sigma-Aldrich, St Louis MO, USA)

Secondary antibody:
- Biotinylated horse anti-mouse IgG (1:500; #31806 Thermo Fisher Scientific)
- Biotinylated goat anti-rabbit IgG (1:500; #31820 Invitrogen, Thermo Fisher Scientific)

Blocking solution reagents:
- Normal horse serum (#7484 Abcam)
- Normal goat serum (#S-100 Vector Laboratories, Inc. Burlingame, CA, USA)

Avidin-biotin complex (ABC) Standard reagent kit (#32020 Thermo Fisher Scientific); (#Vectorstain PK-6102, Vector Laboratories, Inc. Burlingame, CA, USA)

Staining is performed on free-floating sections; all steps are done on a shaker.

Day1
- Wash 6x10min in PBS 0.01M
- Block endogenous peroxidase with 1% H2O2 for 10 min (15min for NeuN) (prepare from 3% stock H2O2 with 0.01M PBS)
- Wash 3x5 min in PBS 0.01M
- Block non-specific tissue binding with 2% blocking solution (1.5% for NeuN) prepared with horse serum (goat serum for Iba-1) in PBST for 1h at room temperature
- Incubate with primary diluted in blocking solution (prepared as in a previous step) for 30min at a room temperature following overnight in fridge (4°C cold room on a shaker)

Day2
- Continue incubation in primary for 30 min at a room temperature
- Wash 3x5 min in PBS 0.01M
• Incubate with secondary diluted in blocking solution (prepared as in day 1) for 1h at room temperature

• Wash 3x5 min in PBS 0.01M

• Incubate with horseradish peroxidase (ACB kit - 2 drops A, 2 drops B in 10ml 0.01M PBST – prepare 1h before use) 1h at room temperature

• Wash 3x5 min in PBS 0.01M

• Incubate in 0.05% diaminobenzidine tetrahydrochloride (DAB, Sigma-Aldrich #D5637) solution. Toxic! Dissolve 10mg DAB, 1 tablet or powder, in 20mL of 0.01M PBS (add 5ml more PBS as will lose later when filtering). Stir for ~15min for a tablet; powder form much faster, and filter through filter paper (Whatman Grade 2/8μm). Add 3% H₂O₂ immediately before use (20μL of 3% stock H₂O₂ in 20mL DAB solution). Dispose properly after.

• Wash 3x5 min in PBS 0.01M

• Mount with 0.3% Gelatin on glass slides and leave to air-dry overnight

**Day3**

• Step done in fumehood. Dehydrate slides sequentially in 50%, 70%, 95%, 100% Ethyl Alcohol (EtOH,) 50/50 EtOH/Xylene for 5 min in each Then 10 min in 100% Xylene. Coverslip with Depex (BDH Chemicals, Poole, UK), remove air bubbles, dry overnight in the fumehood, clean slides the next day.
E3 Luxol Fast Blue Staining Protocol

Solutions:

0.1% Luxol fast blue solution
Luxol fast blue powder, MBS 0.1g
Ethyl Alcohol, 95% 100ml
Glacial acetic acid 0.5ml
Filter after preparation. Can be stored and reused for 5 years.

0.05% Lithium carbonate solution
Lithium carbonate 0.05g
ddH₂O 100ml

Day 1
- Wash 6x10min in PBS 0.01M
- Mount sections on (non ‘+’) slides using water. Do not use gelatin! Fully dry overnight

Day 2
- Step done in fume hood. Dehydrate slides sequentially in 50% → 70% → 95% EtOH for 3 min in each
- Incubate in Luxol fast Blue solution for 16-18h at 56 °C. Make sure the boats are properly sealed to prevent solution evaporation. Better use screw-cap jars, lid should be tightly sealed!

Day 3
- Rinse off excess Luxol fast Blue with 70%EtOH for 1.5-2 min (thicker cut sections use more time), continuously dip and agitate slides in a boat
- Rinse carefully with ddH₂O (2 times x1 min) – dip and agitate in a boat, NOT under running water (sections may detach from slide)
- Differentiate slides in 0.05% Lithium Carbonate solution for 1.5min in the 1st run, then 1min in each following run. Thicker sections require more time (1.5-2min per step). This step takes
as long as necessary to distinguish white matter, can take between 5 and 30 min. On average 3 runs are enough. If in doubt, take out a bit earlier rather than later.

