# CHOLESTEROL METABOLISM IN THE BRAIN AND DEMENTIA

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Submitted to the Graduate Faculty of

the Graduate School of Public Health in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

University of Pittsburgh

2011

UNIVERSITY OF PITTSBURGH

Graduate School of Public Health

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April 21, 2011

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## ABSTRACT

**Introduction:** Cholesterol metabolism in the brain is implicated in the development of Alzheimer's disease (AD). Understanding the relationship between markers of brain cholesterol metabolism and the structural changes occurring in the aging brain has particular public health relevance to the treatment and prevention of AD.

**Methods:** This dissertation consists of a systematic review of the literature and two papers of original research. Our systematic review: critically evaluates the literature regarding brain cholesterol metabolism and AD, identifies gaps in our current understanding, and proposes directions for future research. The two papers of original research were designed to address these gaps in knowledge. We examined the relationship between plasma oxysterol metabolites and cerebrovascular disease, amyloid deposition in the brain, and incident cognitive impairment using two longitudinal cohorts of older adults with extensive characterization of cognition and brain structure. Quantitative marker of brain structure were prior to clinical disease using magnetic resonance imaging (MRI) and positron emission tomography (PET).

**Results:** Our review found inconsistent associations between brain-derived plasma oxysterols and AD. Epidemiological design issues and methodological limitations may explain these conflicting results; these include: residual confounding, lack of temporal of associations, and

inconsistent direction of associations resulting from stage of the disease at which oxysterols were measured. A major methodological limitation is the scarcity of objective measures to quantify underlying structural changes occurring the brain. Our original research examined the longitudinal association between oxysterols, cognition and brain imaging markers in non-demented older adults. We found higher levels of brain-derived oxysterols were associated with MRI markers of cerebrovascular disease and a greater risk of cognitive impairment over 8 years of follow-up. Furthermore, we found that lipid-lowering drugs modify the association between plasma oxysterols levels and amyloid deposition in the brain, visualized using PET.

**Conclusions:** There are important relationships between brain degeneration, cholesterol metabolism and dementia that need to be better understood. Brain-derived metabolites of cholesterol appear to be elevated in the early stages of disease prior to the onset of cognitive impairment. Brain-derived plasma oxysterols may be an important marker of underlying cerebrovascular disease preceding cognitive impairment and risk for developing cognitive impairment.

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# PREFACE

The author would like to acknowledge the work of Rona de la Vega and Beth Hauth in the Heinz Nutritional Research Laboratory for training and assistance in the measurement of oxysterols and lipids. The author would also like to acknowledge the support of his dissertation committee, his wife and his family. Without whom, this work would not be possible.

This research was supported in part by NHLBI Grant/Contract numbers N01-HC-85239, N01-HC-85079 through N01-HC-85086; N01-HC-35129, N01 HC-15103, N01 HC-55222, N01-HC-75150, N01-HC-45133; HL080295, HL-075366; NIA Grant/Contract numbers AG-023269, AG-15928, AG-20098, and AG-027058; University of Pittsburgh Claude. D. Pepper Older Americans Independence Center grant number P30-AG-024827; with additional contribution from NINDS. See also http://www.chs-nhlbi.org/pi.htm. Additional funding was provided by the Alzheimer's Disease Research Center at the University of Pittsburgh (AG05133) and grant funds to O.L.L. (AG020098) and L.K. T.M.H. was supported in part by funds from an NHBLI training grant to the University of Pittsburgh (T32HL083825).

**Key Terms:**
Oxysterols
Brain
24S-hydroxycholesterol
Cerebrovascular disease
Alzheimer's disease
Dementia

# 1.0 REVIEW ARTICLE: CHOLESTEROL METABOLISM IN THE BRAIN AND DEMENTIA

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## 1.1 INTRODUCTION TO REVIEW

Studies examining cholesterol synthesis as an intervention target for dementia make the assumption that altering cholesterol levels in the circulation correlate directly with cholesterol levels in the brain. In fact, the measurement of brain cholesterol *in vivo* remains difficult because the presence of the blood brain barrier (BBB) ensures the near complete separation of cholesterol in the body from cholesterol produced in the brain. Cholesterol's inability to cross the BBB makes total blood cholesterol a surrogate marker with little potential relevance to the brain(Bjorkhem 2006). Other more proximate markers of cholesterol concentrations in the brain exist. The brain-derived oxysterol, 24S-hydroxycholesterol (24-OHC) crosses the BBB directly by diffusion and is the primary means by which excess cholesterol is eliminated from the brain(Bjorkhem 2006). The ability of 24-OHC to cross the BBB, whereas cholesterol cannot, makes 24-OHC a noninvasive and more proximate marker of cholesterol homeostasis occurring in the brain(Leoni 2009).

In the past decade, there has been a growing interest in examining the association between brain-derived oxysterol, 24-OHC and neurodegenerative diseases including vascular dementia, Alzheimer's disease and mild cognitive impairment. Previous studies suggest that 24-OHC is a marker of the number of metabolically active neurons in the brain and is associated with the diagnosis and severity of dementia and other neurodegenerative diseases.

This review critically evaluates evidence as to whether plasma 24-OHC is a suitable marker of dementia and it proposes directions for future studies of objective measures of brain degeneration. Specifically, we propose that: 1) the brain-derived oxysterols, 24-OHC, is a more

proximate marker of cholesterol homeostasis in the brain than the level of total cholesterol in the circulation; 2) excess cholesterol in the brain is likely a by-product of cell membrane disruption; and 3) excess brain cholesterol leads to increased 24-OHC levels; 4) excess brain cholesterol contributes to amyloid plaque formation and the risk of dementia; 5) 24-OHC is a potential marker of the changes in cholesterol homeostasis occurring early in the dementia process. This review is organized into three major sections. First, we review cholesterol synthesis and metabolism in the brain. Then, we provide a detailed overview of the issues regarding the measurement of 24-OHC as a marker of cholesterol metabolism in the context of age-related cognitive impairment. Finally, we discuss these findings in the context of potential mechanisms linking excess cholesterol in the brain to the development of dementia.

## 1.2 CHOLESTEROL METABOLISM IN THE BRAIN

Maintaining cholesterol homeostasis in the brain is vitally important to its proper functioning. Cholesterol deficiency in the brain will inhibit neuronal growth(Mauch and Nagler 2001) and integrity of the connection between neurons(Pfrieger 2003). Conversely, excess cholesterol levels in the body are associated with increased AD risk(Kivipelto and Helkata 2002) and demonstrate AD pathology *in vivo*(Reid, Urano et al. 2007).

The brain is the richest organ in cholesterol content, containing about 25% of the total amount of cholesterol in the body(Snipes and Suter 1997). Efficient cholesterol synthesis and metabolism is critically important to maintaining the function of the entire brain(Snipes and Suter 1997). The vast majority (70-80%) of the cholesterol in the brain resides in the myelin. Axonal myelination lowers the action potential of the nerve, speeding the propagation of the

signal along the length of the axon(Bartzokis 2004) and increasing the connectivity between neurons. Myelin is composed mostly of fat (70%), and 28% of this fat is cholesterol. Thus, a major function of brain cholesterol homeostasis is to maintain the insulatory properties of the myelin(Pfrieger 2003).

Highly efficient mechanisms have evolved for maintaining constant levels of cholesterol in the brain. The most important of these is the isolation of brain-derived cholesterol by the BBB(Bjorkhem 2006). Cholesterol is unable to cross the BBB directly, thus ensuring the complete separation of cholesterol in the circulation from cholesterol synthesized in the brain. The BBB isolates brain cholesterol from fluctuations of cholesterol in the periphery. As a result, both the *de novo* synthesis and the metabolism of cholesterol are solely responsible for cholesterol homeostasis in the brain(Leoni 2009).

### 1.2.1 Synthesis of cholesterol in the brain and transport via ApoE.

Cholesterol synthesis occurs at relatively low rates in the brain, with the majority of production believed to come from the glial cells(Pfrieger 2003; Dietschy and Turley 2004). Following synthesis, cholesterol is secreted from the glial cells and internalized within ApoE for delivery to the neurons(Pfrieger 2003). Cholesterol has been shown to play important roles in central nervous system development and synaptic plasticity. *In vitro* studies indicate cholesterol is a synapse-promoting factor when carried by ApoE(Mauch DH and K 2001). ApoE is involved in the distribution and recycling of cholesterol during neuronal growth and death. Neurons meet their cholesterol requirements via ApoE dependant mechanisms(Pfrieger 2003).

Cholesterol-rich ApoE bind primarily to a group of receptors known as the LDLR family. Over ten receptors have now been identified as members of the LDLR family. They all bind

ApoE and a diverse array of ligands to mediate their signaling and transport across plasma membranes(Bu 2009). LDLR and LRP1 are the most prominent members of the LDLR family and also the main ApoE metabolic receptors in the brain(Abbott, Sharp et al. 1997). As the prototype of this family, LDLR is the main receptor for cholesterol homeostasis(Rapp, Gmeiner et al. 2006). LDLR is more prominently expressed in glia than in neurons. Data from mice gene deletion models suggest that LDLR gene increases ApoE levels in the brain parenchyma and CSF, suggesting that it causes impaired metabolism of ApoE and cholesterol recycling(Fryer, Demattos et al. 2005). LRP1 is expressed highly on the surface of neurons and to a lesser degree in glia. Deletion of LRP1 gene increases ApoE and decreases cholesterol concentrations in the mouse brain(Fryer, Demattos et al. 2005).

Once delivered to the neuron by ApoE, cholesterol is internalized and incorporated into the neuronal membranes, including the myelin. Neurons meet their cholesterol requirements via local synthesis and ApoE-dependant tranport(Pfrieger 2003). Depending on the requirements of the neuron, cholesterol is either catabolized into cholesterol esters for storage in plasma membranes and myelin or metabolized into oxysterols for elimination(Brown and Theisler 2004).

### 1.2.2 The brain's reaction to excess cholesterol and the potential link to Alzheimer's disease.

Experimental animal and cell-culture studies suggest that cholesterol metabolism in the brain can modify amyloid production(Kuller 2007). Bartzokis recently proposed a model of Alzheimer's disease (AD) pathology that is linked to cholesterol metabolism(Bartzokis 2009). The neuropathological profile of AD consists of deposition of beta amyloid (Aβ) in the form of

neuritic plaques and by neurofibrillary tangles in soluble deposits of hyperphospholated tau(Reiss 2005; Blennow and de Leon 2006). Bartzokis' model proposes the breakdown of the myelin as the instigating event of amyloid formation and deposition. This myelin model reframes key observations of the AD process, such as: axonal transport disruptions, formation of axonal swellings/sphenoids, neuritic plaques, and proteinaceous deposits, including Aβ and tau, as by-products of homeostatic myelin repair processes(Bartzokis 2004). *In vitro* studies show that membranes containing high levels of cholesterol are more likely to lead to a primary cleavage of the amyloid precursor protein (APP) by β-secretase leading to increased Aβ production and deposition surrounding the neuron(Fassbender and Stoick 2002). The deposition and aggregation of neuritic plaques and neurofibrillary tangles will in turn lead to widespread loss of neuronal funtion and loss of synapses(Brown and Jessup 2009). As the branching of the neuron decreases, so does the plasma membrane and myelin. Because cholesterol is so abundant in the plasma membrane and myelin, synaptic degeneration will in turn cause excess cholesterol release(Brown and Jessup 2009). We propose that there is a feed-forward relationship connecting myelin breakdown, excess cholesterol in the brain, the formation of Aβ plaques, and neuronal death occurring in the brain (Figure 1).

