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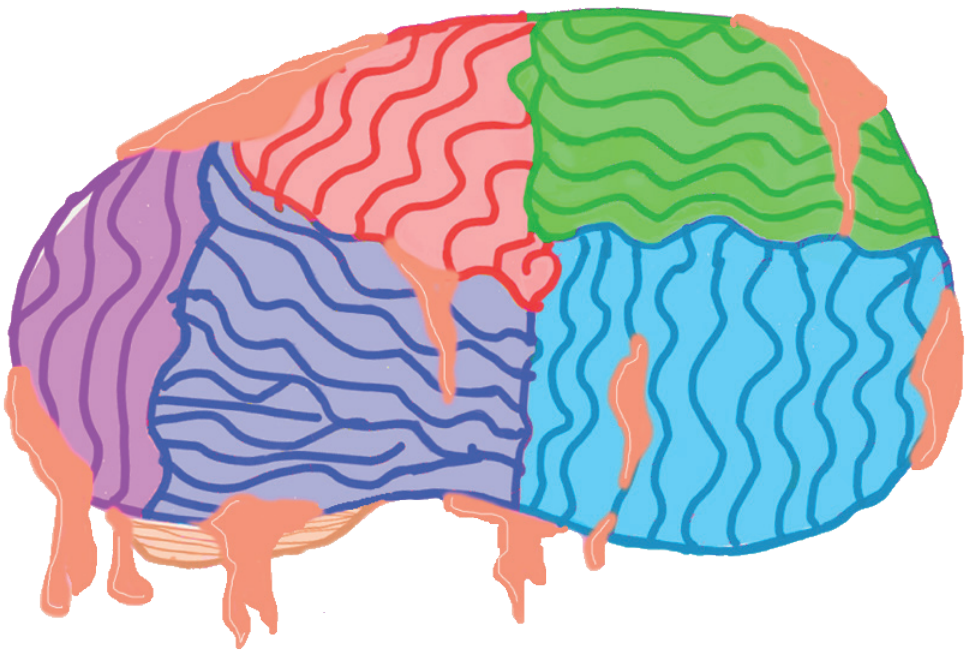
Deciphering the Role of 27-Hydroxycholesterol in Neurodegeneration



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DECIPHERING THE ROLE OF 27-HYDROXYCHOLESTEROL IN NEURODEGENERATION

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On the Cover: An abstract depiction of the brain with excess 27-hydroxycholesterol.
Designed and crafted by my niece, Maryam.

Deciphering The Role Of 27-Hydroxycholesterol In Neurodegeneration

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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In loving memory of the strong, resourceful women whose intelligence, vision and perseverance have shaped not only the future of my parents but the generations to succeed them.
My Great-Grandmother Salha and Grandmother Hayat

FOREWORD

While this thesis is the culmination of a few years, it is based and adds upon the work of many that have dared to tread into the unknown. A path not studded with eureka moments, rather many pondering nights and slaving days to reveal the truths behind these devastating diseases. My journey is not much different from that of my predecessors; its start begins with a curious mind and barely any research skills whatsoever. Having to learn everything, including the somewhat basic ability to pipette, the patience of the people around me catapulted that process tremendously. It was the fun-loving atmosphere that kept that laborious work going especially when the bouts of work got overwhelmingly tedious. The one-team mentality shared among all our group members cemented that environment into one of the most effective and cohesive groups within our department. The members were hand picked and within a field with so many socially awkward disciples, Ángel made sure that none passed his iron curtain of sanity.

Science driven by direct irrefutable empirical evidence was the pursuit we were after, however being bombarded with expert opinions guided by scant research and a dogmatic perspective was more the reality. This was evident since we dared to defy Alzheimer's research by publishing without emphasizing the canonical amyloid beta, thus being honored with the Oscar of most rejections for a single paper being 12. This continued with our successive paper, still struggling with its acceptance 20 months from its first submission. Of course you might say we are biased and possess the mentality that the world is against us. I tell you when you write a second response to the one reviewer (the other two were happy with our second modified manuscript) who persistently rejects not only your results but also that of many highlighted in a rebuttal letter spanning 8 pages and 37 references, only to be further coerced by an editor to reply in a more polite and less combative manner and stating that the acceptance lies in his/her hand, after which the reviewer stubbornly denies the widespread existence of a protein in the brain, then you can't possibly think science is based on empirical evidence.

Yet there is much hope in the field with many audaciously formulating new threads of thought that may very well lead to the better understanding of Alzheimer's disease and ultimately its treatment. To them I salute and acknowledge their uphill march not only in challenging the status quo, but their extensive struggle in achieving great quality science.

ABSTRACT

Cholesterol is constantly attributed to neurodegenerative diseases including Alzheimer's disease (AD). This is due to the association of high blood cholesterol and the consequences of cholesterol metabolism in these conditions. However the brain isolates itself from the peripheral cholesterol with the use of its impervious barrier, therefore how does cholesterol influence the brain?

Converted into the oxidized form known as 27-hydroxycholesterol (27-OH), extracerebral cholesterol is able to circumvent this blood brain barrier and enter the brain's refuge. Some studies point to the effect of this oxysterol on brain cells, indicating that this metabolite is more than a cholesterol intermediate. Herein, we dissect the role this cholesterol metabolite undertakes in the brain both *in vitro* and *in vivo*, while probing its influence on neurodegenerative diseases.

In **Paper I** we elucidated the levels of 27-OH in both the cerebrospinal fluid (CSF) and plasma of mild cognitively impaired (MCI) and AD patients. In addition to correlating it to AD markers, we further investigated its relationship with the angiotensin-converting enzyme (ACE), which was increased in AD as previously described. Delving deeper uncovered 27-OH had more of a causal link than a mere correlation with the renin-angiotensin system (RAS) in the brain highlighted in **Paper I** and **Paper II**. This system, apart from being independent from the renal system, is involved in many processes in the brain including learning and memory. The repercussions of 27-OH on the brain RAS was further investigated *in vivo* in **Paper II**, in mice lacking the ability to produce 27-OH, and in humans. Furthermore, the effects of 27-OH were also observed in mice fed a high-fat diet, a connection that is further investigated in all the papers within this thesis. These results draw to attention the relationship between plasma cholesterol, hypertension and neurodegeneration.

In addition to affecting the spatial memory of mice, 27-OH reduced the levels of glucose uptake in their brains. Emphasized in **Paper III**, the mechanistic link between 27-OH and glucose metabolism was examined, explicating several therapeutic targets. The biomarker suitability of 27-OH was tested in a small proof of concept study utilizing ^{18}F- fluorodeoxyglucose positron emission tomography (^{18}F-FDG PET). Results illustrated a negative correlation, thus having higher levels of 27-OH in the CSF is associated with lower glucose uptake levels in the brain of individuals with ramifications on memory.

Inflammation is an important part of AD and is thought to occur very early in the disease progression. Moreover, peripherally 27-OH plays a part in mediating atherosclerosis via its proinflammatory actions. To this end, we hypothesized that 27-OH may have similar actions in

the brain and the resulting data is presented in **Paper IV**. 27-OH increased the levels of the inflammatory mediator S100A8 in addition to its receptor, Receptor for Advanced Glycation Endproduct (RAGE) and the downstream β-secretase 1 (BACE1) observed in several mice models. S100A8 was previously described by us to be increased in the brains of AD mice models and may accumulate even before amyloid plaque formation. This study therefore accentuates the function of 27-OH in propagating inflammation in the brain, a function recognized peripherally.

Neurodegenerative diseases such as AD are debilitating conditions affecting the lives of millions; however to this date the causes of these overwhelming disorders remains unclear. While the levels of CSF 27-OH are likely to be increased in many AD patients, little is known as to the ramifications of this increase. Herein, we try to bridge this gap and propose several mechanistic links between blood cholesterol and neurodegenerative diseases while underscoring several plausible therapeutic targets, in an attempt to further empower our fight against AD.

LIST OF SCIENTIFIC PAPERS

This doctoral thesis is based on the following original papers, referred to in the text by their Roman numerals.

I. Mateos L, **Ismail MAM**, Gil-Bea FJ, Leoni V, Winblad B, Björkhem I, Cedazo-Mínguez A. Upregulation of brain renin angiotensin system by 27-hydroxycholesterol in Alzheimer's disease. J Alzheimers Dis. 2011;24(4):669-79.

II. Mateos L, **Ismail MAM**, Gil-Bea FJ, Schüle R, Schöls L, Heverin M, Folkesson R, Björkhem I, Cedazo-Mínguez A. Side chain-oxidized oxysterols regulate the brain renin-angiotensin system through a liver X receptor-dependent mechanism. J Biol Chem. 2011 Jul 22;286(29):25574-85.

III. **Ismail MAM***, Mateos L*, Maioli S., Merino-Serrais P., Ali Z., Lodeiro M., Westman E., Leitersdorf E., Gulyás B., Olof-Wahlund L., Winblad B., Savitcheva I., Björkhem I, Cedazo-Mínguez A. 27-Hydroxycholesterol Impairs Neuronal Glucose Uptake Through An IRAP/GLUT4 System Dysregulation. *Submitted* *These authors contributed equally to this work.

IV. **Ismail MAM**, Lodeiro M., Loera-Valencia R., Rodriguez-Rodriguez P., Maioli S., Mateos L, Merino-Serrais P., Björkhem I, Puerta E., Cedazo-Mínguez A. Hypercholesterolemia and 27-hydroxycholesterol enhance S100A8 aggregation and RAGE expression in the brain; a link towards amyloid-beta accumulation. *Manuscript*

OTHER PUBLICATIONS BY THE AUTHOR NOT INCLUDED IN THE THESIS

Lodeiro M, Puerta E, **Ismail MAM**, Rodriguez-Rodriguez P, Rönnbäck A, Codita A, Parrado-Fernandez C, Maioli S, Gil-Bea F, Merino-Serrais P, Cedazo-Minguez A. Aggregation of the Inflammatory S100A8 Precedes Aβ Plaque Formation in Transgenic APP Mice: Positive Feedback for S100A8 and Aβ Productions. J Gerontol A Biol Sci Med Sci. 2016 Apr 30.

Maioli S, Båvner A, Ali Z, Heverin M, **Ismail MAM**, Puerta E, Olin M, Saeed A, Shafaati M, Parini P, Cedazo-Minguez A, Björkhem I. Is it possible to improve memory function by upregulation of the cholesterol 24S-hydroxylase (CYP46A1) in the brain? PLoS One. 2013 Jul 16;8(7):e68534.

Mateos L, **Ismail MAM**, Winblad B, Cedazo-Mínguez A. side-chain-oxidized oxysterols upregulate ACE2 and Mas receptor in rat primary neurons. Neurodegener Dis. 2012;10(1-4):313-6.