- Rinse with distilled water (2 times x 1min) – dip, agitate in a boat
- Dehydrate in 100% EtOH (2 times x 5min), then in Xylene (2 times x 5min)
- Coverslip with Depex resinous medium.
Appendix F: Microscopy and Image Analysis

F1 Image Acquisition on the Microscope

Tissue sections were imaged on the Nikon Eclipse Ni-E upright microscope with a Nikon DS Fi2 color camera head (NIS Elements Imaging; Mississauga, ON) using NIS-Elements Imaging Software Version 4.30.02 (Nikon Instruments Inc., Melville, NY).

Microscope settings:

Lamps (DIA enabled, fixed) at 43-48, LUT function disabled, Exposure 10ms, Analogue gain 1.0, Zoom 1.0x, no Filters on Turet1, FL1 fluorescent shutter is off.

Using 2X objective: Aperture 30.6mm, Field Stop 30.6mm, condenser 5 2/4x; 10X objective: Aperture 11.5mm, Field Stop 10.5mm, condenser 7N1; 20X objective: Aperture 19.1mm, Field Stop 5.2mm, condenser 6N2.

Scanning image:

For every tissue section set white balance using a probe (rectangle icon) on a no-tissue spot on a slide (direct light) and applying AWB (auto white balance) function found on the tool bar at the top.

Adjust focus manually prior to scanning either a single focus for the entire image or using a focus surface which allows to pre-set different focus on multiple points on the tissue, particularly when the slide preparation resulted in uneven surface and requires different focus depth to create a sharp image.

Select on a top tool bar Acquire → Scan Large Image: set scanning objectives (i.e. capture 2x – scan 10x), set area of scanning manually defining number and position of fields, set stitching overlap at 20%, shading off, automatic postprocessing off, select Focus manually at start (or Focus surface). Extended depth of focus (EDF) is available for creating a Z-stacked image scanned at various depth (select steps in um, range) and combining sharp signal from multiple layers into one image.
Capture function can be used for smaller images which do not require stitching (camera button on top tool bar). This option is also available with EDF→ Real time EDF – select range (top and bottom sharp focus) and step length (e.g. 0.9μm) for Z-series acquisition.

Save an image as TIFF for publication purposes, as JPEG for large images and JPEG or TIFF for small image for Image J or microscope software analysis.
F2 Image Acquisition using Aperio

Aperio digital entire-slide scanner (Department of Pathology, Western University, London, Ontario, Canada), allowing 20x magnification, was used to acquire images of entire series of brain sections for initial scanning of activated microglia location and of Luxol Fast Blue stained tissue for myelination analysis. Aperio ImageScope software (Leica Biosystems Pathology Imaging), version 12.3.2.8013 was used to extract images of specific regions of interest for further ImageJ analysis.

Choose magnification (zoom slider) and select Camera button on the top tool bar (Scanshot) to acquire image of the region at a fixed resolution and with a scale bar which can not be removed from the image. Image can be saved as TIFF/JPEG, however, is suitable for analysis and a low magnification full-section image, but not publication. Extract region tool (floppy disk and arrow icon on the top tool bar) allows to obtain an image of a selected region with variable resolution: click and drag on the image, in the opened window define output format – TIFF or JPEG, LZW compression, % extraction (80-100 on average), extract. This function does not capture scale bar. Both methods can be used together to create a high-quality image with defined scale bar.
F3 Image Analysis

White matter analysis

Image analysis was done in the ImageJ program (Version 1.48u4, Wayne Rasband, National Institutes of Health, Bethesda, MD, USA). Prior to quantification set a scale: use a line tool to outline the scale on the image and measure its length in pixels (Analyze→ Measure or Ctrl+M); open Analyze→ Set scale, input measured and known length (in um), set pixel aspect ratio to 1.0 and apply Global to use this scale (pixel/um) for a series of images in one ImageJ session. Then Analyze→ Set measurements: area, min and max gray value, integrated density (include raw integrated density = sum of pixel values), mean gray value and area fraction.