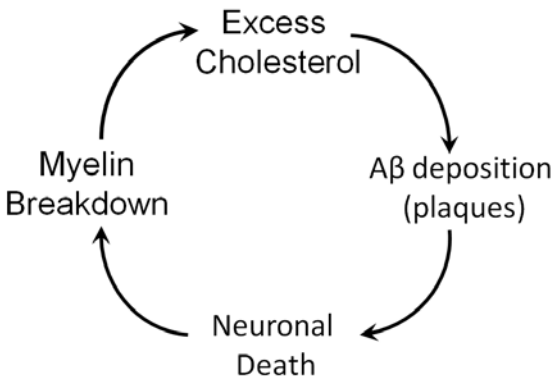


**Figure 1: Hypothesized feed forward loop of myelin breakdown as the initiating event for beta amyloid deposition.**

Another link between cholesterol metabolism and AD pathology rests at the level of the ApoE. Besides transporting cholesterol from the glial cells to the neurons, ApoE is also a Aβ chaperone. There are strong and important consequences of ApoE binding Aβ. First, the region of ApoE that is responsible for Aβ binding overlaps with the cholesterol-binding region(Tamamizu-Kato, Cohen et al. 2008). Second, the binding of Aβ to ApoE also changes the ability of ApoE to bind cholesterol(Tamamizu-Kato, Cohen et al. 2008). Thus, Aβ peptides can interfere with the normal function of ApoE in brain lipid metabolism and contribute to AD pathogenesis. This suggests that the efficient function of ApoE as a cholesterol carrier protein may be involved in its effect on AD risk(Wollmer 2010). Additionally, the capacity of the three isoforms of ApoE to mediate cholesterol efflux to neuronal cells correlates inversely with their effect on AD risk (ApoE-4≤ ApoE-3<ApoE-2)(Michikawa, Fan et al. 2000). Importantly, ApoE-4 allele is the primary genetic risk factor for early age at onset of Alzheimer's disease (AD)(Richard and Amoyuel 2001). The ApoE gene has been estimated to account for 65% of all sporadic cases of AD(Richard and Amoyuel 2001). The discovery that the ApoE-4 allele is the

primary genetic risk factor for early age at onset of Alzheimer's disease (AD) has renewed interest in the relationship between cholesterol metabolism and the risk of AD and dementia.

### 1.2.3 Production of 24 OHC as a means to eliminate excess cholesterol in the brain.

Cholesterol bound by ApoE can be eliminated from the brain via the cerebrospinal fluid (CSF)(Shafaati, Solomon et al. 2007) along the interstitial fluid (ISF) drainage pathway, through the blood-brain barrier (BBB), where it then enters the circulation or undergo proteolytic degradation upon delivery to lysosomes(Bu 2009). Until recently, ApoE-dependant elimination was the only mechanism known to eliminate cholesterol from the brain(Bjorkhem 2006). However, the rate of elimination via this route is less than 1% or approximately 1mg per 24 hours(Bjorkhem and Lutjohann 1998). If this were the only mechanism of elimination, the half-life of cholesterol in the brain would be about 50 years(Bjorkhem and Lutjohann 1998).

The other 99% of excess cholesterol in the brain is eliminated by the enzyme-dependant oxidation of excess cholesterol into oxysterols(Bjorkhem 2006). Oxidization of cholesterol at the 24-position, forming 24S-hydroxycholesterol (24-OHC), occurs in the neuronal cells via the enzyme CYP46A1 (24-hydroxylase)(Bjorkhem 2006). The enzyme 24-Hydroxylase is produced almost exclusively in the neuron and only a subset of neurons express the enzyme, these include: pyramidal cells of the cortex, Purkinje cells of the cerebellum and some neurons of hippocampus and thalamus(Lund, Guikeyardo et al. 1999). Expression of 24-hydroxylase is large confined to the brain in mouse and humans; there is no evidence to date that of its expression in the peripheral nervous system(Russell, Halford et al. 2009). Genetic variation in 24-hydroxylase governs the rate each individual converts cholesterol into 24-OHC(Kolsch 2009). The promoter region of the CYP46 gene bears all the classical hallmarks of a gene with putative housekeeping

functions(Ohyama, Meaney et al. 2006; Brown and Jessup 2009). Therefore, plasma 24-OHC is primarily a neuron derived product and its levels in the circulation likely reflect the number of metabolically active neuronal cells in the brain able to convert cholesterol to 24-OHC(Leoni 2009). This is demonstrated patients with brain death, the levels of plasma 24-OHC demonstrated a 50% decrease within two hours of brain death(Bretillon and Siden 2000).

Seminal work by Bjorkhem *et al.* demonstrates that 24-OHC can cross the blood-brain barrier directly by diffusion and is the primary mechanism by which the brain removes excess cholesterol(Bjorkhem and Lutjohann 1998; Bjorkhem 2006). The ability of oxysterols to traverse the BBB -while cholesterol cannot- results from the addition of the hydroxyl group on the steroid side-chain of the cholesterol molecule. Upon contact with the blood-brain barrier, the hydroxyl group of the oxysterols induces a reordering of the phospholipid acyl chains within the plasma membrane to create a pocket within the plasma membrane which permits the diffusion of the oxysterol across the BBB. The diffusion of 24-OHC across the BBB occurs at a rate of 6-7mg/24 hour(Bjorkhem and Lutjohann 1998). The flux of oxysterols across the BBB appears to be concentration dependant. No regulatory factor residing within the BBB has been described yet in the literature.

Once across the BBB, the oxysterols can be extracted by an acceptor such as a circulating lipoprotein in the blood. Burkhard et al. determined the distribution of esterified and unesterified 24-OHC and 27-OHC in lipoprotein sub-fractions(Burkhard, von Eckardstein et al. 2007). They found oxysterols, 24-OHC and 27-OHC are carried primary in their esterified form by high density lipoproteins (HDL) and low density (LDL) lipoproteins throughout the circulation(Burkhard, von Eckardstein et al. 2007). The percent recovery of oxysterols from lipoprotein sub-fractions appears to be slightly higher from HDL subfractions than from LDL

subfractions(Burkhard, von Eckardstein et al. 2007). Given the lower levels of HDL in the circulation, this suggests that oxysterols may be preferentially bound and transported by HDL throughout the circulation.

The elimination of 24-OHC from the circulation is dependent on rate of hepatic metabolism by three different species of cytochrome P-450 (CYP7A1, CYP39 and CYP27) which conjugate or metabolize oxysterols(Bjorkhem 2006). However, liver disease has not been show to affect levels in the circulation(Bretillon and Lutjohann 2000).

### 1.2.4 The role of 24-OHC in the brain.

The production of 24-OHC not only promotes the clearance of excess cholesterol from the brain, but may also be involved in signaling the homeostatic mechanisms regulating cholesterol production and distribution in the brain. 24-OHC directly suppresses the synthesis of cholesterol, via suppression of HMG CoA reductase in the glial cell(Lund, Guikeyardo et al. 1999). HMG CoA reductase is the rate limiting step in cholesterol synthesis in the body and brain(Lund, Guikeyardo et al. 1999). Oxysterols are also believed to be ligands for the nuclear receptors found on glial cells(Lehmann, Kliewer et al. 1997). Liver X receptors (LXR) beta and alpha regulate sterol transporters ATP-binding cassette A (ABCA1) and G (ABCG1) which regulate transport of cholesterol from the glials cells to the neuron. ABCA1 acts by regulating ApoE levels and ApoE lipidation which directly affects its ability to carry excess cholesterol(Wahrle, Jiang et al. 2004). Studies of combination ABCA1 and ABCG1 knockout mice show simultaneous reduction in the rate of cholesterol synthesis and activation of LXR signaling(Wang, Yvan-Charvet et al. 2008). When bound to LXR, 24-OHC is believed to stimulate ABCA1 to increase the amount of cholesterol carried by ApoE to the neuron.

At extremely high levels, 24-OHC is also believed to have direct neurotoxic effects on undifferentiated human neuroblastoma cells *in vitro*(Kolsch and Luthjohann 1999). High levels of 24-OHC may induce apoptosis in the surrounding cells which is mediated by the generation of oxidative free radicals. Without the presence of antioxidative defense systems, the apoptosis-oxidative stress cycle continues unabated. These oxidative defense systems such as superoxide dismutase are weakened in AD(Richardson 1993). However, studies of 24-OHC kinetic demonstrates diffusion of 24-OHC out of the brain is concentration dependent(Bjorkhem 2006). Increases in brain concentrations of 24-OHC would be expected to lead to increased diffusion across the blood-brain barrier, therein reducing concentrations of 24-OHC in the brain.

### 1.2.5 Sources of excess cholesterol in the brain.

Potential sources of excess cholesterol in the brain may include: excess synthesis from glial cells, plasma membrane breakdown, myelin breakdown, and subsequent neuronal cell loss. While hypercholesterolemia in the blood can come from *de novo* and dietary sources, hypercholesterolemia in brain is only affected by *de novo* production and homeostasis(Bjorkhem 2006). In the brain, cells must produce cholesterol or import the required cholesterol from neighboring cells. This process ensures brain cholesterol is derived locally and is highly regulated by their oxysterol metabolites. According to the current model of cholesterol homeostasis, excessive cholesterol synthesis would stimulate increased production which in turn down regulates cholesterol synthesis through LXR signaling(Bjorkhem 2006).

Moving across the BBB in an opposite flux is the oxysterol 27-hydroxycholesterol (27-OHC). 27-OHC occurs as an extracellular oxidation of cholesterol. Nearly all the cells in the body contain the enzyme 27 hydroxylase (CYP27A1), which is responsible for converting

cholesterol into 27-OHC, while macrophages have the highest levels of CYP27A1. The influx of 27-OHC into the brain has been found to be about 5mg per 24 hours(Meaney and Heverin 2007). The levels in the CSF correspond to levels the circulation, indicating the diffusion of 27-OHC from the circulation across the BBB is most likely concentration dependant(Bjorkhem 2006). Diffusion of 27-OHC has also been suggested to correspond to the integrity of the BBB. Damage to this barrier is believed to result from a higher flux of 27-OHC from the circulation into the brain(Leoni and Masterman 2003).

Disruption of the BBB may consist of gaps within the plasma membrane where systemic cholesterol or 27-OHC may enter. In the normal brain, the influx of 27-OHC from the periphery into the brain through the BBB is believed to be concentration-dependent(Bjorkhem 2006). With increased 27-OHC levels in the circulation as it is found in hypercholesterolemic states, disruption of the BBB may increase infiltration of 27-OHC(Leoni and Masterman 2003; Ghribi 2008). Inside the brain, 27-OHC is metabolized to 7α-hydroxycholesterol (7α-OHC)(Bjorkhem 2006). Excess 27-OHC in the rabbit brain has been shown to lead to Aβ production and deposition(Ghribi 2008). Excess 27-OHC in the brain would not be expected to produce changes in 24-OHC production as there is no direct mechanism know to convert 27-OHC to 24-OHC. The influx of peripheral cholesterol, through a damaged BBB into the brain, may lead to increased 24-OHC production and its diffusion out of the brain. Yet, studies of 24-OHC in stroke patients do not show that 24-OHC levels in the plasma are altered by acute BBB disruption(Holdenrieder and Lutjohann 2004). The inability to quantify the amount of cholesterol or of 27-OHC entering the brain, the relative concentrations of 27-OHC within the brain, and the rate at which 27-OHC is metabolized within the brain remain obstacles impeding further examination of this hypothesis *in vivo*.

Myelin breakdown is a common phenomena of aging(Bartzokis 2004). The most vulnerable myelin are those produced later in life(Bartzokis 2004). Myelin and its components such as cholesterol and myelin proteins are reduced in old age and substantially further reduced in mild cognitive impairment (MCI) and dementia(Bartzokis 2009). *In vitro* evidence demonstrates breakdown of the myelin releases fat, iron, and cholesterol into the extracellular space and CSF leading to excess cholesterol levels and neuronal death (Figure: Feed Forward Loop)(Bartzokis 2004). While all of these instances could lead to dramatically increased cholesterol levels in the brain, they will also trigger conversion of cholesterol into 24-OHC which is in turn cleared through the BBB into the periphery.

### 1.2.6 The role of 24-OHC in amyloidogenesis and Alzheimer's disease.

The conversion of cholesterol to 24-OHC may be indirectly neuroprotective, because it reduces concentrations of excess cholesterol that lead to increased Aβ production in the brain(Lutjohann, Papasotiropoulus et al. 2000). The production of 24-OHC has been shown to be an efficient inhibitor of the formation of Aβ *in vitro*(Brown and Theisler 2004). 24-OHC is believed to play a role in the suppression of amyloidogenesis and acts as a stimulator of ApoE-mediated removal of cholesterol via the CSF(Brown and Jessup 2009). Genetic variation in 24-hydroxylase is associated with an increased load of Aβ and the early onset of AD (Table A1)(Kolsch 2009).