Cedazo-Mínguez A, **Ismail MAM**, Mateos L. Plasma cholesterol and risk for late-onset Alzheimer's disease. Expert Rev Neurother. 2011 Apr;11(4):495-8.

CONTENTS

LIST OF ABBREVIATIONS

^{18}F-FDG	^{18}F Fluorodeoxyglucose
22(R)-OH	22(R)-Hydroxycholesterol
22(S)-OH	22(S)-Hydroxycholesterol
24S-OH	24S-Hydroxycholesterol
27-OH	27-Hydroxycholesterol
7-HOCA	7α-hydroxy-3-oxo-4-cholestenoic acid
ACE	Angiotensin-Converting Enzyme
aCSF	Artificial Cerebrospinal Fluid
AD	Alzheimer's Disease
AGT	Angiotensinogen
Ang	Angiotensin
AP	Aminopeptidase
ApoE	Apolipoprotein E
APP	Amyloid Precursor Protein
Arc	Activity- Regulated Cytoskeleton-Associated Protein
AT1R	Angiotensin II Type 1 Receptor
Aβ	Amyloid Beta
BACE1	β-secretase 1
BBB	Blood Brain Barrier
CSF	Cerebrospinal Fluid
CVD	Cardiovascular Disease
DSM	Diagnostic and Statistical Manual of Mental Disorders
eoAD	Early-Onset forms of Alzheimer's Disease
ER	Estrogen Receptor
GLUT	Glucose transporter
GWAS	Genome-Wide Association Study
HDL	High-Density Lipoprotein
HFD	High Fat Diet
ICD-10	International Classification of Mental and Behavioural Disorders
ICV	Intracerebroventricular
IL-1β	Interleukin-1β
IL-6	Interleukin-6
IRAP	Insulin-Regulated Aminopeptidase
LDL	Low-Density Lipoprotein
LRP1	Low-Density Lipoprotein Receptor-Related Protein 1
LXR	Liver X Receptor
MCI	Mild Cognitive Impairment
MMSE	Mini-Mental State Examination
MRI	Magnetic Resonance Imaging
ND	Normal Diet
NF-kB	Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells
PD	Parkinson Disease
PET	Positron Emission Tomography
PS	Presenilin
RAGE	Receptor for Advanced Glycation Endproduct
RAS	Renin-Angiotensin System
RXR	Retinoid X Receptor
STZ	Streptozotocin
TNF-α	Tumor necrosis factor-α
VaD	Vascular Dementia

1 INTRODUCTION

1.1 NEURODEGENERATION

The definition of neurodegeneration, as you would have already formulated in your mind by now, is roughly the degeneration or loss of function of neurons. While this classification appears to be obvious, the reality is much more complicated. Conditions such as brain tumors, epilepsy and multiple sclerosis, despite the involvement of neuronal damage, are generally not considered neurodegenerative diseases[1]. Therefore when neurodegenerative diseases are mentioned, the more commonly discussed are diseases such as Alzheimer's disease (AD), Parkinson disease (PD) and Huntington disease. According to the EU Joint Programme-Neurodegenerative Disease Research (JPND), the largest research initiative tackling neurodegeneration, neurodegenerative disease is a term that encompasses a range of diseases resulting in incurable and debilitating states from progressive neuronal death, with a focus on movement or memory disorders[2]. Neurodegenerative diseases are approximated to be in the hundreds, and their classification is constantly challenged by the superimposed depiction of the many diseases both clinically and pathologically. While I will not delve into attempting to classify and discuss these myriad diseases, Przedborski *et al.* made a brilliant effort at just that[3]. This thesis will focus more on the memory aspect of neurodegenerative diseases, of which AD is the most prevalent form.

1.2 DEMENTIA

Dementia in itself is also an umbrella term for a spectrum of diseases that afflict a person's memory and lead to cognitive impairment. Even though memory seems to be the major complaint, other cognitive domains such as language and orientation must be impaired for a diagnosis to be given. The progression of these symptoms eventually affects an individual's ability to cope with their daily living and thus cripple their independence. The prevalence of dementia subtypes include 32% vascular dementia, 8% Lewy body dementia, 3% PD associated dementia, 3% Frontal Temporal dementia and the lion share belonging to AD, contributing to 41% with many speculating it to be up to 70%[4,5]. It is important to note that this demarcation between the different dementia subtypes is not strict, with mixed pathologies being more frequent than pure[6].

1.3 ALZHEIMER'S DISEASE

Being characterized by the presence of neurofibrillary tau tangles and amyloid plaques, the loss of synapses and neurons in cortical areas are also important features of the disease. While the cause is still unknown, speculations regarding the etiology are monopolized by the amyloid cascade

hypothesis[7], which states that the disease is driven by the deposition of amyloid beta (Aβ). Even though this hypothesis is partly generated from the genetic data implicating mutations in the Amyloid Precursor Protein *(APP)*, several other genetic risk factors have been determined and are summarized in Fig 1. These genes are considered more common within the population, although their risk in producing the disease dwarfs in comparison to the inevitability of the deterministic *APP* and *presenilin* (*PS*) 1 and 2 mutations[8], known to ultimately cause the early-onset forms of AD (eoAD).

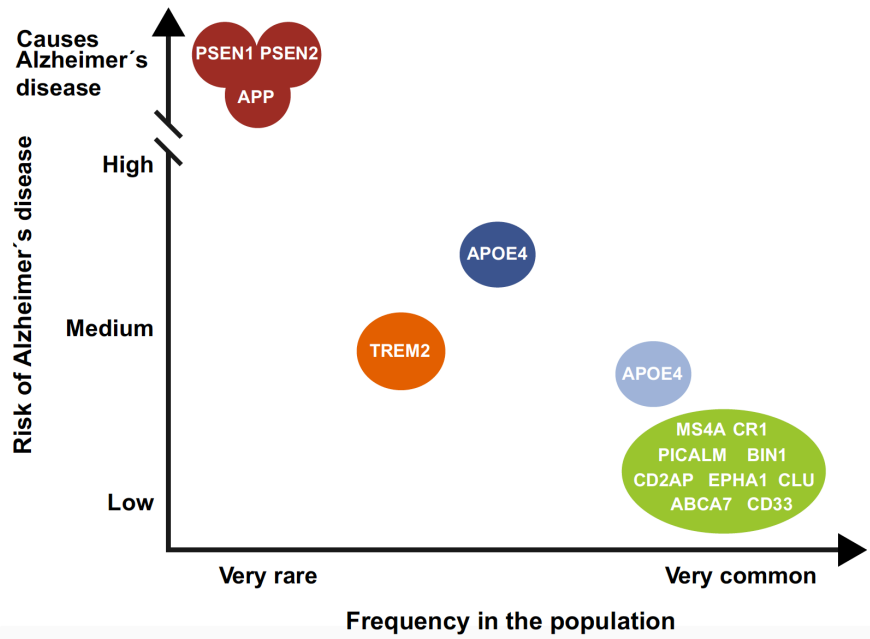

Fig. 1. GWAS determined genes plotted with regards to its relative risk to AD and its frequency in the population. Reproduced from Hardy, J. *et al.* Pathways to Alzheimer's disease. *Journal of Internal Medicine* 275, 296-303, doi:10.1111/joim.12192 (2014)[8] under the Creative Commons license.

These deterministic genes have however been erroneously used experimentally to model the sporadic forms[9], even though genome-wide association study (GWAS) has unveiled that the frequent genes point towards several other mechanisms such as endosome vesicle recycling, cholesterol and the immune system[10], which have been extensively investigated with regards to their mechanistic contribution to the disease. The effects of these systems have been superimposed onto the amyloid cascade, by investigating them in both *in vitro* and *in vivo* eoAD disease models thus the interpretation of this data has to be done with caution when translating the results into sporadic AD. It is equally important to highlight that cognitively normal

individuals exhibiting progressive AD pathology do not develop dementia[6,11]. This incongruence has contributed to the current status in therapeutic targeting of AD, with the vast majority of pharmacological clinic trials being unsuccessful[12]. This predicament accentuates the need for the adequate modeling of the sporadic forms of AD.

1.4 RISK FACTORS

Age is without a doubt the most important risk factor for AD, however so far the second most influential factor is the genetic ε4 allele of the Apolipoprotein E (ApoE). This allele not only increased the risk for AD threefold and fifteen times when homozygous, it also reduces the onset of the disease whether the sporadic form or eoAD[13]. Only 14% of the global population express the ε4 allele, with the vast majority carrying the ε3 allele[14]. Furthermore, studies have shown that in combination with APOEε4, environmental risk factors augmented not only the risk but also the severity of the disease[15,16]. These included chronic diseases such as diabetes, elevated midlife blood cholesterol levels, depression and hypertension in addition to lifestyle factors such as alcohol consumption, physical and cognitive activity[17]. Some of these, identified as modifiable risk factors had a combined attributable risk of half the worldwide cases of AD[18]. The targeting of these factors, exhibited in a recent multimodal clinical trial with at-risk elderly population[19], revealed a 25-150% improvement in cognitive outcome in comparison to the control group. These findings emphasize the importance of these risk factors in AD or indeed cognitive decline. Far from being an extensive review, the following section encapsulates a short account of some of these modifiable factors.

1.4.1 Hypertension

The relationship between hypertension and AD or dementia has been vastly covered with many considering it a risk factor for all types of dementia[20]. Although inconsistencies exist between the various studies, age appeared to be the contradicting factor[21,22]. Late life hypertension was not associated with the risk for AD or dementia; on the contrary hypotension, as well as patients taking antihypertensive medication have been associated to the risk for AD[20]. Furthermore, the effect of having high blood pressure in midlife was assessed in several longitudinal studies, which asserted that cognition was consistently negatively influenced[22]. The use of anti-hypertensive drugs reduced the risk of dementia[23,24], though this was not observed in a recent meta-analysis conducted on several randomized control trials[25]. Nonetheless they noticed significantly less cognitive decline in the treated group.

Explaining the mechanistic link between hypertension and dementia is still controversial, proposed mechanisms include atherosclerotic lesions and/or hemodynamic insufficiency[22].

Blood pressure is regulated by complex mechanisms, most importantly by the systemic endocrinal renin angiotensin system (RAS). The injection of angiotensin (Ang) II intracerebroventricularly caused brain-orchestrated hypertension and ultimately led to the belief that RAS is present centrally[26]. This brain RAS, entirely autonomous from the peripheral system, is known to regulate hypertension and is currently targeted for the control of peripheral blood pressure[27]. Apart from its regulatory power over blood pressure, central RAS modulates cerebral blood flow, the autonomic system and more importantly within the context of this thesis, memory processing[28]. This central system is also presently recognized in several diseases involving the brain such as AD, stroke and depression[29]. One proposed interaction between central RAS and AD is based on the binding of ACE to $A\beta_{1-42}$, and long-term treatment with ACE inhibitors increased the deposit of $A\beta_{1-42}$ in old age AD mouse model. Furthermore, hypertension and memory impairment were seen in mice chronically infused with AngII[30], whereas the use of an angiotensin receptor blocker reduced the Aβ-induced cognitive impairment in an AD mouse model[31]. Taken together, these data further implicates brain RAS with neurological diseases, though more studies are required to discover both the underlying basic mechanism and new therapeutic targets.