To create selection for the region of interest (ROI), use the stereotaxic atlas to define ROI’s boundaries. Outline the ROI with a polygon tool avoiding edges (DAB accumulates) and excluding artifacts overlapping with the ROI. To add selection to the ROI Manager use Edit→ Selection→ Add to manager (Ctrl+T), then save all ROIs for an animal by selecting all and ROI Manager→ More→ Save. ROI shape and size can be changed by manipulating individual points on selection (hold and drag, add point Shift+left mouse click, remove point Ctrl+left click) and ROI can be updated in the Manager (ROI should be selected in the manager list prior to changes). If you click outside of the selection, it will disappear from the image. To bring it back, use Edit→ Selection→ Restore selection (Ctrl+Shift+E). Do not crop the image to minimize the field to ROI, as such ROI saved will not match the region boundaries on the original image when re-opening the image.

If a part of the original full selection is needed for a separate analysis (e.g. PVCC regions from the entire corpus callosum selection), use rectangle/polygon outline tool to draw a selection around the region to be cropped out, and add this new selection to ROI Manager. Select a newly added ROI and Edit→ Selection→ Make inverse (note a fine colored line at the perimeter of the image) program window), add to manager, select both full and inverse selections in the Manager→ More→ AND→ this will generate a new selection with undesired region cropped from the full selection. In the case of bilateral analysis (e.g. fimbria), combine several individual ROIs by selecting those ROIs in Manager→ More→ OR (combine). Add newly created ROI to the Manager and save.
Convert image to black-white format: Image ➔ Type ➔ 8-bit. Measure ROI area. Process ➔ Subtract background (select “light” as the signal is dark; rolling ball radius 50 pixels) and finally Image ➔ Adjust ➔ Threshold (Ctrl+Shift+T) to an adequate maximum (233-248 depending on the stain). Do not apply threshold, limit measurement to threshold (area should not be 100%) and do the measurement. Copy all measurements and paste into excel file to calculate weighted average for each of the ROIs per animal and a final sum.

**Automatic cell count**

Neuronal cell count was done using the NIS-Elements Imaging Software Version 4.30.02 (Nikon Instruments Inc., Melville, NY). Follow the following steps: select Binary ➔ Spot detection: dark spots (spot 9 pixels, typical diameter 13.5um or size of cell body, contrast 20.1); methods – select dark, clustered, blue channel, apply to current frame, preview. ROI ➔ Move binary to ROI ➔ see the number appearing when right-click on image – number of selected ROIs represent number of cell bodies. If only a selection from entire image area needs to be analyzed, go to measure using rectangle tool (record the area and dimensions of the selection for consistent application), crop at the end of a measured box, delete the box and do same later.
Curriculum Vitae

Nadezda Ivanova

Education and Degrees

PhD, Schulich School of Medicine and Dentistry, The University of Western Ontario 2014-2019
PhD research student in the Department of Anatomy and Cell Biology
Supervisor: Dr David Cechetto

Medical Residency, Kemerovo State Medical Academy, Kemerovo, Russia 2012 – 2013
Residency training in General Internal Medicine

Doctor of Medicine, Kemerovo State Medical Academy, Kemerovo, Russia 2006 – 2012
Graduated with High Distinction

Academic Experience

Teaching assistantship, Anatomy and Cell Biology Department, The University of Western Ontario

ANATCELL 3309 Mammalian Histology 2014-2019

This 3rd year undergraduate course is a full year course with an average enrollment of 200 students. The course program includes a detailed study of the principal organization of various tissues in the human and other mammals’ body using microscopy with a following integration of basic cellular and tissue knowledge to study organ systems structure. I have been a TA for the online (2014 – 2015, full year) and face-to-face (2015 – 2019, full year TA) sections. My responsibilities were to assist students during the weekly laboratory practice, answer their questions and guide in completing lab assignments. Further duties included grading laboratory assignments and quizzes, proctoring and grading mid-term and final exams. I had also given several 40-minute introductory talks at the beginning of the laboratory on the following topics: supporting tissue, female reproductive system, ear, digestive (gastrointestinal part) system. In the 2017-2018 academic year, I have worked as a Head TA. In addition to the regular responsibilities, my duties included assistance with the lectures (i.e. recording system set up, managing students’ questions), monitoring email correspondence and online platform Echo 360 and responding to students’ questions and concerns. As a Head TA, I have supervised the online TAs team and led marking sessions ensuring consistency in assignment and quiz grading. I have contributed to the composition of the list of TA duties improving the allocation of TA duties and efficiency of the use of contract hours.