Alzheimer's disease is characterized by deposition of beta amyloid (Aβ) in the form of neuritic plaques and by neurofibrillary tangles in soluble deposits of hyperphospholated tau(Reiss 2005; Blennow and de Leon 2006). In biochemical studies of AD, total tau is used as a marker of neurodegeneration, β-amyloid 42 ($A\beta_{42}$) is used to reflect aggregation and subsequent

plaque deposition of β-amyloid, and phosphorylated tau is used as an indicator of hyperphosphorylation of tau and formation of neurofibrillary tangles. Among AD and MCI patients, CSF levels of 24-OHC are positively and significantly correlated total tau and phosphorylated tau in the CSF(Leoni, Shafaati et al. 2006; Solomon, Leoni et al. 2009). The ratio of plasma 24-OHC to total cholesterol (24-OHC/Chol) is associated with CSF levels of Aβ42, total and phosphorylated tau after controlling for age, sex, ApoE-4 status, and statin therapy(Solomon, Leoni et al. 2009). The levels of 24-OHC in the plasma and CSF are highly correlated with ApoE levels. In the CSF, the levels of 24-OHC increase additively with the number of ApoE-4 alleles. In the plasma, ApoE is an independent predictor of levels of 24-OHC and the ratio of 24-OHC/Chol. An in-depth discussion of oxysterols, their links with tau and their role in tauopathies is presented by Leoni, Solomon and Kivipelto(Leoni, Solomon et al. 2010).

### 1.2.7 Variation in plasma oxysterol levels across the lifespan.

More than 90% of the 24-OHC in the circulation is of cerebral origin; less than 10% of plasma 24-OHC is believed to come from the adrenals, bone marrow, and other sources(Bjorkhem 2006; Kolsch 2009). The half-life of 24-OHC in the circulation is approximately 12 hours(Bjorkhem and Lutjohann 1998). There appears to be neither diurnal or post-prandial variation in levels of 24-OHC. The lack of post-prandial variation confirms that 24-OHC concentrations are independent of time of nutritional intake(Bjorkhem and Lutjohann 1998). Variation in plasma levels of 24-OHC appear to vary by age, gender, cholesterol concentrations in the brain, metabolism of cholesterol in the gray matter, and genetic variation in the family of genes coding for cytochrome P450 enzymes. The levels of 24-OHC tend to be higher in women; whereas levels of 27-OHC tend to be higher in men; however neither

oxysterols has been shown to vary with the menstrual cycle (Burkhard, von Eckardstein et al. 2007).

Variation in plasma concentrations of 24-OHC occurs over the life-span. Concentrations are highest in the circulation during the first two decades of life. This is postulated to be a consequence of higher production during active myelination relative to a lower degree of hepatic metabolism in youth. It has been suggested this is due to a greater head size to liver size ratio in youth(Bretillon and Lutjohann 2000). In the absence of neurodegeneration, concentrations of 24-OHC are relatively stable between the third and seventh decades of life. An age related decline appears to be initiated in the sixth and seventh decades of life, which parallels the decline in gray matter and total brain volume beginning in the fifth decade of life(Fotenos and Snyder 2005). Thelen *et al.* examined cholesterol, including its precursors and metabolites, in the brains of autopsied decedents of various ages that had no history of cognitive or mental disease(Thelen and Falkai 2006). The autopsied brains showed that while the amount of total cholesterol was the same in young and older participants (<38 versus >38 years of age), the precursors of cholesterol were significantly higher in the young participants. The concentration of 24-OHC were significantly lower in cerebrospinal fluid of hippocampal sections in older adults compared to younger brains(Thelen and Falkai 2006). However the authors did not correlate the levels of cholesterol metabolites with the volume or weight of the autopsied brains.

### 1.2.8 Challenges to obtaining accurate markers of brain cholesterol metabolism: interaction with statins.

Studies of oxysterols and neurodegenerative disease express plasma levels of 24-OHC in three ways: as an absolute value, as a ratio to total cholesterol, and as a ratio to 27-OHC. As

outlined above, absolute levels of plasma 24-OHC are regarded as surrogate marker of the number of metabolically active neurons located in the gray matter of the brain(Leoni 2009). When expressed as a ratio to total cholesterol (24-OHC/Chol), the ratio represents cholesterol metabolism to oxysterols in the brain relative to total cholesterol in the circulation (periphery). This ratio places potential changes in absolute levels of 24-OHC in the context of changes in plasma cholesterol that may be occurring later in life. Longitudinal studies of changes in cholesterol levels show that total cholesterol tends to increase with age in young or middle-aged adults but decreases in late life(Hershcopf, Elahi et al. 1982; Abbott, Sharp et al. 1997; Ferrara, Barrett-Connor et al. 1997; Solomon, Kareholt et al. 2007). In younger participants, potential elevations in 24-OHC occurring as a result of neurodegenerative disease may not be evident in ratio of 24-OHC to total cholesterol if the levels of total cholesterol remain unaffected.

The metabolism of cholesterol to oxysterols is likely dependent on cholesterol concentrations and affected by statin use. Small studies of patients with normal and elevated cholesterol levels have examined the effect of statins on 24-OHC levels in the CSF and plasma(Botti and Triscari 1991; Locatelli and Lutjohann 2002; DeKosky 2005; Thelen and Laaksonen 2006). The ability of the statin to alter cholesterol synthesis in the brain and the subsequent levels of 24-OHC appears to be largely dependent on the ability of the statin to cross the BBB. Statins that cannot cross the BBB, appear to depress total plasma cholesterol levels without producing reductions in brain cholesterol synthesis or its metabolism to oxysterols; in effect, creating a higher ratio of 24-OHC/Chol. Potent lipophilic statins atorvastatin and simvastatin can cross the BBB and appear to suppress cholesterol synthesis in the body and brain leading to subsequent decreases in the levels of plasma 24-OHC(Locatelli and Lutjohann 2002). Conversely, pravastatin is considerably more hydrophilic, and less likely to cross the blood brain

barrier. As a result, the ratio of plasma 24-OHC/Chol is expected to be higher patients treated with pravastatin compared to patients treated with the BBB penetrating statins, atorvastatin and simvastatin. These studies demonstrated that high-dose statin therapy using statins able to penetrate the BBB resulted in decreased absolute levels of 24-OHC in the circulation; yet, the ratio of 24-OHC to circulating total cholesterol, remained unchanged(Locatelli and Lutjohann 2002).

## 1.3 METHODS OF REVIEW

### 1.3.1 Search strategy

Original articles and reviews were identified by search of Ovid Medline for articles published between 1950 and the first week of March 2011. Searches utilized MeSH terms ("24S-hydroxycholesterol" or "24-hydroxycholesterol") and ("Brain" or "Cognitive"). The relative sensitivity of this search strategy was evaluated by reconditioning the search to include addition of the keyword "oxysterol". The search was repeated using alternative keywords ("oxysterols" and ("Brain" or "Cognitive")) not ("24S-hydroxycholesterol" or "24-hydroxycholesterol"). This search provided no additional articles relating 24-OHC levels to neurodegerative disease.

### 1.3.2 Selection criteria

All observational and experimental studies in humans investigating cholesterol metabolism in the brain and its relationship to neurodegeneration and cognition were considered.

We chose to limit our review to studies evaluating 24S-hydroxycholesterol in human subjects and cell lines (n=93). Only original articles involving human subjects were considered for this review; we eliminated 17 *in vitro* and 20 review articles which left 56 suitable articles. These 56 articles were evaluated and categorized according to the following criteria. Pertinent studies included in this review focused on: 1) hydroxycholesterols and neurodegenerative diseases (AD, MS, Huntington's, etc); 2) population based genetics studies of CYP family hydroxylases; 3) Extracellular metabolism of cholesterol in the brain; 4) interventions modifying hydroxycholesterol synthesis or metabolism. The 26 articles investigating the relationship between hydroxycholesterol levels in human subjects with neurodegenerative disease were evaluated for the utilization of magnetic resonance imaging modalities. Only five studies were found which utilized MRI when evaluating the association between levels of oxysterols and neurodegenerative diseases. The selection of articles for review was then limited to just articles pertaining to age-related neurodegeneration, including: dementia, Alzheimer's disease, mild cognitive impairment and studies with cognitively normal participants. All review articles were assessed for references potentially missed by OvidMEDLINE searches. One reviewer (TMH) prepared searches, applied the selection criteria and collected articles relevant to the topic.

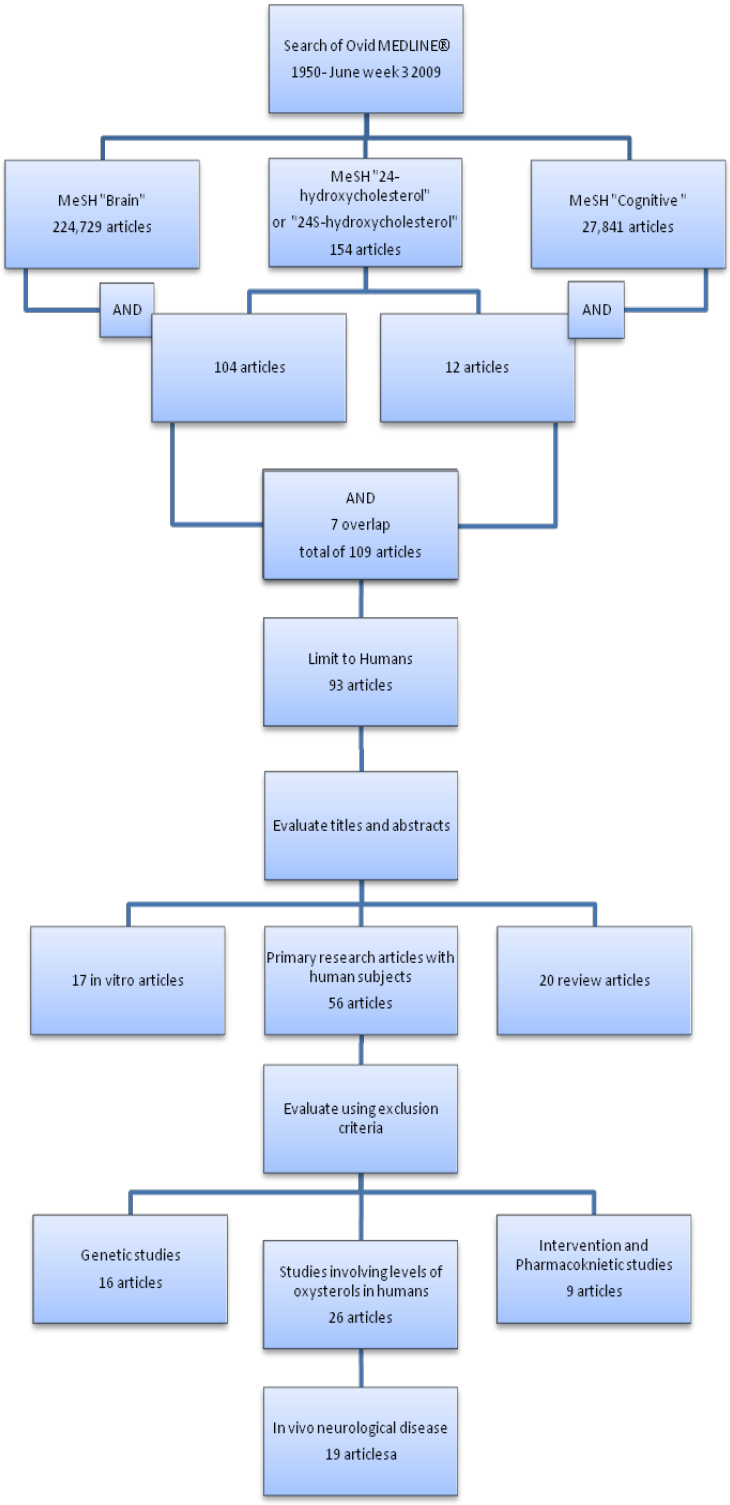


**Figure 2: Flow chart detailing literature review of oxysterols and neurodegenerative diseases**

## 1.4 OXYSTEROLS AND NEURODEGENERATIVE DISEASE

Results of the OVID Medline search produced 19 articles addressing plasma or CSF levels of 24-OHC in neurodegenerative diseases [multiple sclerosis (MS) = 3; Alzheimer's disease (AD) = 9; Huntington's disease = 1; dementia, mild cognitive impairment (MCI), or cognitive performance = 4; stroke = 2; and multiple neurodegenerative diseases = 2] Of these 19 studies (summarized in Tables A1-A4), only five correlate 24-OHC with structural measures of brain disease. Three articles utilize MRI to produce volumetric measures of the brain regions for comparison between diseased and normal patients(Leoni and Mariotti 2008; Koschak and Lutjohann 2009; Solomon, Leoni et al. 2009). Another study, conducted in MS patients, assessed the presences of lesions indicative of neurodegenerative disease processes using MRI(Karrenbauer and Leoni 2006). However, no study has quantified the extent or severity of brain lesions and levels of oxysterols in the brain.