1.4.2 Diabetes

With several groups calling it diabetes type 3, AD has many features in common with the metabolic disease. Advanced AD patient brains revealed decreased levels of both insulin and its receptor despite the patients not having diabetes[32]. This is especially important as the signaling of insulin, shown to be impaired in AD, plays a crucial role in tau phosphorylation, the key protein in the development of neurofibrillary tangles present in several tauopathies[33]. Insulin signaling is also vital for the survival and function of both neurons and oligodendrocytes[34]. Moreover, streptozotocin (STZ) brain injections revealed AD neuropathological features including brain atrophy and increased immunoreactivity towards Aβ, in addition to higher tau mRNA levels in the STZ-injected rats[35]. Furthermore, the intranasal injection of insulin in a triple transgenic AD mouse restored insulin signaling, increased synaptic proteins and reduced $A\beta_{1-40}$ levels[36], accentuating the role insulin signaling has in AD.

An important feature of diabetes is hyperglycemia, however no data exists to suggest that excess glucose is present in AD brains, a critical point that would rebuke the diabetes type 3 hypothesis[37]. On the other hand, evidence exists to link hypoglycemia to cognitive dysfunction, therefore supporting glucose hypometabolism as an important feature of AD[38,39]. AD-associated glucose hypometabolism is thought to occur relatively early in the course of the

disease[40]. In fact, increasing the presence of glucose in the brain potentiates memory processing[41]. While dysfunctional insulin signaling, a complex cascade in itself, is not enough to explain the pathology observed in AD, glucose transporters (GLUT) have been shown to be implicated[42].

Several GLUTs are present in the brain including GLUT1, with two isoforms expressed in endothelial cells lining the blood brain barrier (BBB) and astrocytes, is mainly involved in mediating glucose into the brain[43]. GLUT3, the main neuronal transporter, is also present in glia and is expressed throughout the brain, exhibiting a higher affinity to glucose than both GLUT1 and GLUT4[44]. A lingering misconception is that the insulin sensitive-GLUT4 is only expressed in the hippocampus, however this transporter is observed throughout the brain including the hypothalamus, motor cortex, piriform and entorhinal cortices, most neocortical areas, as well as in different nuclei of the limbic and motor systems, pituitary, olfactory bulb and basal ganglia[45-50]. Furthermore, the glucose transport capability of neurons (via GLUT3 and GLUT4) surpasses that of astrocytic GLUT1, based on the kinetic characteristics and the cellular concentration of glucose in both neurons and glia[51]. This is consistent with the notion that synaptic connectivity and communication bears the largest demand for energy in the brain[52].

Not many studies have investigated GLUT4 in AD, though a recent study observed decreased levels in neurons treated with exogenous $A\beta_{1-42}$[53]. Although one could argue that glucose alterations are seen earlier than amyloid deposition, a recent study revealed that decreased levels of low-density lipoprotein receptor-related protein 1 (LRP1), implicated in the pathogenesis of AD, led to insulin resistance and GLUT4 reduction[54]. In AD, GLUT1 and GLUT3 are also decreased, suggesting that this reduction in GLUTs may possibly contribute to the observed reduction in glucose metabolism[55]. This reduction not only correlates with the progression of AD, it could also contribute to diagnosis[56]. Remarkably, population-based studies have also observed the link between diabetes and AD, where some studies have correlated diabetes to an increased risk for AD by approximately 50%, independent of vascular dementia[57].

1.4.3 Hypercholesterolemia

Cholesterol was first featured in dementia as a differential diagnostic marker between AD and multi-infarct dementia[58]. In the decades to follow, a plethora of epidemiological data correlated high cholesterol levels in the blood to the increased risk of AD[59]. A few studies have also shown a positive correlation of higher cholesterol with the risk for dementia in older individuals[60,61]. This was further strengthened by studies that affirmed the risk of AD with higher cholesterol was

most significant in mid-life or more recently early AD[62-64]. This relationship between blood cholesterol and AD was confirmed *in vivo* where feeding a high fat diet to animals led to AD neuropathological features[65,66]. Furthermore, the cholesterol transporter APOEε4, as previously mentioned, increased the risk for AD[13] and is associated with increased low-density lipoprotein (LDL) cholesterol in the blood[67]. The metabolic syndrome, composed of high blood pressure, blood glucose and abnormal blood cholesterol levels, was also seen to increase the risk of AD among other diseases[68]. Nonetheless, these conditions are also considered to be modifiable risk factors for dementia, and targeting these factors is now considered part of the prevention of AD[5], hence solidifying the contention that peripheral cholesterol may have a possible role in mediating AD. Despite all these evidence pointing to the importance of blood cholesterol in AD, cholesterol does not cross the BBB, therefore how does it impose its action on the brain? This will be further discussed in the next section.

1.5 CHOLESTEROL AND THE BRAIN; AN INTIMATE AND COMPLEX RELATIONSHIP

The brain is isolated from the rest of the body by the function of the BBB. This barrier, with its tight junctions, prevents many substances present in the blood from entering the brain's refuge, including cholesterol. Therefore, how does the brain, which contains 25% of the body's cholesterol[69], obtain its cholesterol? All the cholesterol in the brain is thus produced locally, with synthesis being considerably high during development and substantially reduced during adulthood[70,71]. Interestingly, this reduction is thought to increase the half-life of brain cholesterol to span five years[72].

Cholesterol is synthesized from acetyl-CoA through an arduous and energy-taxing series of conversions that takes place mainly in oligodendrocytes and astrocytes[73,74]. Although neurons are also capable of producing cholesterol, they postnatally mainly rely on astrocytes for its cholesterol, while oligodendrocytes provide myelin its cholesterol during axon coating[73]. It is worth mentioning that brain cholesterol is predominantly unesterified and the primary component of both myelin and the plasma membranes lining neurons and glia[74]. Astrocytes are able to provide neurons with cholesterol not only due to their cholesterol synthesizing capability but also via their production of ApoE, the cholesterol transporter[75]. ApoE is secreted by astrocytes together with cholesterol, which allows receptor-mediated uptake of cholesterol containing-lipoproteins by neurons. This also allows cholesterol homeostasis within the brain through the excretion of excess cholesterol, achieved with the release of 1-2 mg of ApoE-bound cholesterol into the CSF per day[76]. More significant is the elimination by conversion to 24S-hydroxycholesterol (24S-OH), estimated to be 6-7 mg/day, making it the major cholesterol

elimination pathway[72]. This is evident as 24S-OH, mainly produced in the brain, is able to traverse the BBB with a net outflux observed[72].

Cholesterol is vitally important for the normal functioning of the brain. The structure of the brain is determined by cholesterol, including neuronal membranes and the myelin sheath that enables its conductive properties[74]. Cholesterol impacts neurotransmission through its influence on the function and release of synaptic vesicles, which is dependent on the cholesterol content[77]. It was observed that altering the amount of cholesterol postsynaptically caused the loss of dendritic spines and synapses, therefore undermining neurotransmission[78]. Moreover, the importance of cholesterol in the brain is additionally evident from the various diseases impacting cholesterol synthesis and causing neurological deficits. As discussed in the previous section, the relationship between blood cholesterol and AD has led to several theories on the influence cholesterol in the brain can have on the development of AD, which have been summarized in Fig. 2. This is in addition to the evidence that reducing cholesterol in membranes favored alpha-cleavage of APP and reduced the formation of Aβ, thereby motivating the use of statins in the treatment of AD[79,80]. While clinical trials with statins have only yielded inconsistent results[81], and that the cholesterol content in the brain of AD patients is seemingly low, it remains a puzzle how targeting cholesterol in the brain might help prevent AD.

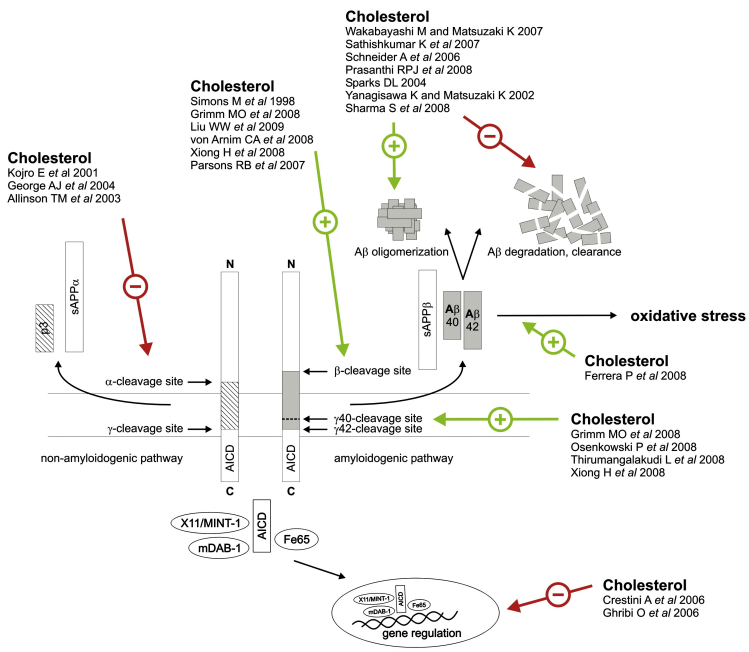

Fig. 2. The different mechanisms of how cholesterol influences APP processing. Reproduced from Grimm, M. O., Rothhaar, T. L. & Hartmann, T. The role of APP proteolytic processing in lipid metabolism. *Exp Brain Res* **217**, 365-375, doi:10.1007/s00221-011-2975-6 (2012)[82] under the Creative Commons license.