ANATCELL 4451 Integrative Neuroscience 2018 (Fall term)

This one-term course combines 4th year undergraduate and graduate students. The program repeats basic neuroscience concepts during lectures and practical laboratories, focuses on the integration of this
knowledge to understand the connection of behavior with brain processes in health and disease and introduces the principals of experiment design and planning. My primary role as a TA was to participate in designing the histology laboratory assignment and to plan and lead the histology laboratory. I have also graded the assignments and held office hours for students, as well as proctored final examination.

**Supervising experience, Anatomy and Cell Biology Department, The University of Western Ontario**  
2015 – 2018

Supervised 7 undergraduate students working as volunteers in the research laboratory assisting with my research project. Encouraged and hands-on learning of experimental techniques, data analysis, analytical and critical thinking.

**Scholarships and Awards**

Western Graduate Research Scholarship (5x)  
2014-2019

Anatomy and Cell Biology Travel Prize, 500  
2015

Gold Medal for high school graduation with distinction  
2006

**Conference presentations**

"Negative impact of Ketoprofen-lysine treatment on cerebral pathology and cognition in Hypercaloric diet-induced metabolic syndrome and Alzheimer's disease rat study "

Co-authors: Weishaupt N, Whitehead SN, Cechetto DF. Poster presentation.

- Anatomy and Cell Biology Research Day, 2017. Western University, London, ON, Canada

"Hypercaloric diet-induced metabolic syndrome increases white matter inflammation and cognitive deficits in a transgenic rat model of Alzheimer's disease"

Co-authors: Weishaupt N, Whitehead SN, Cechetto DF. Poster presentation.

- Anatomy and Cell Biology Research Day, 2017. Western University, London, ON, Canada
- Society for Neuroscience Conference, 2016. San Diego, CA, USA

"Interactions between Alzheimer’s disease and Metabolic Syndrome” and updates

Co-authors: Weishaupt N, Whitehead SN, Cechetto DF. Poster presentation.

- Annual Canadian Neuroscience Meeting, 2016. Toronto, ON, Canada
- Society for Neuroscience Conference, 2015. Chicago, IL, USA
- Southern Ontario Neuroscience Association Conference, 2015. McMaster University, Hamilton, ON, Canada

**Annual provincial scientific conference of young researchers, Kemerovo, Russia**

**Oral and poster presentations**

- "Legal aspects of medical services provided on a paid basis". Medicine in Kuzbass, 2011
- "Training in medical legislation for pediatricians as the guarantor for formation of healthy generation". Co-authors: Gracheva T, Oliferchuk M, Medintex, 2009
- "Healthy lifestyle teaching begins with the medical legislation knowledge". Co-authors: Gracheva T, Oliferchuk M, Medintex, 2009.
- "Healthy eating for students". Co-authors: Mukhinova E, Medicine in Kuzbass, 2008.

**Community engagement**

**Chief steward for Graduate Teaching Assistants (GTA) and Postdocs Union** 2017-2018

I served as a chief steward to the Biosciences division to the GTA and postdoc Union (PSAC Local 610) at the University of Western Ontario. I managed a team of 25 departmental stewards, established trusted and supportive environment to ensure an excellent service for the members. I have organized regular team meetings to educate stewards on their roles and provide necessary information to disseminate among TAs. As a part of my duties I have delivered oral presentations on the Union organization, role, duties and benefits to members and departmental faculty at departmental orientation meetings, and trained new chief stewards elected for the term following mine. I have also been actively involved in planning, organizing and running solidarity events for members to update them on the Union’s work, provide information on their rights and benefits, collect work-related concerns as well as discuss acting strategies. I have been an active participant in the grievance process including communication with the member and the employer including meetings, which led to a successful resolution. Being on the executive committee, I participated in major Union decision making and execution, have promoted and arranged sponsorship for the biology graduate research forum, a student organized conference the University of Western Ontario, to support graduate research. As a member of scholarship committee, I reviewed applicant’s submission packages, rated candidates and chose the recipients of union’s scholarships. I was also an elected member of the Bargaining team and mobilization committee, negotiating a new collective agreement with the University over several months.

**Medical student council, Kemerovo State Medical Academy, Russia** 2006-2007
Participated in planning, organization of student social events and recreational stage performances.

**Volunteer to Russian Red Cross Society**

2011

Helped at the donation center with organizing donated items, compiling gift packages for events at children’s shelter.