The use of 24-OHC as a biomarker of neurodegeneration has been investigated in the context of various neurodegenerative diseases. Early reports relied strictly on clinical definitions of disease processes(Bretillon and Siden 2000; Lutjohann, Papasotiropoulus et al. 2000; Papassotiropoulus and Lutjohann 2000; Schonknecht and Lutjohann 2002; Teunissen, Lutjohann et al. 2003; Heverin and Bagdanovic 2004; Kolsch and Heun 2004; Leoni, Masterman et al. 2004; Leoni, Shafaati et al. 2006; Shafaati, Solomon et al. 2007) and did not apply imaging modalities for structural confirmation or quantification of disease severity. More recent publications have aimed to correlate levels of 24-OHC with structural or biochemical imaging techniques of the brain(Leoni and Masterman 2002; Karrenbauer and Leoni 2006; Thelen and Falkai 2006; Leoni and Mariotti 2008; Koschak and Lutjohann 2009; Solomon, Leoni et al. 2009). The latter publications appear to provide direct parallels between oxysterol levels and

structural changes in the brain as well as more sensitive staging of disease. A brief summary of these studies follows.

### 1.4.1 Association with non-dementia neurodegenerative diseases.

Demyeliating diseases degrade the myelin of the neurons, where the vast majority of brain cholesterol is stored; therefore, these diseases provide unique insight into cholesterol homeostasis and metabolism during the neurodegenerative disease process (detailed in Table A2). Studies of patients with acute demyeliating diseases (Guillian-Barre syndrome and inflammatory demyeliating polyradiculoneuropathy) indicate the levels of 24-OHC are increased in the patient's CSF and plasma compared to controls(Leoni, Masterman et al. 2004). The ratio of 24-OHC to 27-OHC (24-OHC/ 27-OHC) is also elevated in patients with acute demyelinating diseases compared to controls. The literature investigating oxysterol concentrations in patients with multiple sclerosis (MS) suggest levels of 24-OHC experience a transitory increase in both the plasma and CSF during remitting-relapsing and early stages of disease(Leoni, Masterman et al. 2004). Progressive and later stages of disease suggest consistent declines in levels of 24-OHC in the circulation(Leoni and Masterman 2002; Leoni, Masterman et al. 2004; Karrenbauer and Leoni 2006). Among MS patients, levels of 24-OHC are higher in younger patients and tend to decrease with increasing age and disability scores(Leoni, Masterman et al. 2004; Karrenbauer and Leoni 2006). Both absolute levels of plasma 24-OHC and the ratio 24-OHC/Chol were found to be significantly lower in both primary progressive (PPMS) and relapsing-remitting (RRMS) disease states than normal controls(Karrenbauer and Leoni 2006). The current model of oxysterols as markers of neurodegenerative disease suggests that plasma and CSF levels depend upon stage of disease at which the 24-OHC is measured. Concentrations of plasma 24-OHC may

be elevated (early stages), normal (middle stage), or decreased (later and progressive stages)(Leoni 2009). This hypothesized trajectory of 24-OHC over the neurodegenerative process is used below to evaluate results from cross sectional studies of 24-OHC and dementia.

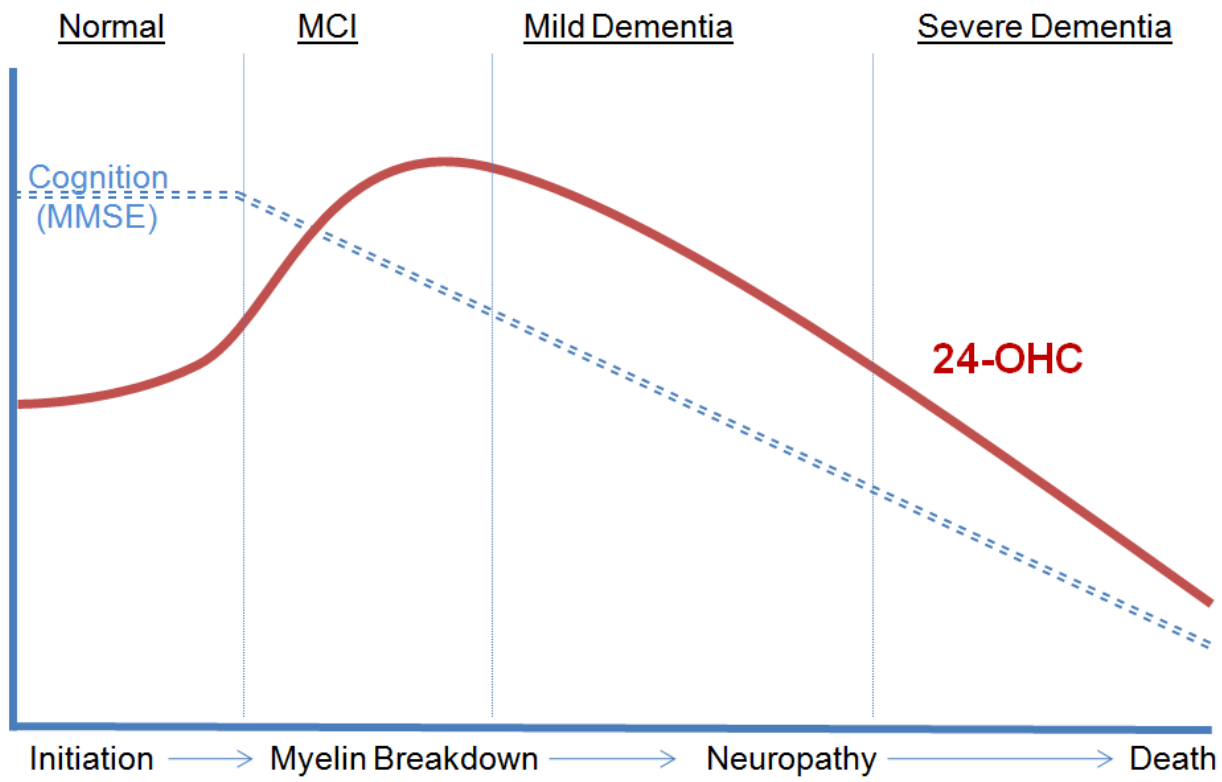


**Figure 3: The hypothesized trajectory of 24-OHC and cognition across stages of age-related neurodegeneration.**

### 1.4.2 Neuroimaging studies of oxysterols and neurodegenerative diseases.

Magnetic Resonance Imaging (MRI) has recently been utilized in the study of 24-OHC and neurodegenerative disease states (Table A3). To date, only one study of dementia and 24-OHC utilized MRI measures(Solomon, Leoni et al. 2009). MRI has been implemented in studies of other neurodegenerative diseases, including MS and Huntington's disease(Leoni, Masterman et al. 2004; Karrenbauer and Leoni 2006; Leoni and Mariotti 2008). Results from these studies can provide insight into what we can expect to see from MRI studies of dementia-type lesions.

Gadolinium-enhanced (Gd) lesions visible on MRI are used in MS to identify primary progressive (PPMS) and relapsing-remitting (RRMS) disease states. Gd-positive lesions on MRI are believed to indicate areas of active disease, where BBB disruption and ongoing perivascular disease may be occurring(Karrenbauer and Leoni 2006). Increased levels of plasma 24-OHC was found only in patients with lesion confirmed active disease indicated by Gd-positive MRI scans, specifically in PPMS patients(Leoni, Masterman et al. 2004; Karrenbauer and Leoni 2006). The presence of hypointense and hyperintense lesions on $T_1$ and $T_2$ weighted MRI have been examined in relation to levels of plasma 24-OHC in MS patients. The volume of $T_2$–weighted hyperintense lesions is a marker of cumulative disease extent representing a spectrum of events, ranging from demyelination and edema to gliosis and axonal loss(Karrenbauer and Leoni 2006). In both RRMS and PPMS disease states, decreases in ratio 24-OHC/Chol were associated with increases in disease extent as indicated by the volume of $T_2$–weighted hyperintense lesions. In RRMS patients alone, the ratio 24-OHC/Chol was positively associated with the volume of $T_1$ hypointense lesions which are believed to represent axonal loss(Karrenbauer and Leoni 2006). These data suggest increases in plasma 24-OHC would be expected in early states of

neurodegenerative diseases where demyelination is initiated; whereas decreases in plasma 24-OHC would be expected in latter stages of disease.

Plasma levels of 24-OHC were significantly higher in controls than Huntington's disease patients at all stages of the disease. The levels of 24-OHC and 27-OHC paralleled large decreases in caudate volumes. Lower levels of plasma 24-OHC were also seen in later more advanced stages of disease(Leoni and Mariotti 2008). The correlations between oxysterols and caudate volumes were also seen in cognitively normal controls without neurodegenerative disease(Koschak and Lutjohann 2009).

MRI studies of 24-OHC and brain volume show the metabolite of cholesterol is differentially associated with fractions of the brain. Segmentation of the brain into CSF, gray matter and white matter shows that the ratio of 24-OHC/Chol was positively associated with larger grey matter fractions across all subjects with AD, MCI and subjective cognitive impairment(Solomon, Leoni et al. 2009). Interestingly, the ratio of 24-OHC/Chol was associated with grey matter volume and total brain volume in subjects with subjective cognitive impairment, but not in AD and MCI. Due to the high concentration of neurons in the grey matter, these findings support the hypothesis that 24-OHC is a marker of the number of metabolically active neurons in the brain, but suggest this may only be true for individuals without neurodegenerative diseae. Among cognitively normal middle-aged adults, lower plasma levels of 24-OHC were also associated with smaller brain volumes(Solomon, Leoni et al. 2009) and smaller hippocampus(Koschak and Lutjohann 2009). Participants with both high levels of 24-OHC and 27-OHC had larger hippocampal volumes.(Koschak and Lutjohann 2009)

Solomon et al. found associations between absolute levels of oxysterols and brain volume. They did not find an association between white matter volume and fraction with the

ratio of 24-OHC/Chol(Solomon, Leoni et al. 2009). Unfortunately, the investigators did not use standard MRI procedures to distinguish normal white matter from white matter hyperintense areas visible on MRI. Hyperintense white matter on MRI is indicative of damage to the white matter occurring from ischemia and maybe associated with cholesterol metabolism in the brain. With the volume of white matter hyperintensities included in the total white matter volume interpretations regarding this tissue may be inaccurate.

### 1.4.3 Cross-sectional studies of 24-OHC and dementia

#### 1.4.3.1 Associations with plasma 24-OHC

Case-control studies have found significant associations between 24-OHC and various forms of dementia, specifically severity of disease state; yet, they indicate inconsistencies in the direction of association (Table A4). The levels of 24-OHC in the plasma are expected to increase in the early stages of the neurodegenerative disease process(Leoni 2009) (Figure 3). Evidence for this initial increase in plasma 24-OHC is seen in dementia: AD, vascular dementia (VaD), and MCI.(Lutjohann, Papasotiropoulus et al. 2000; Papassotiropoulus and Lutjohann 2000) Three studies indicate increased levels of plasma 24-OHC are associated with dementia and MCI. These three studies utilize patients referred to memory clinics with memory complaint. Absolute levels of plasma 24-OHC were found to be significantly higher in participants with AD, VaD, and MCI patients compared to both controls and depressed participants(Lutjohann, Papasotiropoulus et al. 2000; Papassotiropoulus and Lutjohann 2000). No significant differences were observed between dementia subtypes (AD and VaD) or between depressed participants and controls(Lutjohann, Papasotiropoulus et al. 2000). Only one study reported no association between absolute plasma levels of 24-OHC in AD compared controls(Schonknecht and

Lutjohann 2002). This study found that absolute plasma 24-OHC levels appeared to be higher in AD cases compared to controls, but differences were not statistically significant(Schonknecht and Lutjohann 2002).