1.6 THE CHOLESTEROL METABOLITE 27-HYDROXYCHOLESTEROL

Cholesterol metabolism in the brain is much more complex then described above[83], and many questions remain unanswered. Similar to the 24S-OH elimination of cholesterol from the brain, peripheral cholesterol is additionally reversely transported from the body to the liver in the oxidized form, 27-hydroxycholesterol (27-OH)[84]. 27-OH is produced by the enzyme sterol 27-hydroxylase (CYP27A1) present in the mitochondria and distributed throughout the body though in extremely low levels in the brain[92]. This oxysterol is the most abundant cholesterol metabolite in the plasma of humans and rodents[85]. 27-OH also possesses the ability to cross lipophilic membranes more easily than cholesterol and is therefore able to traverse the BBB[86,87]. Despite substantial uptake of this oxysterol into the brain, its levels in the brain remain low due to its efficient metabolism[92]. This is performed by the action of several enzymes including oxysterol 7α hydroxylase (CYP7B1) and 3β-hydroxy-C27-steroid dehydrogenase/isomerase (HSD3B7) forming the major 27-OH metabolite, 7α-hydroxy-3-oxo-4-cholestenoic acid (7-HOCA), which is excreted via the BBB and has a net outflux from the brain into the circulation[88,89].

27-OH has also been shown to influence the synthesis of cholesterol *in vitro*[90-92],thus having important repercussions on the homeostasis of brain cholesterol *in vivo,* to which Ali *et. al.* have shown that 27-OH has a slight inhibitory effect on cholesterol synthesis in the brain[93]. In light of the effect of high blood cholesterol on AD, the levels of 27-OH correlates with plasma cholesterol levels[94], where feeding a high fat diet to rabbits markedly increased the levels of this oxysterol in the plasma[95]. As expected, an accumulation of this oxysterol was present in the brains of eoAD and sporadic AD[88,96]. This increase was also evident in the CSF of AD patients[97]. Additionally, the plasma levels of 27-OH was observed in several neurodegenerative diseases to be reduced and its potential as a biomarker was postulated, though more extensive studies need to be performed to accurately assess its suitability[98].

In view of its increased presence in the brain of AD, does 27-OH have a role in the disease? Treating cells and tissue slices with 27-OH resulted in higher production of amyloid beta and phosphorylated tau[99-101]. Additionally, aged APP mice exhibited higher levels of 27-OH in their brains[88]. 27-OH treatment on neuroblastoma cells also resulted in endoplasmic reticulum stress mediating the reduced regulation of leptin, which is associated to AD[102]. Apart from the previously described effects, 27-OH has a potential to affect the BBB by increasing the expression of cholesterol transporters ATP Binding Cassette Subfamily A Member 1 (ABCA1) and ATP binding cassette transporter G1 (ABCG1) in endothelial cells lining the brain capillaries and brain pericytes[103-106]. Whether or not these transporters are important for the integrity and

function of the blood-brain barrier *in vivo* has not been established. The BBB is also responsible for regulating the cerebral pool of amyloid beta by preventing its entry or allowing its elimination. In theory, 27-OH may affect this process by increasing the expression of ABCB1 thereby reducing the entrance of amyloid beta into a brain, though not much is known about how it influences the elimination of this peptide from the brain[104,105,107]. Interestingly, the metabolite of 27-OH processing, 7-HOCA was increased in patients with dysfunctional BBB and therefore stipulated to be a marker of the BBB integrity[108].

Despite the lack of *in vivo* evidence describing the effects of 27-OH on the brain, this oxysterol influences several systems peripherally. This cholesterol metabolite has been shown to be a risk factor for breast cancer due to its action on the estrogen receptor (ER)[109,110]. As a selective estrogen receptor modulator, 27-OH mediated the growth of ER-positive high-grade tumors. In addition, it further acts by influencing liver X receptor (LXR) signaling that may stimulate metastasis[111]. Bone homeostasis is also another system that is affected by this cholesterol metabolite, where 27-OH is the linking factor between metabolic diseases and osteoporosis via its action on ER, thereby causing higher bone resorption with complementary reduced bone formation and ultimately decreased mineral density in bones[112]. All these data indicate that 27-OH has a prominent role in disease mechanisms much more then a sheer reverse cholesterol transporter.

A recent publication has highlighted the lack of evidence of the relationship between blood cholesterol and cardiovascular mortality. Here they underplayed the effect cholesterol had in cardiovascular disease (CVD), however interestingly patients with advanced atherosclerosis often had increased levels of 27-OH in their circulation[94,113]. Furthermore, 27-OH was the most prevalent oxidized cholesterol in atherosclerotic plaques, with its accumulation seemingly correlated to severity of the lesion and the affluence of macrophages[114]. This oxysterol may mediate an inflammatory cascade via macrophages and endothelial cells with increased release of cytokines such as TNF-α, IL-1β mRNA and IL-6[115]. These cytokines also contributed to the instability of atherosclerotic plaques by causing matrix degradation following the increased expression of the matrix metalloproteinases 9, which was also increased with 27-OH treatment[116].

The effects of 27-OH on many of these diseases may be specific to their respective affected tissue, though the mechanism of how it exerts its action may be somehow similar in the brain. Thus more evidence is needed to affirm what this peripheral oxysterol accomplishes in the brain and how it does so. With this regard, this thesis expands the current state of knowledge of the

influence of 27-OH on the brain, attempting to bridge the many gaps present and illuminating a role for this oxysterol beyond a mere cholesterol intermediate.

2 AIMS

The cholesterol metabolite, 27-OH, is known to cross the BBB. While many have correlated its presence in the brain with neurological diseases, little is known about its function within this cerebral refuge. Thus, this thesis aims to investigate whether 27-OH does have an active role in propagating pathological states in the brain.

Specifically, the aims include:

- Examining the level of 27-OH in Alzheimer's disease and its relationship with other pathological markers.
- Investigating the role of 27-OH on the brain renin angiotensin system.
- Analyzing and scrutinizing the effects of 27-OH on cognitive function and glucose metabolism.
- Exploring the relationship between inflammation and 27-OH in the brain.

3 METHODOLOGY

This thesis is generated with the use of many methods, spanning both cellular and molecular, in probing the brain's machinery. Here, a brief account of some of the methodologies is discussed. However, please refer to the respective papers for a more comprehensive presentation of the applied models and methods.

3.1 EXPERIMENTAL MODELS

3.1.1 Mice Models

All the mice models used throughout the thesis are based on the C57BL/6 mice. These mice are widely used and are ideal as a general multipurpose model, diet-induced model and for the development of transgenic/knockout models. For our purposes we have used only male mice, and while eliminating hormonal differences, variations were inevitable. When using genetically modified mice, wild-type littermate controls were the control of choice in all experiments. All the animals were housed in a Makrolon type II cage with a wire top (Tecniplast, Sweden) and lived on a 12-h light-dark cycle with controlled humidity and temperature. The food (normal chow feed) and water were provided ad libitum.

3.1.1.1 High fat induced mice

Mice were purchased from B&K, Sweden. For nine months, 5-6 week-old mice were separated into two groups; one fed a normal diet (ND)(R34) while the other a high fat diet (HFD) consisting of 21% fat and 0.15% cholesterol (R638, Lactamine, Sweden). The level of plasma lipids was analyzed after 18 weeks to monitor the effects of the diet on the mice. At this time-point, the cholesterol plasma level was almost double in the HFD-fed mice compared to their ND controls (2.78 ± 0.55 vs. 1.51 ± 0.56 mmol/L, $P = 0.0011$).

3.1.1.2 CYP27A1-overexpression mice (Cyp27Tg)

CYP27A1-overexpression mice were bred from our own colony within our animal facilities. The colony was initiated with founder mice provided by Eran Leitersdorf, who has developed and characterized these transgenics[117]. This mouse provides a useful model to study the *in vivo* effects of higher levels of 27-OH, mimicking the complexity that cannot be imitated in a petri dish. This model is also beneficial as there are no significant differences in total plasma cholesterol, triglycerides and phospholipids between these transgenic mice and their counterpart littermate controls. Furthermore, these mice exhibited 11-fold higher brain levels of 27-OH than controls[93]. It is important to mention that despite this strikingly higher level of

27-OH in the brain, it still constitutes only 10% of the level of 24S-OH, whereas in wild-type mice 27-OH is less than 1% of 24S-OH levels. Since 27-OH is predominantly produced extracerebrally, its effects in the periphery may influence the brain in a manner that is indistinguishable in our paradigm. We therefore have addressed this concern with the addition of another mouse model, i.e. 27-OH injected intracerebroventricularly described below. Characterizing the behavior of these mice was performed and is described in **paper III**.

3.1.1.3 Cyp27a1 Knockout mice (Cyp27$^{-/-}$)

Being a valuable model in studying the effects of increased 27-OH; the Cyp27Tg model does come with certain biases, allocating any finding to that single variable. Thus the *Cyp27a1* knockout mouse offers another perspective in probing the validity of these findings and their association to 27-OH. The generation of the original knockout mouse was previously described in detail[118]. The expansion of these Cyp27$^{-/-}$ mice in our Swedish facilities along with its challenges were also detailed earlier[119]. Noteworthy, these mice surprisingly exhibit normal levels of cholesterol despite a significant decrease in the rate of bile acid synthesis, which consequently leads to the reduced absorption of intestinal cholesterol[118]. In **paper III**, a subset of the Cyp27$^{-/-}$ mice were fed 0.05% cholic acid for 16 weeks. The behavioral characterization of these mice revealed no significant changes in spatial memory compared to controls and feeding these knockout mice a high fat diet, while detrimental to the wild-type, did not modify their behavior either[120].

3.1.1.4 Intracerebroventricular 27-OH injected mice (ICV27-OH)

As previously explained, this mouse model provides the added benefit of ensuring that the 27-OH-mediated responses are purely due to its central actions within the brain, thus eliminating offshoot peripheral effects. Seven-eight week-old C57BL/6 mice were fixed on a stereotaxic frame (David Kopf Instruments) with a heated pad (37°C) to maintain normal body temperature. Anesthesia was administered by inhalation of isoflurane/oxygen mixture at 4-5% and maintained throughout the duration of the surgery at 1.5-2%. The injections were performed bilaterally into the lateral ventricle using the following coordinates: -0.9 mm anteroposterior, ± 1.4 mm laterally from the bregma and 2.0 mm from the skull surface, in accordance to the Paxinos mouse brain atlas[121]. The injected 27-OH, was bound to the high-density lipoprotein (HDL) protein at a concentration ratio of 1:3 and incubated for 1 h at 37°C. This bound 27-OH was then diluted in artificial CSF (aCSF) to a concentration of 5 and 10 µM. The control group was injected under similar conditions with HDL diluted in aCSF. A 10 µl gauge Hamilton syringe was used to deliver 1 µl of the solution into each hemisphere.

3.1.2 Human Subjects

The use of animal models allows manipulation in elucidating mechanisms important in disease pathogenesis/progression. Findings from animals however are limited in its translation or even mirroring of disease states in humans. It is thus imperative to be cautious when extrapolating results from animals, especially when trying to implement or even correlate these to humans. To this aim, we have tried to bridge this gap by utilizing human subjects to implement a more translational approach. The patient cohorts used in this thesis were specifically sampled based on the proposed scientific questions and are therefore grouped based on the individual respective papers.