Plasma levels of 24-OHC correlate with severity of dementia symptoms and genetic risk factors for AD. Levels of 24-OHC are negatively correlated with severity of dementia using mini mental state exam (MMSE) to assess global cognition scores(Lutjohann, Papasotiropoulus et al. 2000). Severe AD and the ApoE genotype have been independently associated with both lower levels of absolute 24-OHC and a reduced ratio of 24-OHC/Chol in the plasma(Papassotiropoulus and Lutjohann 2000). When stratified by early and late stage of AD, levels of 24-OHC were found to be slightly higher in the early stages of AD(Papassotiropoulus and Lutjohann 2000).

Two other cross-sectional studies found that absolute levels of 24-OHC were significantly lower in patients with AD compared controls. The first study only included AD patients with a long duration of disease. The authors noted that the AD patients were diagnosed at least four years before the blood draw and decreases in 24-OHC may represent a progressed course of disease and greater disease severity(Bretillon and Siden 2000). The second study reporting levels of 24-OHC were lower in cases of AD and MCI compared to controls with subjective cognitive impairment(Solomon, Leoni et al. 2009). All participants, including both cases and controls, were referred to a hospital memory clinic with memory complaint. Plasma levels of 24-OHC and the ratio 24-OHC/Chol were significantly lower in patients with MCI and AD compared to controls with subjective cognitive impairment. As in other studies the levels of 24-OHC were not significantly different between patients with MCI and AD. The authors noted that the subjective cognitive impairment patients used as controls presented with concerns of some cognitive impairment, but only did not meet a clinical threshold for cognitive impairment.

Mean MMSE scores for subjective cognitive impairment, MCI and AD were 29.1, 28.1 and 23.6, respectively. The lower levels of 24-OHC corresponded with lower MMSE scores across the groups. No direct comparisons to cognitively normal controls could be made from this study.

#### 1.4.3.2 Associations with ratio 24-OHC to total cholesterol and other oxysterols.

While absolute levels of 24-OHC appear to increase in the early stages of dementia, the ratio of 24-OHC to total circulating cholesterol may decrease or remain unchanged in AD, MCI and VaD. The ratio of 24-OHC to total cholesterol is significantly lower in AD, VaD and MCI compared to controls(Kolsch and Heun 2004). The ratio of plasma 24-OHC/Chol correlates negatively with severity of disease and is associated with ApoE-4, Aβ42 and tau related proteins(Lutjohann, Papasotiropoulus et al. 2000; Papassotiropoulus and Lutjohann 2000; Solomon, Leoni et al. 2009). It appears that the ratio of 24-OHC/Chol may only be elevated in mild versus severe states of the disease(Papassotiropoulus and Lutjohann 2000). A paper by Teunissen *et al* reported the ratio of 24-OHC to total cholesterol was not significantly different patients with various neurological disease compared to controls(Teunissen and De Vente 2003; Teunissen, Lutjohann et al. 2003). The author's combination of clinical syndromes (Alzheimer's disease, obsessive compulsive disorder, and Parkinson's disease) into a comprehensive 'neurological patients' group produced no significant differences in the ratio of 24-OHC to total cholesterol in cases compared to controls(Teunissen, Lutjohann et al. 2003). The investigators noted, but didn't show, that results from the analysis of variance test indicated that the ratio of 24-OHC to total cholesterol differed significantly across the groups.

One study of oxysterols and AD investigated oxysterol formation in the brain relative to oxysterol formation in periphery as a ratio of 24-OHC to 27-OHC. This study showed the ratios of both 24-OHC/Chol and 27-OHC/Chol appear to be lower in patients with AD, VaD and MCI

compared to controls. Interestingly, the plasma ratio of 24-OHC/27-OHC was significantly elevated in the AD, VaD and MCI participants compared to controls(Kolsch and Heun 2004). These findings suggest higher cholesterol metabolism to oxysterols occurs in the brain versus the periphery early in the dementia process; furthermore these results show higher cholesterol metabolism in the brain versus the periphery in patients with mild cognitive impairment compared to cognitively normal controls(Kolsch and Heun 2004).

#### 1.4.3.3 Associations with 24-OHC in the cerebrospinal fluid.

Measurements of 24-OHC in the CSF, taken from spinal fluid, are more consistent than those measured from the plasma. 24-OHC in the CSF is believed to indicate active and acute neurodegeneration(Leoni and Masterman 2003). CSF levels of 24-OHC are significantly increased in MCI and AD patients, specifically in the frontal cortex of AD patients(Papassotiropoulus and Lutjohann 2002; Heverin and Bagdanovic 2004; Shafaati, Solomon et al. 2007). CSF concentrations of 24-OHC are significantly, yet slightly, elevated in MCI patients compared to controls(Papassotiropoulus and Lutjohann 2002). In participants with normal cholesterol levels, CSF 24-OHC levels is significantly higher in AD patients compared to controls(Schonknecht and Lutjohann 2002). The number of ApoE-4 alleles additively increased the levels of 24-OHC in the CSF(Papassotiropoulus and Lutjohann 2002); however levels of 24-OHC were not correlated to beta amyloid and tau protein in the CSF(Shafaati, Solomon et al. 2007). In autopsied brain tissue samples, the ratio of 24-OHC/27-OHC in the CSF is elevated in AD patients compared to controls. Despite having autopsied brain samples, the authors did not control for brain volume or weight and no designation was made for the amount of healthy or diseased brain tissue(Heverin and Bagdanovic 2004).

### 1.4.4 Longitudinal studies of 24-OHC with cognitive decline

Cognitive decline can be viewed as the overarching clinical manifestation of the dementia process. In dementia patients, plasma 24-OHC is associated with global cognitive performance (MMSE) often used as a marker of disease severity. Table A5 details the two studies assessing the relationship between 24-OHC and longitudinal cognitive decline(Teunissen and De Vente 2003). The results from these two studies are either incomplete or insufficient (due to small sample size) when taken alone, but complimentary when examined together. The first study attempted to determine if cholesterol and oxysterols concentrations are associated with cognitive performance in the healthy aging population over six years of follow-up using a relatively small sample size (n=65 participants at both baseline and follow-up)(Teunissen and De Vente 2003). Absolute levels of both 24-OHC and 27-OHC are correlated with individual cross-sectional cognitive performance at baseline and follow-up. Longitudinally, only the ratio of 24-OHC/Chol was related to significantly slower speed of processing at follow-up and not other cognitive tests. This study suffered from many limitations, including: the small number of participants had both baseline and follow-up measures of cognition; little variation was seen over 6 years in the cognitive scores of participants; also the failure to adjust for, or exclude, individuals that were currently taking statins during the observation period.

A recent prospective study collected longitudinal cognitive testing over 6 years of follow-up in over 1000 participants, 65 and older, who were not taking statins and cognitively normal at baseline(van den Kommer, Dik et al. 2009). The authors found that low total cholesterol in participants 65 years and older is an independent predictor of cognitive declines over six years. The ratios of 24-OHC/Chol and 27-OHC/Chol were significantly lower in ApoE-4 carriers. ApoE-4 status, the ratio of 24-OHC/Chol and the ratio of 27-OHC/Chol showed no association

to cognitive decline over six years. Only the ratio 27-OHC/Chol was inversely associated with performance on MMSE and immediate recall tests in ApoE-4 carriers. While the association between the ratio 24-OHC/Chol and cognitive performance over six years was not statistically significant, an interaction between the ratio of 24-OHC/Chol and the ApoE-4 allele suggested that ApoE-4 is a potential moderator of association between ratio 24-OHC/Chol and speed of processing. Unfortunately, the authors did not report the cross-sectional associations between cognition and 24-OHC or 27-OHC at baseline or follow-up. These results support the hypothesis that decreased serum cholesterol in late-life may reflect ongoing pathological processes and may represent a risk marker for cognitive impairment and dementia.

In summary, 24-OHC is believed to be a marker of the number of metabolically active neurons in the brain able to convert excess cholesterol to 24-OHC and has been used extensively as a biomarker in studies of neurodegenerative diseases. However, its associations with age-related cognitive impairment and dementia have been inconsistent, partially due to factors including: study design; point of the disease process at which 24-OHC is assessed; and how it is expressed as an absolute value or ratio compared to total cholesterol levels. Studies of oxysterols show 24-OHC is higher in AD, MCI and VaD early in the disease process when compared to cognitively normal controls. The levels of plasma 24-OHC correlate with ApoE genotype, scores of global cognition and severity of disease.

## 1.5 DISCUSSION OF RESULTS FROM SYSTEMATIC REVIEW

The presence of the blood brain barrier makes cholesterol in the circulation a distant and poor marker of cholesterol concentrations in the brain. The metabolism of cholesterol to 24-OHC

accounts for 99% of the cholesterol eliminated from the brain and makes 24-OHC a more proximate marker of cholesterol homeostasis in the brain. Excess cholesterol in the brain can come from either the excess synthesis of cholesterol or the breakdown of the cholesterol-rich myelin during neuronal death. Both sources of excess cholesterol in the brain will increase the elimination of cholesterol as 24-OHC and levels of 24-OHC in the blood. The conversion of excess cholesterol to 24-OHC occurs in the neuron; therefore it is also considered a marker of number of metabolically active neurons in the brain. Previous studies demonstrate that 24-OHC is associated with the diagnosis and severity of dementia and other neurodegenerative diseases. The concentration of 24-OHC in relationship with dementia may depend upon the stage of disease at which the 24-OHC is measured. Specifically, we propose (in Figure 3) the concentrations of plasma 24-OHC may be elevated (in the early stages of dementia, due to myelin loss), normal (in middle stages, an equilibrium may be reached through increased conversion of cholesterol to oxysterols and subsequent elimination), or decreased (in later and progressive stages, when the neuronal loss that accompanies the neurodegenerative process causes lower concentrations of CYP46, the enzyme that converts cholesterol to 24-OHC). Early stage myelin breakdown is expected to increase levels of free cholesterol in the brain and its primary metabolite, 24-OHC in the blood. A progressive course of disease -characterized by pervasive synaptic and neuronal loss leading to progressive atrophy- will result in increases in initial increases in 24-OHC. This process will continue until the number of metabolically active neurons in the brain cannot keep pace with excess cholesterol levels in the brain resulting in declines in 24-OHC levels in step with severity.

When measured in the CSF, 24-OHC is a marker of active neurodegenerative disease. Increased levels of 24-OHC in the CSF are believed to indicative of active neurogenerative

disease(Leoni 2009). CSF levels of 24-OHC have been shown to be elevated in AD and MCI patients. These associations appear to be independent of circulating cholesterol levels. Evidence from MS supports this assumption(Leoni and Masterman 2002; Karrenbauer and Leoni 2006).

When measured in the plasma, the associations between dementia and 24-OHC appear to be less consistent. However, these inconsistencies likely result from two design issues: the stage of the disease process (severity) at which 24-OHC is assessed; and whether levels of 24-OHC are expressed as an absolute measure or as a ratio of 24-OHC to total cholesterol. The levels of absolute 24-OHC are expected to increase in the early stages of the neurodegenerative disease process, and decline with severity of disease. Evidence for this increase is seen not just in MCI, but also in early stages of AD and VaD. With increased duration and severity of disease, both the absolute levels of 24-OHC and the ratio of 24-OHC/Chol appear to decline. Studies showing a lower ratio of 24-OHC/Chol in participants with neurodegerative disease also demonstrate that total cholesterol levels do not differ between controls and cases of AD and MCI(Solomon, Leoni et al. 2009). Studies reporting no association or lower levels of plasma 24-OHC in AD patients compared to controls fail to account for the severity or stage of disease. One study reporting lower levels of plasma 24-OHC in AD patients compared to controls noted that the AD patients all were diagnosed with disease a minimum of 4 years before blood draw. As demonstrated by the increase ratio of 24-OHC to 27-OHC seen in dementia patients, elevations in 24-OHC appear to occur relative to 27-OHC levels.