3.1.2.1 Paper I

CSF samples were collected from patients recruited at the Karolinska University Hospital memory clinic. A cross-sectional cohort consisting of 25-28 Controls, 8-10 MCI and 20-21 AD patients were enrolled. The control group consisted of subjective cognitively impaired individuals. These individuals are generally considered old and have non-specific memory complaints, revealing no memory-related deficits upon objective testing.

Mild cognitive impairment (MCI) was described by Winblad *et al.* and is defined upon these three criteria[122]:

i. Not demented, however are not considered normal either. These patients do not fulfill the criteria for a demented diagnosis according to either the Diagnostic and Statistical Manual of Mental Disorders, fourth edition (DSM-IV) or the International Classification of Mental and Behavioural Disorders (ICD-10).

ii. Experience cognitive decline assessed either subjectively by the patient and/or an informant and additionally exhibiting impairment on objective cognitive tasks or the declined performance over time in objective cognitive tasks.

iii. The patient has minimal impairment in complex instrumental functions alongside intact basic daily living activities.

While the term generally refers to a transitional stage before the onset of clinical AD, it encompasses far more than just a prodromal AD zone[122]. This heterogeneous group includes different classifications where some revert to normal cognitive capabilities and others progress to AD. It is important to note that all the patients described as MCI in our study developed AD during the following 3 years. Finally, the DSM-IV was used in the diagnosis of the included AD patients in the study. They were further assessed based on a comprehensive clinical protocol

including clinical examination, detailed psychological assessment, magnetic resonance imaging (MRI), single-photon emission computed tomography brain imaging, electroencephalography and the analysis of CSF and blood (total-tau, phospho-tau and $A\beta_{1-42}$), all detailed in Table 1 in **paper I**.

With regards to the brain samples used in the study, the postmortem material was acquired from the brain bank at the Karolinska Institutet. The patients included 4 AD and 3 age-matched controls. The AD samples consisted of two males, 79 and 89 years-old and two females, 76 and 80 years-old whereas the controls were two males, 66 and 83 years-old and one 83 years-old female. As for the AD patients, they exhibited a Mini-Mental State Examination (MMSE) score between 2 and 15 and were clinically diagnosed as probable AD (DSM-IV) in addition to the CERAD neuropathological criteria. The postmortem delay was between 24-48 h and the temporal cortex was used.

3.1.2.2 Paper II

A human model, depicting the Cyp27Tg mouse model, in having excessive levels of 27-OH in the blood was used here. These patients suffer from hereditary spastic paraplegia 5 (SPG5), having an autosomal recessive mutation in the *Cyp7b1* gene[123]. This encodes for the oxysterol 7α-hydroxylase, which facilitates an important step in the synthesis of bile acids from cholesterol by acting on 27-OH. A consequence of this mutation is the accumulation of 27-OH in the blood, hence the levels of this oxysterol is 6-9 fold higher compared to controls[124]. The CSF of these patients have extremely high levels of 27-OH ranging a 30-50 fold increase. While 25-OH, another form of oxysterol was also increased in serum, this was not mirrored in the CSF of these patients. It is also worthy to note that the total cholesterol levels in the plasma are unchanged, a finding also present in the Cyp27Tg mouse model. These patients are clinically characterized by a progressive type of spastic paraplegia of a variable onset, while pure in most cases, it can however express mild cerebellar ataxia and optic atrophy. Even though these patients have normal MRI scans, this subset of hereditary spastic paraplegics is distinguished by the primary demyelination of the pyramidal tracts in the absence of peripheral neuropathy[123]. Despite the myriad degenerative signs present in these patients no changes were seen in the CSF tau and phospho tau protein levels[124].

3.1.2.3 Paper III

This study tests the hypothesis that higher CSF levels of 27-OH are associated with reduced glucose uptake in humans. To this aim, we recruited 75 individuals who have undergone ^{18}F-FDG PET imaging at the Radiology department at the Karolinska University Hospital. No

criteria were employed when selecting these individuals other than having underwent PET scanning. From these subjects only 34 had CSF samples collected which is needed to determine the levels of 27-OH. The randomness of the selection process enabled the assembly of a heterogeneous group of neurological diseases adding to the strength of our cohort despite the small number. These patients all underwent a comprehensive cognitive evaluation by a neuropsychologist according to the procedures of the memory clinic at the Karolinska University Hospital. The diagnoses were set according to the ICD-10 and are displayed in supplemental Table 2 in **paper III**. The clinical setting of these PET scans, forced the implementation of tighter research criteria to eliminate inaccurate observations. Thus the time between injection of the radioligand and scanning should not be less than 30 and over 45 minutes, thereby excluding 11 patients from our cohort. The levels of 27-OH may be slightly modulated by cholesterol modifying drugs[125] therefore two further patients were excluded from the study. This led to the remainder of the sample having normal cholesterol levels despite the large variation in the levels of 27-OH in the CSF. Highlighting this hidden population and the importance of monitoring the levels of 27-OH, which need not mirror that of cholesterol.

3.1.3 Cell cultures

The simplicity of cell cultures being their advantage over *in vivo* models is ironically also its limitation. Their flexibility in applications and ease of use allows their continual survival as an experimental model. Throughout this thesis when probing cellular mechanisms we used to a large extent rat primary cultures in addition to neuroblastoma (SH-SY5Y). Rat primary neurons and astrocytes were prepared from unborn embryos. These cells enable the mirroring of brain processes in a more convenient and easy to handle method. Being either predominantly neuronal or astrocytic cultures, the communication between the different cell types is a major obstacle in these cultures. They do however readily allow genetic manipulation in a reproducible fashion, which we have employed here with the use of siRNAs. Primary neuronal cultures are post-mitotic whereas neuroblastomal neurons are not. Neuroblastomas are derived from a neuronal tumor and therefore may differ from healthy neurons. They however need no ethical permit to use and therefore have the added benefit of accessibility over primary cultures.

3.2 ETHICAL CONSIDERATIONS

Research is governed by morals that form the code of conduct. These codes which researchers must adhere to, allow scientific advancement not only through collaboration but enabling the reproducibility of data to name a few. Accordingly, any research being conducted must be examined and approved by an ethical review board. These boards comply with the much-

recognized Helsinki Declaration. In this thesis, the use of humans and animals were approved by the appropriate regional ethical committee for Stockholm and are detailed below:

Human CSF: DNR: 2007/697-31/1

Human Brain: DNR: 024-01; DNR: 2011/692-31/1

Rat primary cultures: DNR: S16-09; DNR: S194-11; DNR: S45-15

Mice: DNR: S139-10; DNR: N578-11; DNR: S51-13; DNR: S141-12; DNR: S57-15

With regards to the use of rat primary cultures, when possible was substituted with neuroblastoma cells in compliance with the replacement aspect of the 3R principles of animal research. Furthermore, all the patients involved in this thesis granted their consent.

4 RESULTS AND DISCUSSION

4.1 ALZHEIMER'S DISEASE AND ITS PATHOLOGIES

Alzheimer's disease although being considered a multifactorial disease is restricted to two characteristic pathologies, amyloid plaques and tau tangles. The changes of these proteins reflected in bodily fluids such as the blood and CSF are considered biomarkers for the disease. Within our cohort, the CSF levels of total and phosphorylated tau were increased and $A\beta_{1-42}$ decreased in the AD patients compared to controls (**Paper I**). Cholesterol levels surprisingly did not exhibit any changes in either plasma or CSF, however its metabolites 27-OH and 24S-OH were modified. The plasma levels of both these oxysterols were significantly reduced, with this decrease being observed even earlier within the MCI group. The CSF 27-OH levels were elevated while the 24S-OH levels revealed an increased trend in the AD patients. These findings have been previously described and the potential use of oxysterols as biomarkers for neurodegeneration, although proposed, has not gained momentum[98,126].

In concurrence with prior findings, ACE levels in the CSF were not only increased in the AD patients but also in the MCI sample[127]. Our study also highlighted the previously unknown increase of the precursor angiotensinogen (AGT) in both MCI and AD patients. ACE and AGT constitute an important rate-limiting enzyme and the main precursor of the renin angiotensin system respectively. This system, completely independent from its renal counterpart, is known to regulate cerebral blood flow, influence the autonomic system and processing of memory[28]. Furthermore, the involvement of this system is not only being recognized in AD but also in other neurological disease such as stroke and depression[29].

In AD the levels of the cholesterol metabolites in addition to members of the brain RAS are modified, and whether these are unrelated coincidences or have some sort of connection was formerly unexplored. To further examine this relationship, we compared the levels of 27-OH and 24S-OH with the CSF levels of ACE. A positive correlation in both plasma and CSF levels of 27-OH existed with ACE in the MCI-AD group even after correcting for age and gender, though this relationship was not seen in the control group. Moreover, no correlation between plasma or CSF levels of 24S-OH and ACE was observed. This relationship forged a new path of research that we set out to investigate featured in the subsequent chapter.

4.2 BRAIN RAS IS MODULATED BY 27-HYDROXYCHOLESTEROL

It is important to state that the correlation study only indicates a relationship where having higher levels of 27-OH shows a tendency of exhibiting higher CSF ACE levels in AD patients. Thus to address the issue of whether 27-OH alters ACE levels causatively, we treated primary neurons with 27-OH. This resulted in the increased expression of ACE in addition to the precursor AGT and the downstream angiotensin II type 1 receptor (AT1R) (**Paper I and II**). At the protein level, the secreted AGT was higher in both neurons and astrocytes, while higher cellular amounts were only observed in neurons. To probe whether this increase led to a functional outcome with regards to cellular signaling of the RAS cascade, the downstream AT1R-mediated JAK/STAT activation was assessed[128]. The phosphorylated STAT3 (Tyr-705), indicating an active STAT3, was elevated in neurons treated with 27-OH. This heightened phosphorylation was however inhibited when losartan, a specific AT1R antagonist, was used.

The effects of 27-OH observed thus far are within the *in vitro* paradigm and may not translate to the complex situation within the human body. Therefore, in **Paper II** we employed the $Cyp27^{-/-}$ mouse, incapable of synthesizing 27-OH, these mice showed no significant increase in ACE expression. Additionally, lower levels of AGT and phosphorylated STAT3 were seen in the cortices and hippocampi of these mice. Forming a larger translational picture the SPG5 patients, having a 30-50 fold increase in 27-OH CSF levels, expectedly had contrary findings to the $Cyp27^{-/-}$ mouse. These patients revealed higher levels of both the precursor AGT and the AngI/II in their CSF.

In view of the current data, we speculated that 27-OH leads to the observed increase in ACE and AGT. Levels of CSF 27-OH are seen to be higher in AD patients while plasma levels show a reduction, which is evident even earlier within the prodromal AD stage. We have also observed that these patients have elevated levels of both ACE and AGT. The obvious question of whether 27-OH initiates this elevation in patients is crucial to answer and our investigation in both *in vitro* and *in vivo* models points towards an influential role as displayed in Fig. 3. In fact, the use of statins, although controversial in improving cognition, showed some reduction in the incidence of dementia[129], while the use of ACE inhibitors enhanced cognitive performance in addition to improving learning and retaining memory in animal studies[130,131]. Within CVD, reaching the cholesterol-lowering goal with statins alone still had some residual risk for CVD, a risk that was decreased with the combined use of ACE and AT1R antagonists[132]. This is an important finding that may have some resounding implication for the brain.

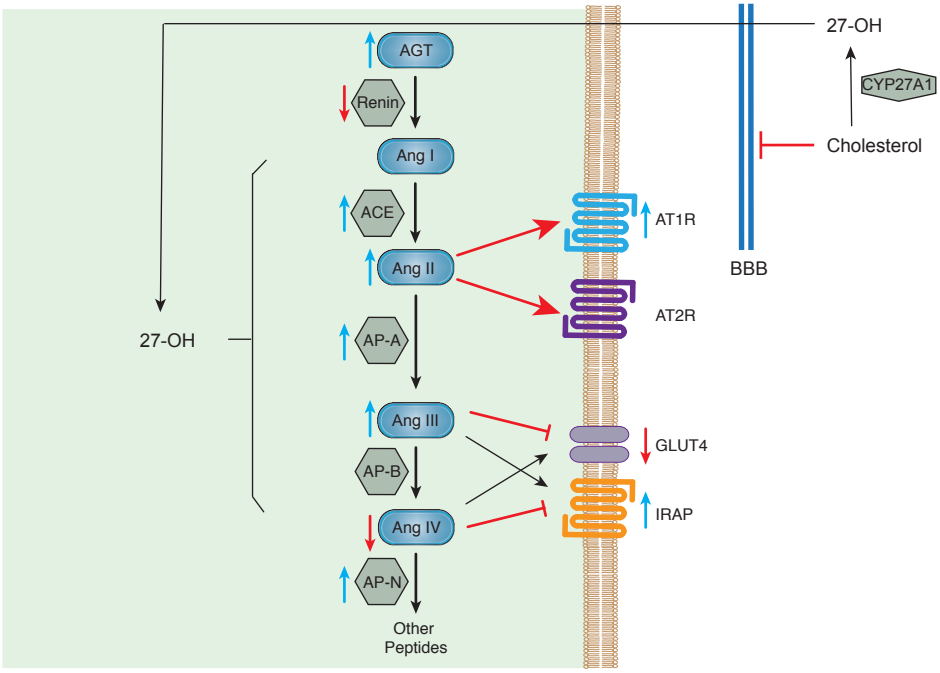

Fig. 3. A Schematic overview of the mechanism by which 27-OH influences the Brain RAS.

4.3 THE ROLE 27-HYDROXYCHOLESTEROL PLAYS IN COGNITION AND GLUCOSE UPTAKE

The upregulation of brain RAS due to 27-OH was observed on ACE, AGT and AT1R, which are the rate-limiting enzyme, the precursor substrate and the receptor respectively. These are important players within the brain RAS, however, the memory related action of RAS was found to revolve around the AT4R and its ligand AngIV. AngIV affects learning and memory through its inhibitory control of AT4R, also known as the insulin-regulated aminopeptidase (IRAP)[133,134]. This increase in cognition is believed to be associated to either heightened neuronal glucose uptake[135] or the reduction of the receptors-cleaving action on memory-associated neuropeptides, among other hypotheses[136,137].

In our paradigm, 27-OH increased both the expression of IRAP and its activity (**Paper III**). This was not only seen *in vitro* the Cyp27Tg mouse in addition to ICV27-OH also exhibited this increase. The effect of 27-OH extended to the enzymes converting AngII to AngIII and the degradation of AngIV, i.e. increasing aminopeptidase (AP) A and AP-N respectively. To further clarify the roles these enzymes play in maintaining the levels of AngIII and AngIV and consequently their effects on both IRAP and glucose uptake, we knocked down their expressions. Treating neurons with AngIII alone led to similar effects as 27-OH i.e. increased

IRAP activity and reduced glucose uptake. Thus siRNA against AP-A caused less production of AngIII, which led to the negation of the 27-OH-mediated effects. On the contrary, AngIV treatments maintained the catalytic activity of IRAP at control levels, although elevating its expression and surprisingly increased glucose uptake even more than insulin. Knocking down AP-N led to the accumulation of AngIV, which reduced the effects of 27-OH on IRAP and glucose uptake. Furthermore, the use of Bestatin, an inhibitor of a range of metallo-aminopeptidase leading to the accumulation of AngIII while reducing AngIV production, resulted in comparable effects on glucose uptake and IRAP activity as 27-OH.

These series of experiments consolidate a new function for AngIII on both glucose uptake and IRAP activity along with mediating the action of 27-OH within the brain. They also emphasize the modulation by 27-OH of the levels of AngIII and AngIV by altering the expression of the enzymes important in maintaining a balance between the function of both these RAS mediators (Fig. 3). Although insightful these experiments do not elucidate the *in vivo* consequence of the previously discussed alterations. Thus scanning the Cyp27Tg mouse using ^{18}F-FDG PET revealed the reduced uptake of glucose generally across the entire brain of these animals (**Paper III**). This was associated with lower levels of GLUT4, while the levels of GLUT3 and GLUT1 remained unchanged. The testing of spatial memory of these mice revealed longer escape latencies and less time in the correct quadrant than their counterpart WT-littermates. A marker for long-term memory consolidation, activity-regulated cytoskeleton-associated protein (Arc) was also decreased. While it is difficult to assess causation of 27-OH in reducing glucose uptake in humans, we have showed a negative relationship between both with findings in a random sample of patients (**Paper III**). These patients had various diseases and thus not limited to a certain disease scope, and more importantly all had normal levels of cholesterols. They also displayed varied cognitive ability as disclosed by the MMSE scores and the memory-related diagnoses. This cohort emphasizes that measuring 27-OH may be more relevant than cholesterol within neurodegenerative diseases, especially since cholesterol during later stages of life has a controversial relationship with dementia risk[138]. Additionally, cholesterol does not traverse the BBB and that cholesterol produced in the brain is formed within this refuge[86].

4.4 27-HYDROXYCHOLESTEROL MEDIATES BRAIN INFLAMMATION VIA S100A8

The role of inflammation within neurodegenerative disease has gained wide support in the past several years. Reviewed here, there are many mechanisms for the induction and persistence of inflammation within these diseases[139]. Even though 27-OH has not been described to have a role in inflammation within the brain, it propagates inflammatory processes seen in other diseases

such as atherosclerosis and chronic obstructive pulmonary disease[115,140]. Previously, we have shown that a HFD leads to the upregulation of the genetic expression of S100A8 in the brains of mice[141]. Thus in **Paper IV**, we investigated the effects of 27-OH, which revealed an increase in the expression of S100A8 in astrocytes. S100A8 is a member of the S100 calcium-binding proteins, which are involved in inflammatory response in addition to other cellular processes and seen to be elevated in AD[142]. We have established previously that S100A8 is increased and aggregated in the brains of several mouse models overexpressing APP[143]. This accumulation occurs earlier than the development of Aβ plaques. Furthermore, S100A8 binds to, RAGE, which is also elevated in the presence of 27-OH. The activation of this receptor leads to the downstream induction of the expression of BACE1, an enzyme that takes part in producing Aβ[144]. Therefore, increased levels of BACE1 by 27-OH indicated that RAGE is activated. This activation is also elucidated by the nuclear translocation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) by 27-OH. RAGE is known to activate NF-kB, promoting the induction of both itself and BACE1[145]. Moreover, the use of a NF-kB inhibitor prevented the increase of RAGE initiated by 27-OH. Both our *in vivo* mouse models, with increased levels of 27-OH, had elevated levels of S100A8, its receptor RAGE and the downstream BACE1 (**Paper IV**). These findings illuminated in Fig. 4 implicate 27-OH in mediating inflammation within the brain and suggests a role in the accumulation of Aβ plaques in AD.

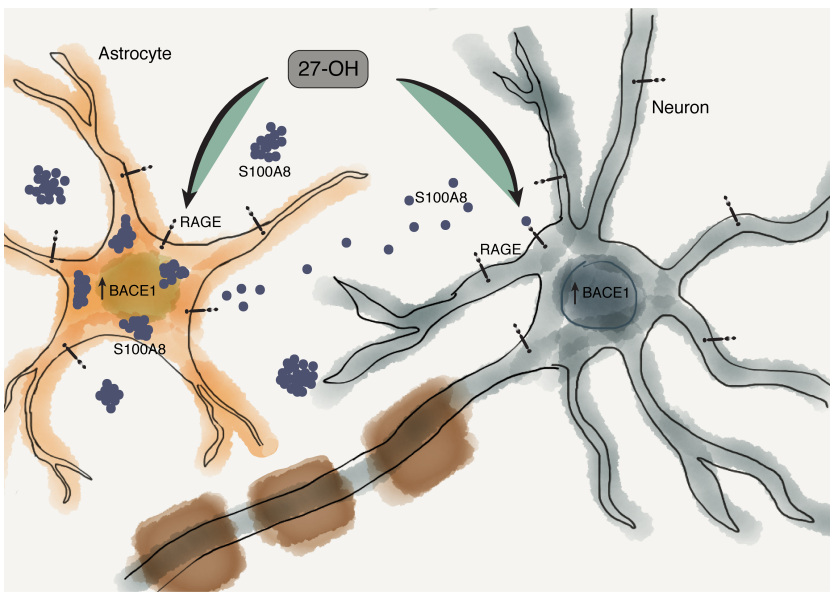

Fig. 4. A Schematic overview of the mechanism by which 27-OH induces inflammation in the brain. 27-OH via RXRγ (explained in more detail in section 4.6) increases the expression and aggregation of S100A8 in astrocytes. Through RXRγ 27-OH also increases the levels of RAGE in both astrocytes and neurons respectively. RAGE signaling mediated via NF-kB is increased which in turn raises the levels of both RAGE and BACE1.

4.5 DOES 27-HYDROXYCHOLESTEROL MEDIATE THE NEGATIVE CHOLESTEROL EFFECTS ON THE BRAIN?

The risk of dementia due to increased cholesterol and dysregulated cholesterol metabolism has been studied tremendously and while still some controversy exists as to how, there is no doubt that it does contribute to neurodegenerative diseases[86]. Even though cholesterol does not cross the BBB, it is implicated in different pathways leading to AD[146]. However, the levels of the BBB-traversing 27-OH correlate with the circulatory cholesterol levels[94]. Furthermore, production of 27-OH seems to be an alternative method for the reverse transport of peripheral cellular cholesterol, having a large influx to the liver[84]. This led to the concept that 27-OH may mediate negative effects on the brain, a hypothesis given by others and us[86,95,147].