Plasma levels of 24-OHC lacks the specificity to differentiate AD patients and other neurodegenerative diseases, such as Parkinson disease(Teunissen, Lutjohann et al. 2003). However, it appears 24-OHC is unable to differentiate dementia patients (AD and VaD) from MCI patients. This suggests that the changes in cholesterol metabolism in the brain occur long

before cognitive impairment becomes severe. The mechanism is responsible for the elevation in 24-OHC in the early stages of the neurodegenerative disease process remains uncertain.

Cholesterol levels and statins clearly modulate APP processing *in vitro* and *in vivo*(Reid, Urano et al. 2007). Statin trials (using statins known to cross the BBB) have been unable to demonstrate a reduction in dementia risk calls into question the hypothesis that cholesterol synthesis alone is involved in the Alzheimer's disease pathology. Studying factors regulating cholesterol homeostasis in the brain (both synthesis and elimination) are essential to understanding hypercholesterolemia in the brain. Early in the disease process, excess cholesterol in the brain could lead to both increases in Aβ deposition in the brain, and increases in 24-OHC. Aβ deposition can now be visualized *in vivo* by positron emission tomography using the Pittsburgh compound B (PiB-PET). States of excess cholesterol in the brain would be expected to show positive correlations between 24-OHC and PiB-PET.

Studies involving 24-OHC and dementia fail to consider the underlying pathology which could account for states of excess brain cholesterol in dementia studies, or whether 24-OHC is a plausible marker of cholesterol metabolism and dementia pathology. More importantly, studies of 24-OHC and dementia appear to overlook the importance of myelin breakdown: 1) as a potential source of excess cholesterol in the brain; 2) as a potential source of increased levels of 24-OHC in the circulation; and 3) as a potential initiating event in the development of neurodegeneration and dementia. *In vivo* tools exist to quantitatively measure the integrity of the myelin and white matter of the brain; however, these tools remain underappreciated in the oxysterol literature. The inconsistencies of the initial associations between plasma 24-OHC and AD underscore the importance of examining 24-OHC in the context of the structural abnormalities involved in the dementia process. These processes occur at different points in the

dementing process or may occur in isolation. Understanding these relationships show 24-OHC either a marker neuronal activity or a sensitive, early marker of initiating events in the pathology of dementia.

Long standing and recent advances in Magnetic Resonance Imaging (MRI) have enabled visualization of abnormal white matter as well as quantitative means to evaluate the integrity of the brain's white matter. MRI sequences needed to visualize and quantify structural changes in the brain of vascular nature are readily available. Standard sequences (T1, T2, PD and FLAIR) provide insight into hypo- and hyper-intense lesions within the white matter. The techniques exist to quantify the integrity of the white matter (healthy white matter fraction), the extent of white matter disease (white matter lesion grade or volume) and the relative health of the brain tissue. Studies have yet to investigate the relationship between these lesions, commonly seen in cerebrovascular disease and dementia, and their relationship to oxysterols concentrations. In addition, Magnetic Transfer Imaging (MTI) and Diffusion Tensor Imaging (DTI) techniques can provide measures of white matter integrity and measures of axonal diffusion, respectively.

Volumetric MRI indicates plasma 24-OHC was significantly associated with total brain volume and volume of the grey matter in participants with subjective and clinical cognitive impairment(Solomon, Leoni et al. 2009). The association between 24-OHC and grey matter volume supports the theory 24-OHC as a marker of the number of metabolically active neurons in the brain. Unfortunately this study did not evaluate the integrity of the white matter measured. The report that 24-OHC was not significantly associated with white matter volume is not surprising because early white matter disease (visible as hyperintensities on MRI) may not necessarily result in loss of white matter volume. What was measured in this study was total white matter volume, which contains two compartments: volume of diseased white matter

(undergoing myelin breakdown) and volume of healthy white matter (white matter integrity). Understanding the relationship between 24-OHC and white matter integrity may be essential to our understanding of Alzheimer's disease and its initiating events. Volumetric MRI can be used to calculate brain volume and assess areas of relative atrophy. What has been reported regarding the association between 24-OHC and brain volume is potentially incorrect if studies do not account for active demyelination of the white matter.

Plasma and CSF levels of 24-OHC have been associated with vascular dementia, brain volume, and cognitive performance at the cross-sectional level. With respect to AD development, the temporality of changes in oxysterols and onset of dementia has not been assessed. Associations between 24-OHC and 'pre-AD' states (e.g. MCI, and cognitive decline) suggest that higher levels of 24-OHC are related to changes earlier stages of dementia process. No studies have directly evaluated relationship between 24-OHC or 27-OHC and direct markers of the dementia process. The myelin model of AD development suggests that white matter disease could be the preceding events to AD development. MRI sequences can visualize and quantify structural changes in the brain; however, no study has investigated these lesions in relation to oxysterols. MRI can be used to assess the integrity of the white matter and extent of white matter disease *in vivo*. A comprehensive study utilizing longitudinal measures of cholesterol measures (total cholesterol, 27-OHC and 24-OHC) and longitudinal measures of cognitive performance and assessment of white matter integrity using MRI is greatly needed.

Epidemiological and *in vivo* studies show that 24-OHC is a direct and non-invasive marker of cholesterol homeostasis in the brain, and 24-OHC may be associated with AD. However, the associations between 24-OHC to amyloid deposition remains unknown. This association can be examined by visualizing amyloid deposition *in vivo* using PiB-PET.

Understanding this association in participants with AD and in the cognitively normal is essential to our understanding of the AD process and the validity of 24-OHC as a marker of AD. Studies that examine dementia: evaluate controls in the same manner as cases; provide cognitive comprehensive examinations as well as neuropsychological examinations to all participants; obtain ApoE genotyping, and utilize MRI for differential diagnostic purposes.

## 2.0 OXYSTEROLS SHOW STRONGER ASSOCIATIONS WITH CEREBROVASCULAR DISEASE THAN ALZHEIMER'S DISEASE

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## ABSTRACT

**Background:** Cholesterol is believed to play a role in the development of Alzheimer's disease (AD). Although total cholesterol levels have been associated with incident dementia in elderly subjects, the cholesterol in the blood has little relevance to cholesterol in the brain due to its inability to cross the blood brain barrier (BBB). However, two enzymatically formed metabolites of cholesterol, 24S-hydroxycholesterol (24-OHC, a brain-derived oxysterol) and 27-hydroxycholesterol (27-OHC, a peripherally derived oxysterol), cross the BBB and have been associated with AD. We investigated whether 24-OHC is associated with markers of cerebrovascular disease prior to the onset of cognitive impairment.

**Methods:** We quantified oxysterols, 24-OHC and 27-OHC, on a sub-set (n=105) of participants from the Pittsburgh Cardiovascular Health Study Cognition Study (CHS-CS) who remained cognitively normal (CN) in 1998-9 and had MRI in 1992 and 1998, blood drawn in 2002, and annual cognitive exams between 1998 and 2010. Diagnosis of incident AD and mild cognitive impairment (MCI) between 1998 and 2010 was made by consensus conference.

**Results:** Participants included in this sub-study had a mean age of 80±4 years in 2002 and were followed for 7.4 years. Forty-three participants developed AD and 36 MCI. In participants with incident cognitive impairment (AD and MCI) compared to CN: plasma levels of 24-OHC were higher (CN=39, MCI=43, AD=43, p=0.05); while the levels 27-OHC tended to be lower (CN=268, MCI=235, AD=227, p=0.18); thus, making the ratio of 24-OHC to 27-OHC (24-OHC/27-OHC) significantly higher (CN=0.17, MCI=0.19, AD=0.20, p=0.02). Higher levels of 24-OHC, but not 24-OHC/27-OHC, were associated with higher white matter hyperintensity (WMH) grade (WMH<2=39 vs. WMH≥2=45, p=0.01) and the presence of MRI-defined infarcts

(no infarcts=41 vs. infarcts=47, p=0.05) at prior MRI in 1997-8. Plasma 24-OHC and 24-OHC/27-OHC were not associated with ApoE-4 status.

**Conclusions:** The higher ratio of 24-OHC/27-OHC, suggesting increased oxidative cholesterol metabolism in the brain versus the periphery, was associated with incident cognitive impairment over 8 years of follow-up. 24-OHC was also significantly higher in participants with higher WMH grade and infarcts at least four years before the onset of cognitive impairment. Measurement of oxysterols may provide information about cholesterol metabolism and brain disease preceding clinical manifestations of cognitive impairment.

## 2.1 INTRODUCTION

High total cholesterol levels are associated with cardiovascular and cerebrovascular disease(Goldstein, Bushnell et al. 2011), and with incident dementia and mild cognitive impairment (MCI)(Kivipelto, Helkala et al. 2001) in elderly subjects, suggesting that brain cholesterol plays a role in the development of Alzheimer's disease (AD). The vast majority of cholesterol in the brain resides in the myelin (70-80%) where is provides insulation to the axon essential to signaling between neurons(Snipes and Suter 1997). *In vitro* evidence suggests that excess cholesterol in the plasma membrane of brain cells initiates: cleavage of amyloid precursor protein by β-secretase, production of beta amyloid (Aβ), and Aβ deposition as plaques(Fassbender and Stoick 2002). However, cholesterol levels in the blood have little relevance to cholesterol levels in the brain because cholesterol cannot cross the blood brain barrier (BBB) (Reiss 2005).

Two enzymatically formed metabolites of cholesterol, 24S-hydroxycholesterol (24-OHC, a brain-derived metabolite) and 27-hydroxycholesterol (27-OHC, a peripherally derived metabolite), can cross the BBB directly by diffusion and can be measured in the blood(Bjorkhem 2006). 24-OHC is the primary metabolite of cholesterol in the brain and is associated with both risk of AD and brain volume(Leoni 2009; Solomon, Leoni et al. 2009). However, associations between plasma 24-OHC levels and AD have been inconsistent in cross-sectional analyses(Leoni 2009). Little is known about the relationship between 24-OHC and structural changes in the brain prior to the onset of cognitive impairment. We investigated whether brain-derived, 24-OHC is associated incident cognitive impairment and with markers of cerebrovascular disease prior to the onset of cognitive impairment. Specifically we: determined the reproducibility and reliability of plasma oxysterol measurements from stored blood samples; quantified the retrospective associations between plasma oxysterol levels and MRI markers of cerebrovascular disease and markers of AD; and evaluated the prospective associations between oxysterols and incident cognitive impairment.

## 2.2 METHODS

*Subjects*

The Pittsburgh Cardiovascular Health Study Cognition Study (CHS-CS) is a sub-study of the Cardiovascular Health Study. Eligibility for the CHS-CS (n=532) participants included: MRI of the brain and detailed cognitive exam in 1992-93 and a second MRI or detailed cognitive exam in 1998-99. Participants were non-demented in 1998 and followed to 2010 with repeat cognitive tests to determine the incidence of dementia and mild cognitive impairment (MCI). For

the purpose of this study we selected 105 participants from this CHS-CS who were cognitively normal in 1998-99 and 2002, had blood drawn in 2002, and had repeat cognitive exams between 2002 and 2010.

*Assay of oxysterols and cholesterol*

The general blood collection and laboratory methods have been previously published (Cushman, Cornell et al. 1995). Single tubes for analysis of total plasma lipids and oxysterols were thawed once for each assay. Oxysterols (24S-hydroxycholesterol and 27-hydroxycholesterol) were assayed by isotope dilution using gas chromatography/mass spectroscopy (GC/MS)(Dzeletovic and Breuer 1995). Reproducibility and reliability of oxysterol measures were determined using a random selection of 14 samples with assay repeated one month apart. We obtained direct measures of lipids (total cholesterol, HDLc, triglycerides) and calculated LDLc from the same sample tubes collected in 2002.

*Diagnosis of cognitive impairment*

The diagnosis of dementia and MCI was based on cognitive and neuropsychological batteries (Lopez, Kuller et al. 2007). The diagnosis of dementia was based on a deficit in performance in two or more cognitive domains that was of sufficient severity to affect the subject's activities of daily living and on history of normal intellectual function before onset of cognitive abnormalities(Lopez, Kuller et al. 2007). An adjudication committee classified diagnosis of AD (probable and possible) using NINDS-ARDA criteria.