Indeed, an enriched cholesterol diet and 27-OH has led to the increase in Aβ production in the brain of rabbits[148]. In our studies, HFD mice showed an increase in several RAS components, mainly ACE and AGT. Additionally, the downstream signaling of the AT1R leading to the phosphorylation of STAT3 was also increased in these mice (**Paper II**). These changes were apparent in the *in vitro* treatment with 27-OH, but were reversed in the $Cyp27^{-/-}$ mice. The behavioral changes that we have witnessed with the Cyp27Tg mice were also present in HFD-fed mice. Reported in Heverin *et. al.*, the deficits seen in spatial learning were propagated by the enriched cholesterol diet and consequently were absent in $Cyp27^{-/-}$ mice fed with the same diet[120]. This was also confirmed with the reduced levels of Arc in the WT mice fed with a HFD, similar to the Cyp27Tg mice. On the contrary, normal levels were observed in the $Cyp27^{-/-}$ mice, whether fed a normal or HFD diet.

The effects of 27-OH on IRAP catalytic activity were also mimicked in the brains of mice on an enriched cholesterol diet, indicating higher activity (**Paper III**). While we did not show how this diet affected glucose uptake, it led to a reduction albeit for a short period due to the rebound of GLUT1[149]. The brain immune system does not seem to be spared either as a HFD also led to the increase in S100A8, an inflammatory mediator, a phenomenon also seen in 27-OH treated cells or upon overexpressing the 27-OH-producing enzyme in mice (**Paper IV**). Activated microglia and the release of pro-inflammatory cytokines were also among the other features a HFD mice elicited[150]. Taken together these evidences emphasize that 27-OH may indeed arbitrate the negative effects of high cholesterol.

4.6 THE ROLE OF NUCLEAR RECEPTORS IN PROPAGATING THE ACTIONS OF 27-HYDROXYCHOLESTEROL

It is well known that 27-OH is a ligand for LXR, and that the nuclear receptor plays an important role in mediating cellular functions. LXR regulates cholesterol metabolism and here we show that its ligand 27-OH functions beyond the known control of cholesterol metabolism, it also upregulates several components of the brain RAS. Therefore it seemed appropriate to investigate if these effects are also propagated via this receptor, which we did using both a pharmacological and siRNA approach.

TO-901317, a potent synthetic LXR agonist with no selectivity for either LXR isoform[151], caused a significant increase in the expression of ACE, AT1R and secreted AGT (**Paper II**). Surprisingly, it also led to a decrease in the intracellular expression of AGT, unlike both oxysterols. Blocking LXR with 22(S)-OH, an inactive isomer of 22(R)-OH[152], eliminated the effects of 27-OH on the expression of ACE and AGT in addition to its secreted levels. Although 22(S)-OH did not completely abolish the increased expression of AT1R by 27-OH, knocking-down the expression of LXRβ did. Furthermore, siRNA of LXRβ eliminated the action of 27-OH on the expression of ACE, renin and AGT. We chose to knockdown LXRβ as it is more widespread within the brain than the LXRα isoform[153]. The effect mediated by 27-OH via LXR on RAS extended further downstream to include AT4R/IRAP, a receptor important in cognition in addition to AP-A and AP-N, disrupting the balance between AngIII and AngIV (**Paper III**).

However, LXR did not seem to propagate the proinflammatory action of 27-OH. 22(S)-OH did not block the action of 27-OH on the expression of both S100A8 and RAGE (**Paper IV**). Since LXR forms heterodimers with the retinoid X receptor (RXR) we hypothesized that 27-OH may also bind to RXR[154]. By using siRNA to reduce the expression of RXRγ, the 27-OH-mediated increase of both S100A8 and RAGE were ameliorated. 27-OH also mediates the release of inflammatory cytokines in atherosclerosis via ER, we have not tested this alternative hypothesis within our paradigm however 27-OH being a SERM may exert a different action in the brain via ER. Within our experiments we have seen that indeed RXRγ and not LXR mediated the proinflammatory effects of 27-OH.

5 CONCLUSIONS AND FUTURE CONSIDERATIONS

Here in this thesis, we propose that 27-OH is a common thread connecting many of the mechanisms identified through the GWAS implicated genes[10] highlighted in Fig. 5. Alzheimer's disease and other dementias are multifactorial in etiology. Many factors such as oxidative stress, inflammation, protein aggregation, insulin/glucose metabolic dysfunction, hypertension and indeed cholesterol metabolic deficits may play a role in either initiating or propagating these crippling diseases.

We show that CSF and plasma 27-OH is pathologically altered not only in AD, but also earlier within the prodromal phase MCI. This increase in CSF 27-OH is not limited to AD as it was seen in patients diagnosed with other diseases with various forms of cognitive impairment. Although all of these individuals are normocholesterolemic, some observed higher levels of CSF 27-OH, highlighting a subgroup exhibiting higher production or reduced metabolism of 27-OH. The number of patients included, though validating a proof of concept, restricts this study. Thus it must be further investigated on a larger cohort to consolidate whether the levels of 27-OH can mirror a decreased brain glucose metabolism/cognitive state or hint at a diseased state that must be interjected. While the use of 27-OH as a diagnostic marker is still debatable, a meta-analysis by Wang *et. al.* regarding CSF levels of this oxysterol shows its additive benefit[126].

Our studies also add to the evidence regarding the use of statins. Statin use within dementia is extremely controversial with many doubting their benefits[155]. In addition, high blood cholesterol levels itself, though correlating with the above diseases, have not revealed any links to causation or mechanism[156]. Thus it does not make sense for statins, which are capable of crossing the BBB, to be used in reducing the production of brain cholesterol, an important player in the plasticity and synaptic ability of the brain. Furthermore, plasma cholesterol does not cross the BBB and is unable to cause any harm to the brain while 27-OH does and its levels correlates with cholesterol in the blood[94]. Thus statins peripherally may have a role in reducing 27-OH levels, as most of this oxysterol emanates from the circulation. Some statins have shown beneficial effects in reducing the incidence of dementia[129], which may be explained by the reduction of 27-OH levels which closely accompanies decreases in cholesterol levels. Statins such as simvastatin can cross the BBB and cause negative memory complaints as seen in patients on high doses, these side-effects would be less expected with the use of polar statins such as fluvastatin. Moreover, cholesterol heavily linked to CVDs is now also under scrutiny, recent research has weakened the link between the two in elderly people[157]. Importantly, the most abundant form of cholesterol present in atherosclerotic plaques is 27-OH, which was also greatly implicated in the atherosclerotic

processes[158,159]. Therefore it maybe more pertinent to monitor this oxysterol and attempt to reduce its levels rather than control the levels of cholesterol in the blood. The choice of Cyp27a1 as a drug target to reduce the levels of 27-OH has only been featured in one paper, revealing that 27-OH can be decreased without altering cholesterol metabolism[160], which has an important practical significance in the development of a more potent and selective inhibitor. The use of Cyp27a1 inhibitors against breast cancer has been patented, in anticipation of the publication elucidating the deleterious effects of 27-OH[110,161].

The function of the brain RAS goes beyond the control of cerebral blood flow and hypertension as it is also involved in mediating memory by the action of AngIV[28]. This thesis emphasizes the role 27-OH plays in upregulating the entire system, affecting AngIV levels and altering both cognition and glucose uptake in mice. It mechanistically does this by increasing AP-A, AP-N and IRAP expression thus increasing the catalytic power of the latter. This leads to the reduction in the levels of AngIV and higher amounts of AngIII, accentuating the previously unknown action of AngIII in inhibiting glucose uptake and increasing IRAP activity. The interplay between IRAP and GLUT4 is important, within our paradigm they seem to have an opposite effect i.e. IRAP activation occurs simultaneously with GLUT4 inhibition and vice versa. GLUT4 is described to contribute largely to the memory-involved cognitive function by taking up the much-required glucose[162,163]. Therefore both these molecules contribute in some form to memory-related tasks.

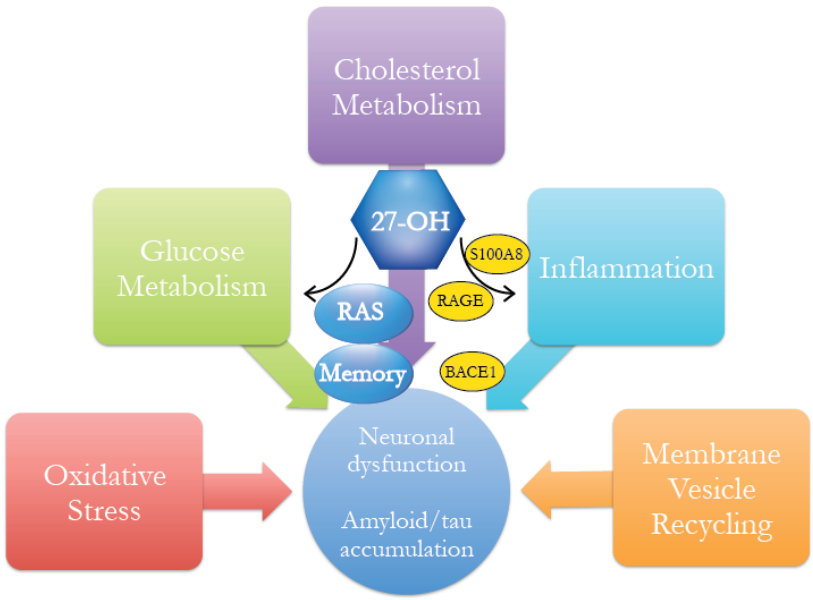

Fig. 5. The effects of 27-OH on features driving the pathology of AD. 27-OH upregulates the Renin-Angiotensin system (RAS) and decreases glucose metabolism thereby influencing memory. This oxysterol also mediates inflammation by increasing S100A8 and RAGE. Together these actions propagate neuronal dysfunction an important part of AD.

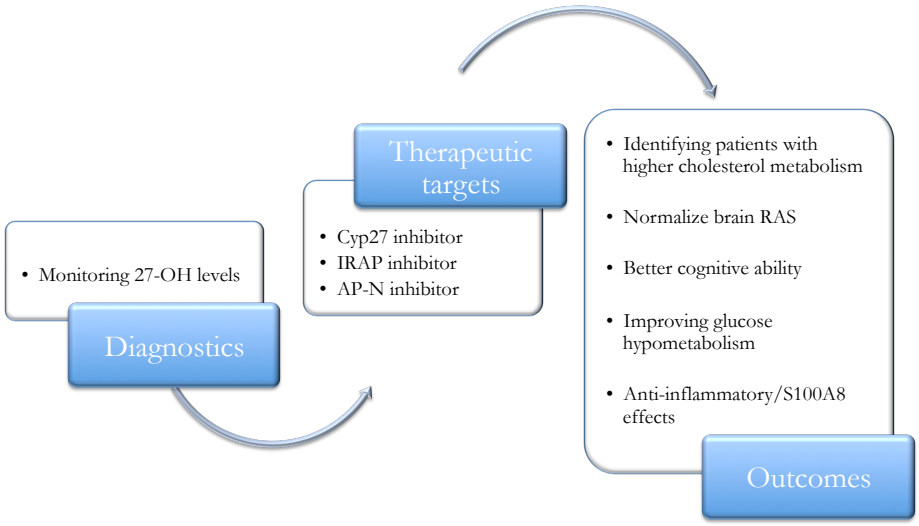

Fig. 6. The proposed goals in limiting the negative effects of 27-hydroxycholesterol.

In fact, the translocation of both of them is initiated by certain stimuli including insulin[135]. Previous studies have observed that intranasal insulin exerts beneficial effects on memory[164]. Currently large multicenter phase II and III clinical trials are underway to determine the effectiveness of the therapeutic application of intranasal insulin in modifying memory (ClinicalTrials.gov Identifier: NCT01767909). Although some mechanisms of how insulin improves memory are described, no conclusive evidence is reached[164]. Here, our results provide some mechanistic insight on how insulin may mediate its action centrally, and we show that AngIV has an even larger effect in mediating glucose uptake than insulin. Therefore strategies modulating this system to allow for the actions of AngIV to linger longer may be advantageous for memory, and thus cognition. Several animal studies have shown that intracerebroventricular injections of AngIV improved learning and memory[165,166], though to date or unbeknown to us, no study has ever intranasally introduced AngIV into the human brain.

While exogenous delivery of AngIV may be one route in achieving better cognition, our data also supports the use of IRAP or AP-N inhibitors. IRAP inhibitors mimicking the effects of AngIV are currently being developed, with a few non-peptide based inhibitors being identified[167]. The same group previously clarified that IRAP inhibitors improve cognition by increasing dendritic spines[168]. Furthermore, targeting the AngIV-degrading enzyme AP-N is widely used in the treatment of cancers. AP-N is a zinc-dependent metalloproteinase that plays an important role in

tumor invasion and angiogenesis by cleaving extracellular matrix proteins. A naturally occurring AP-N inhibitor is curcumin, which has been shown to inhibit tumor invasion and angiogenesis[169]. Remarkably, curcumin is also featured as beneficial against AD due to its anti-inflammatory and antioxidant effects[170]. Further investigations would be required to determine if curcumin or other AP-N inhibitors have an effect on memory, however our study illuminates a possible new therapeutic pathway in the fight against AD.

Astrocytes play an important role in AD, from the consequences of astrocytic atrophy due to the lack of clearing of Aβ[171]. Here we show another method by which astrocytes orchestrate its action in AD via the secretion of S100A8 triggered by 27-OH. This increased S100A8 expression was absent in neurons, thus cementing astrocytic role in propagating inflammation in the brain. 27-OH also increased the receptor of this inflammatory mediator in both astrocytes and neurons, suggesting that there may be communication between both cell types in mediating inflammation in the brain. Though not covered in this thesis, this relationship should be investigated to elucidate the role each of these cells play within the current mechanism described.

The binding of 27-OH to RXRγ thereby mediating the S100A8 and RAGE increase was another finding that we have elucidated in this thesis. This was surprising as beneficial effects leading to decreased amyloid plaques and enhanced soluble Aβ clearance were found with the RXR agonist bexarotene[172]. Strikingly these results were not completely reproducible[173,174]. This may be due to the fact that bexarotene is not isoform selective and also targets RXRγ, which we indicate mediates inflammation and possibly amyloid deposition. Previously, we have observed that S100A8 aggregation occurs in AD mice models before amyloid plaques. This strengthens the conception that mid-life hypercholesterolemia is considered a risk factor for AD[62], as we have shown that a HFD also led to S100A8 aggregation. In addition, inflammation is now being considered an event that precedes amyloid deposition[175]. Thus the suggestion that 27-OH may be a driving force in neuroinflammation leading to the development of AD may not be far fetched, though much work is further needed to investigate this.

The cost of dementia must not be measured by the amount of money spent on the care and treatment for patients (which has reached 487 billion USD in 2015)[5], but the real cost is the life of the single individual, whose own existence has forever changed, affecting the many people surrounding them. While it is estimated that this devastating disease afflicts 47 million people, the number that this disease truly affects might be tripled or even quadrupled. So far no pharmacological intervention targeting amyloid beta has been very promising and the need to have a multi-target treatment is currently imperative[5]. The expansion of our current working hypothesis to include pathophysiologies that might indeed have a causative role in the disease

mechanism could prove to be of great importance in the fight against dementia. This thesis is part of that initiative; an impetus to search beyond the restrictive boundaries of the current AD hypotheses held steadfast, and to dare to wander into the unknown emptiness, currently taking hold of the minds of our nearest and dearest.

6 WHAT DOES ALL THIS MEAN AND WHY YOU SHOULD CARE

For all you non-scientist people, here is a summary of the important points discussed in the thesis.

Cholesterol as many of you know has been and still is considered by many as the "bad guy". Being depicted throughout the past several decades as the cause of cardiovascular diseases, stroke, diabetes and even Alzheimer's disease. The reality of this relationship, as all relationships, is much more complicated then it seems to be. The brain, which is the focus of this thesis, fortifies itself from the cholesterol in the blood with the help of a very tight barrier. Thereby preventing any cholesterol from entering this refuge. Ironically, the brain contains the most cholesterol in the body, so where does this cholesterol come from and how does the blocked cholesterol in the blood affect the brain?

The brain has very efficient machinery that manufactures cholesterol from small building blocks called Acetyl-CoA, which are able to cross this barrier. All the cholesterol in the brain comes from these factories, allowing it to be completely independent from the cholesterol in the blood. The brain uses this cholesterol in many things such as creating the outer walls of brain cells and coating nerve cells to allow the proper conduction of nerve signals. It is cholesterol that enables the brain its flexibility allowing us to be resilient in learning new things. It is therefore imperative for the brain to maintain a constant amount of cholesterol. This is achieved by balancing the production, usage and the removal of excess cholesterol.

The elimination of cholesterol from the brain is accomplished by converting the excess into another form of cholesterol known as 24S-Hydroxycholesterol (24S-OH), which is then discarded outside the barrier into the blood. The foreign counterpart of 24S-OH that is also able to pass over this barrier is 27-Hydroxycholesterol (27-OH). I use the adjective "foreign" because this oxysterol (oxidized cholesterol; i.e. when oxygen combines with cholesterol) originates predominantly from the rest of the body and enters the brain's sanctuary. The levels of 27-OH corresponds to that of cholesterol in the blood, meaning that higher amounts of cholesterol will lead to more 27-OH that is able to traverse the barrier into the brain. Hence, we hypothesized that 27-OH maybe how cholesterol is able to get around the barrier and enter the brain. Not much is known about what 27-OH does in the brain therefore we took it upon ourselves to embark on a journey to discover if 27-OH is truly more than just excess cholesterol waste. This thesis is a recollection of this journey.

Blood pressure is controlled by a system called the renin-angiotensin system (RAS) that is generally found in the kidney. However the brain has a similar system that is separate from that of the kidneys. The brain RAS also has a say in tuning the blood pressure but is more involved in processes that adjusts memory and the function of nerve cells. What we have found is that 27-OH modifies this system by increasing the levels of many of its key players. These key players include Angiotensinogen (AGT)(which is the raw material), Angiotensin-Converting enzyme (ACE)(the factory worker) and Angiotensin II (AngII)(the product). We found that the levels of these players are elevated in Alzheimer's disease and they correspond to the amount of 27-OH present in the fluid surrounding the brain known as cerebrospinal fluid (CSF). The product, Angiotensin II is further modified giving rise to two other forms, Angiotensin III (AngIII) and IV (AngIV). This modification process is heightened by 27-OH. In fact what 27-OH specifically does is create an imbalance between AngIII and Ang IV. This unevenness prevents the beneficial effects of AngIV, which is more glucose entering your brain cells and better memory. In mice that we genetically engineered to produce more amounts of 27-OH, we observed worse memory and less glucose in the brain. Furthermore, we put individuals into a scanner to measure the amount of glucose in their brains and determined how much 27-OH each individual had in their CSF. We discovered that the higher the amount of 27-OH an individual has, the less glucose that person has in their brains. Trying to understand why this is the case, we realized that 27-OH reduces the quantity of a glucose transporter (GLUT4) and consequently uncovered how it does this.

Another feature of Alzheimer's disease is inflammation. It is now considered to be an early event in the development of the disease. Though 27-OH is not known to cause inflammation in the brain it does propagate it in atherosclerosis. 27-OH is the most abundant form of oxidized cholesterol in the plaques that block arteries. It also aids in the release of molecules that promotes the dislodgement of those clogs. Within the brain we have seen that 27-OH also leads to the accumulation of a promoter of inflammation, S100A8. This promoter ultimately leads to the increase in β-secretase 1 (BACE1), the factory worker who produces amyloid beta, which is one of the characteristics of Alzheimer's disease.

All the evidences we unveiled point to that 27-OH is much more than a random form of cholesterol. It bypasses the barrier that the brain upholds and executes the negative effects that cholesterol is thought to cause. Thus reducing cholesterol with medications such as statins that are widespread may have some impact in reducing the harmful effects of 27-OH. Though statins have not yielded consistent results and have many adverse effects. Some statins even cross the brain barrier affecting cholesterol production in the brain and leading to memory disturbances. We believe that statins that do not cross may therefore be more beneficial due to their fewer side

effects in the brain. However, it is much more worthwhile to target 27-OH directly through curbing its production. We have also illuminated several points that can be targeted therapeutically to improve glucose metabolism and memory. Moreover, we believe that it is crucial to monitor the levels of not just cholesterol but also its counterpart 27-OH.

The current concepts that we portray within this thesis may be out of bounds of the current rhetoric for the development of diseases such as Alzheimer's disease. However, we believe that it is essential to tread into new areas and create new threads of thought that connects the many factors that together, cause these diseases. Diseases that affect millions worldwide, crippling not only their lives but the lives of the many people that they touch with their hearts. In an attempt to provide them with hope that their future is not futile. That they can take solace in knowing that we and others are working hard to understand and help prevent the shadow that is taking a hold of their minds.

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