*ApoE genotyping*

ApoE allele 4 (ApoE-4) carrier status was determined in CHS using genotyping procedures previously described(Kuller, Shemanski et al. 1998).

*Brain Imaging*

Because not all participants had an MRI of the brain in 2002-03, we examined the relationship between oxysterols levels in 2002 and MRI measures in 1997-98. All MRI data acquired in 1998-1999 using a 1.5-T GE Signa system (LX version; Milwaukee, WI) were previously published(Yue, Arnold et al. 1997). White matter hyperintensity (WMH) grade was estimated as the total extent of periventricular and subcortical white matter signal abnormality on spin-density-weighted axial images that successively increased from no or barely detectable hyperintensities (grades 0 and 1, respectively) to almost all white matter involved (grade 9)(Yue, Arnold et al. 1997). Abnormalities interpreted as representing areas of large-vessel cerebral infarction or small-vessel lacunar infarction were coded separately in the database as infarct-like lesions(Kuller, Longstreth et al. 2004). The degree of ventricular enlargement was also assessed on a 10-point scale from 0 to 9, according to an atlas of predefined visual standards(Yue, Arnold et al. 1997).

*Statistical Analysis*

Reproducibility and reliability of repeated oxysterol measures were estimated by calculating the intra-class correlation coefficients (SAS, PROC VARCOMP) and evaluated outliers and systematic bias using scatter plots and Bland-Altman plots. Both 24-OHC and 27-OHC were found to have approximately normal distributions. Differences in potential covariates

between the analytic subset (n=105) and the eligible CHS-CS participants (n=524) were evaluated by *t*-tests and $\chi 2$ tests.

The associations between oxysterols, potential covariates and prior MRI measures were estimated using multivariable linear regression. The prospective associations between oxysterols and incident cognitive impairment were estimated using multinomial logistic regression. Findings from logistic models were confirmed considering time to cognitive impairment by quartiles of oxysterols concentrations using log-rank tests, Kaplan-Meier curves and Cox proportional hazards models. Models were first estimated adjusting only for age and then controlling for age, gender and total cholesterol levels in 2002. In time to event models, the proportional hazards assumptions were met for oxysterols predictors of time to cognitive impairment; however the inclusion of age did not meet proportional hazards assumptions. All analyses were conducted using SAS *v*9.2 and level of statistical significance was set at $p<0.05$.

## 2.3 RESULTS

Participants included in this sub-study were to be younger and more educated than the rest of the participants in CHS-CS (Table 1). The mean age of this sub-set was 80±4 years in 2002, 55% were women (58 of 105), and 66% (68 of 105) had greater than high school education. All (n=105) participants underwent MRI-1 in 1992-93 and 93 had a second MRI in 1998-99. No significant differences were observed between this sample and the CHS-CS participants (n=532) with regard to gender, race, cholesterol levels in 1992, and cardiovascular disease. Nineteen percent of the participants were ApoE-4 carriers. Mean cholesterols measures (mg/dL) measured in 2002 were: 187 for total cholesterol, 49 for HDLc, and 109 for LDLc.

**Table 1: Characteristics of analytic subset in 2002**

| | CHS-CS cohort (n=524) | | Analytic sample (n=105) | | p-value |
|---|---|---|---|---|---|
| Age, mean, std | 82.1 | 3.9 | 80.2 | 3.8 | <0.001 |
| Women, n, % | 265 | 62 | 58 | 55 | 0.199 |
| Education >12 years, n, % | 228 | 53 | 68 | 65 | 0.035 |
| White, n, % | 335 | 79 | 80 | 76 | 0.764 |
| ApoE-4 allele*, n, % | 87 | 23 | 19 | 20 | 0.499 |
| Coronary Heart Disease, n, % | 79 | 19 | 16 | 16 | 0.435 |
| Cholesterol in 1992 (mg/dL), mean, std | 213.0 | 38.8 | 214.9 | 38.5 | 0.683 |
| Cholesterol in 2002 (mg/dL), mean, std | | | | | |
| Total | -- | -- | 186.7 | 40.0 | |
| HDLc | -- | -- | 49.2 | 13.0 | |
| LDLc | -- | -- | 109.3 | 32.0 | |
| Triglycerides | -- | -- | 140.9 | 66.6 | |
| Oxysterols in 2002 (ng/mL), mean, std | | | | | |
| 24-OHC | -- | -- | 42.2 | 11.8 | |
| ratio 24/27 | -- | -- | 0.2 | 0.1 | |
| 27-OHC | -- | -- | 238 | 81.1 | |

*ApoE genotyped on n=382 in CHS-CS and n=97 in analytic subset.

Measures of plasma oxysterols obtained from 105 samples averaged 42 ng/mL for 24-OHC and 238 ng/mL for 27-OHC. Measures of 24-OHC and 27-OHC were highly reproducible and reliable, when repeated one month apart, with intra-class correlation coefficients of 0.85 and 0.73, respectively. Evaluation of Bland-Altman plots of repeated measures for each oxysterol indicated no relative bias or outliers between sample preparations.

Higher plasma levels of 24-OHC in 2002 were associated with female gender, and with higher cognitive scores on digit-symbol substitution test, higher total cholesterol and higher LDLc as measured at study entry in 1992-93 (data not shown). The use of lipid lowering

medications (at year 9) resulted in significantly lower levels of plasma 24-OHC (p=0.028) but not of 27-OHC or MRI outcomes. An inverse correlation between 27-OHC and age was present (r=0.19, p=0.05); however no correlation between 24-OHC and age was indicated (r=0.05 p=0.39). Oxysterols 24-OHC and 27-OHC were highly correlated with concurrent measures (2002) of total cholesterol (r>0.25, p<0.01, both) and LDLc (r>0.25, p<0.01, both) but not with HDLc levels (r<0.04, p>0.45, both). Education, race, MMSE, and white matter grade at MRI-1 (1992-93) were not associated with plasma oxysterols (data not shown).

Higher levels of 24-OHC in 2002 were significantly associated with higher WMH grade (WMH<2=39 vs. WMH≥2=45, p=0.01) and the presence of MRI-defined infarcts (no infarcts=41 vs. infarcts=47, p=0.05) at MRI-2 in 1997-1998. Associations between 24-OHC and WMH grade were independent of age, gender, and total cholesterol levels in 2002(Table 2). Additional adjustment for dementia diagnosis over the study period (1998-2010) and statin use had little effect on these associations (p=0.04, data not shown). The inclusion of ventricle size and ApoE-4 carrier status produced mild attenuation of these relationships (p=0.06, data not shown). The 17 participants with MRI-defined brain infarcts at MRI-2 also had significantly elevated 24-OHC and 27-OHC in 2002 (Table 2). These associations were minimally modified by adjustment for age, gender, cholesterol and additional adjustment for dementia diagnosis over the observation period (1999-2010) and size of the ventricles on MRI-2 (data not shown). Plasma 24-OHC and 27-OHC were not associated with age-related size of the ventricles on MRI or ApoE-4 carrier status (Table 2).

**Table 2: The associations between plasma oxysterol levels and MRI measures of cerebrovascular disease and AD**

| | | Oxysterol measured in 2002 (ng/mL) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | **24S-hydroxycholesterol** | | | **27-hydroxycholesterol** | | | **Ratio 24-OHC/27-OHC** | | |
| | n | mean | std | p-value | mean | std | p-value | mean | std | p-value |
| **MRI-defined markers of cerebrovascular disease (1998)** | | | | | | | | | | |
| White Matter Grade, n=93 | | | | | | | | | | |
| <2 | 41 | 39.2 | 10.8 | 0.014 | 225.7 | 74.2 | 0.053 | 0.18 | 0.05 | 0.907 |
| ≥2 | 52 | 44.8 | 11.4 | | 254.3 | 77.6 | | 0.18 | 0.05 | |
| Infarcts, n=93 | | | | | | | | | | |
| No infarcts | 76 | 41.3 | 11.3 | 0.059 | 232.6 | 70.5 | 0.013 | 0.18 | 0.05 | 0.463 |
| Infarcts | 17 | 47.0 | 11.1 | | 282.3 | 93.2 | | 0.18 | 0.04 | |
| **Markers of AD (1998)** | | | | | | | | | | |
| Size of ventricles on MRI, n=93 | | | | | | | | | | |
| <5 | 82 | 42.3 | 11.6 | 0.363 | 245.7 | 78.5 | 0.936 | 0.18 | 0.05 | 0.255 |
| ≥5 | 11 | 44.9 | 9.7 | | 237.7 | 68.1 | | 0.20 | 0.06 | |
| ApoE genotyping, n=105 | | | | | | | | | | |
| e4 - | 86 | 42.7 | 11.8 | 0.664 | 244.3 | 80.2 | 0.179 | 0.18 | 0.05 | 0.393 |
| e4 + | 19 | 41.6 | 11.3 | | 219.6 | 60.9 | | 0.19 | 0.04 | |

*p-values adjusted for age, gender and total cholesterol levels in 2002.

Between 1998 and 2001, 6 participants (5.7% of the analytic sample) were diagnosed with prevalent AD (prior to blood draw in 2002) and were excluded from analyses of incident cognitive impairment. During mean follow-up of 7.4 years, 37 participants went on to develop incident AD and 36 developed MCI between 2002 and 2010. Only 26 participants remained cognitively normal over the entire observation period (1998-2010) and had a mean age of 85.6 years in 2010. Incident AD was associated with older age, gender and greater ventricular grade in 1998-99. Incident MCI (n=36, between 2002-2010) was associated with age and having an increased WMH grade in 1992-93, but not WMH grade in 1998-99. Neither 24-OHC nor 27-OHC was significantly associated with prevalent AD, incident MCI and incident AD when considered as separate groups compared to normal controls. When combined as a single group as incident cognitive impairment, participants developing incident CI had significantly higher levels 24-OHC (CN=0.39, MCI=0.43, iAD=0.43, p=0.05), slightly lower levels of 27-OHC (CN=268, MCI=234, iAD=227, p=0.18) and a significantly higher ratio of 24-OHC to 27-OHC (CN=0.17, MCI=0.19, iAD=0.19, p=0.02). The trend in the ratio of 24-OHC to 27-OHC was also significant across these three groups (cognitively normal, MCI and AD, p=0.021) (Table 3).

**Table 3: Oxysterols as predictors of incident cognitive impairment (n=99)**

| | | Normal (1998-2010) (n=26) | | Mild Cognitive Impairment (2002-2010) (n=36) | | Alzheimer's disease (2002-2010) (n=37) | | | Incident CI (AD + MCI) | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | mean | std | Mean | std | mean | Std | age-adjusted trend p-value | age-adjusted p-value | Model 2 p-value |
| 24-OHC | ng/mL | 39.1 | 10.5 | 43.4 | 13.0 | 43.3 | 11.5 | 0.153 | 0.054 | 0.155 |
| 27-OHC | ng/mL | 268.7 | 114.7 | 234.9 | 66.1 | 227.4 | 63.8 | 0.821 | 0.185 | 0.478 |
| 24-OHC/27-OHC | | 0.17 | 0.05 | 0.19 | 0.06 | 0.19 | 0.05 | 0.021 | 0.019 | 0.159 |

*Prevalent cases of dementia in 2002 (n=6) excluded from analysis of incident cognitive impairment (CI).
Model 2 - p-value adjusted for age, gender and total cholesterol.

Results from time to event analyses (Figure 4) confirmed results from logistic regression models. Quartiles of 24-OHC were marginally associated with time to cognitive impairment over follow-up period (log-rank test, p=0.065). The lowest quartile of plasma 24-OHC was significantly less likely to develop dementia over the follow-up period in unadjusted proportional hazards models [HR(95%CI) = 0.52(0.27-0.98)]. This difference in survival time held after adjustment for age; however, inclusion of age caused to model to fail proportional hazards assumptions. Quartiles of 27-OHC were not associated with differential time to cognitive impairment (log-rank test, p=0.876). The ratio of 24-OHC/27-OHC was marginally associated with time to cognitive impairment in log-rank tests (log-rank test, p=0.055); however, individual quartiles were not significantly different in unadjusted proportional hazards models.

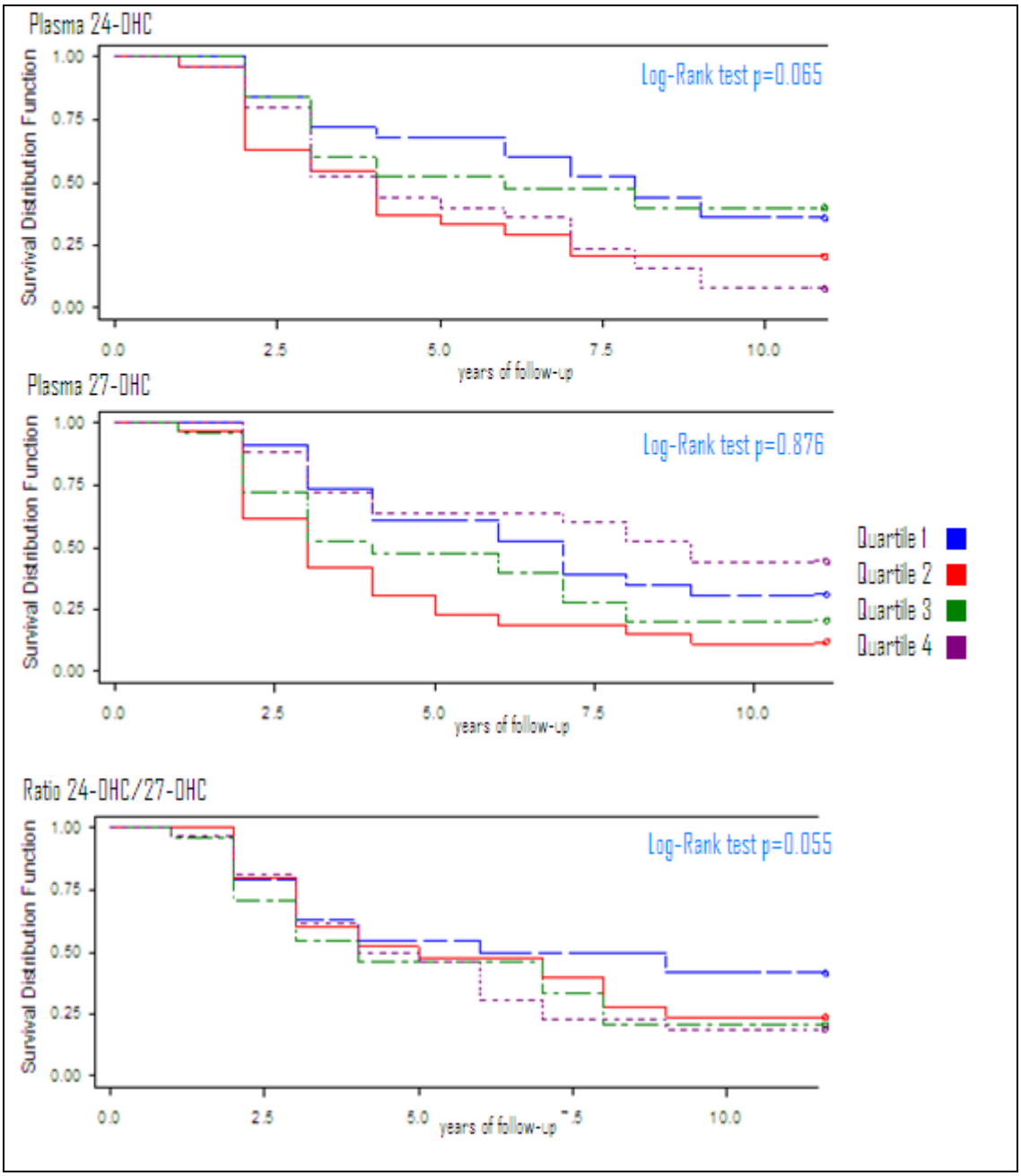


**Figure 4: Kaplan-Meier survival curves for time to cognitive impairment by plasma 24-OHC, 27-OHC and ratio 24-OHC/27-OHC**

## 2.4 DISCUSSION

Measures of oxysterols were highly reliable and reproducible when measured in stored plasma. Levels of 24-OHC were significantly higher in 2002 among participants with large vessel infarcts and high white matter grade at MRI-2 (1998-99), at least 4 years prior to the onset of cognitive impairment. Furthermore, higher levels of 24-OHC in 2002 were associated with the development of incident cognitive impairment over 8 years of follow-up. Higher concentrations of circulating levels of 24-OHC also occurred relative to levels of 27-OHC in participant who went onto develop incident CI, resulting in a significantly higher ratio of 24-OHC/ 27-OHC, indicating a higher production of oxysterols in the brain versus the periphery. This association was independent of age, gender and total cholesterol measured in 2002. These results suggest increased cholesterol metabolism to oxysterols occurs in the brain versus the periphery in participants who went on to develop incident CI. Interestingly, levels of both plasma 24-OHC and 27-OHC were higher in participants with cerebrovascular disease on MRI prior to blood draw. We show that elevated plasma oxysterols are associated with both cerebrovascular disease and incident CI. However, the discrepancies in the direction of associations between 27-OHC with cerebrovascular disease and with incident CI suggest cholesterol metabolism to oxysterols in the periphery differs between participants with cerebrovascular disease versus those who go onto develop to incident CI and these relationships are independent of concurrent circulating cholesterol levels.

To our knowledge, this is the first study to show that higher levels of plasma oxysterols are seen in cognitively normal participants with evidence of small vessel cerebrovascular disease. The differences occurred in cognitively normal individuals at least four years prior to the onset of cognitive impairment. However, these findings complement what we know about the structure and metabolism in the brain and may have implications to understanding the underlying metabolic changes occurring in the aging brain. White matter holds vast reserves of cholesterol (~28% of the dry weight)(Snipes and Suter 1997). Cholesterol provides insulation to the myelin surrounding the length of the axon(Snipes and Suter 1997). The integrity of the myelinated axon is essential to the speed of communication between neurons(Snipes and Suter 1997). Degradation of the myelin is believed to be a normal component of aging and is accelerated in cerebrovascular disease and AD(Bartzokis 2004). Myelin breakdown releases excess cholesterol into the extracellular space(Bartzokis 2009). Excess cholesterol in the brain stimulates its own conversion to 24-OHC in the neuronal body. The enzymatic oxidative metabolism of cholesterol to 24-OHC and its direct diffusion across the BBB is the primary means by which the brain eliminates excess cholesterol(Bjorkhem 2006). This process leads to higher levels of 24-OHC in the circulation(Bjorkhem 2006). Therefore, myelin breakdown resulting from cerebrovascular disease would provide a biologic basis for increasing cholesterol metabolism in the brain and higher levels of 24-OHC in the plasma. Plasma 24-OHC may be a sensitive marker of increased cholesterol metabolism occurring early in the neurodegenerative process.

Additionally, excess cholesterol in the cell membrane has been shown to lead to the production of Aβ and its deposition as plaques *in vitro*(Fassbender and Stoick 2002). Therefore the brain must eliminate excess cholesterol to prevent Aβ formation. Our findings suggest increased oxidative cholesterol metabolism to 24-OHC production occurs prior to the onset of

cognitive symptoms in individuals who go onto develop incident cognitive impairment (MCI and AD). This result supports findings from studies of 24-OHC and neurodegenerative diseases as well prior cross-sectional studies of oxysterols and AD, MCI and dementia in the literature(Lutjohann, Papasotiropoulus et al. 2000; Kolsch and Heun 2004; Leoni 2009). This increased oxysterol formation is also seen in MCI, suggesting a shared pathway. Furthermore, our study examines the relationship between oxysterols, 24-OHC and 27-OHC, and the longitudinal development of cognitive impairment. Our findings suggest a temporal elevation in plasma 24-OHC occurs prior to the development of incident MCI and AD. These findings support the hypothesized trajectory of the 24-OHC across the neurodegenerative disease process(Leoni 2009) (Figure 3).

Strengths of this study include the use of extensive measures of neurological disorders and cognition and the long follow-up time. Further, the CHS-CS participants were followed with repeat cognitive testing from 1998 to 2010. The small sample size of this study limits interpretation of the data. This study did not measure change in oxysterols over time. The hypothesized change in oxysterols over the dementia process is essential to assessing its validity as a marker and has yet to be investigated. We are currently completing the evaluation of the longitudinal change in oxysterols and its relationship to amyloid deposition in brain measured *in vivo* by PET scan using the Pittsburgh compound B (PiB-PET)(Price, Klunk et al. 2005).

Measurement of oxysterols may provide information about cholesterol metabolism and brain disease preceding cognitive impairment. As a marker of excess cholesterol metabolism in the brain, plasma 24-OHC may also have applications to understanding beta amyloid neuropathology occurring early in the cognitive impairment process. It is possible that measures of oxysterols can provide a tool to investigate both drug and non-pharmacological interventions

that may affect cholesterol metabolism in brain and risk of AD. These novel associations need to be replicated in other larger elderly populations. Future research investigating oxysterols as potential markers of AD should consider the structural and metabolic changes occurring in the brain, in particular during the development of cerebrovascular disease and amyloid deposition.

## 3.0 OXYSTEROLS AND AMYLOID DEPOSITION IN THE BRAIN

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*Submitted for review by*
*Gingko Evaluation of Memory Study P&P Committee*

## ABSTRACT

**Introduction:** Excess brain cholesterol is a potential risk factor for Alzheimer's disease (AD) and has been shown to lead to beta-amyloid (Aβ) formation and deposition as Aβ plaques. Cholesterol metabolism remains a target for dementia prevention and treatment with by lipid lowering drugs. We investigate the associations between markers of cholesterol metabolism in the brain and periphery with *in vivo* amyloid deposition and brain volumes

**Methods:** Amyloid deposition and volumetric MRI were assessed in the brains of 200 non-demented elderly participants from the Gingko Evaluation of Memory Study (GEMS). Plasma oxysterol levels and lipid profiles were measured in 183 participants within one year of imaging study.

**Results:** At time of the neuroimaging sub-study participants had a mean age of 85 years, 41% were women, 19% were diagnosed with MCI and 55% were PiB-positive. Oxysterol metabolites of cholesterol from the brain and the periphery were not associated with volumetric MRI of the brain or PiB-PET *in vivo*. We found significant interaction between PiB-status and statin use on oxysterol levels, and also with gray matter volume. Among statins users, oxysterols from the brain were higher for PiB-positive as compared to PiB-negative participants.

**Conclusions**. Future studies would need to examine the role of statins in mediating the relationship between oxysteorl and amyloid deposition.

## 3.1 INTRODUCTION

The mechanisms underlying AD pathology are not well understood. Excess cholesterol and its metabolism in the brain remain a major research interests in dementia. Currently, intervention trials show statins have little promise in the treatment(McGuinness, O'Hare et al. 2010) and prevention(McGuinness, Craig et al. 2009) dementia. The metabolism of cholesterol in the brain is a current important topic in dementia research. Oxysterol metabolites of cholesterol have been proposed as potential biomarkers of dementia and AD; yet their relationship with Alzheimer pathology remains unknown. The oxysterol, 24S-hydroxycholesterol (24-OHC) is the primary metabolite of cholesterol in the brain(Bjorkhem 2006). 24-OHC has been associated with various neurodegenerative diseases(Leoni 2009). Plasma levels of 24-OHC have been associated with AD(Bretillon and Siden 2000; Papassotiropoulus and Lutjohann 2000), VaD(Lutjohann, Papasotiropoulus et al. 2000) and MCI as well as brain volume(Solomon, Leoni et al. 2009). However, the direction of the associations between AD and plasma 24-OHC are inconsistent.

Recently, our group found that oxysterols are higher in cognitively normal participants with evidence of cerebrovascular disease visible on MRI and higher in those who developed cognitive impairment over 8 years of follow-up (Hughes, et al. *unpublished* 2011). The relationship between plasma 24-OHC and amyloid deposition in the brain remains unknown. Understanding the relationship between cholesterol metabolism in the brain in the context of underlying structural and biochemical changes occurring in the dementia process will: provide insight into the unknown underlying pathology of dementia and determine the usefulness of

oxysterols as surrogate markers and targets for interventions aimed at the prevention or treatment of dementia. Here we determine whether 24-OHC, a marker of cholesterol metabolism in the brain, is associated with amyloid deposition in the brain *in vivo* in cognitively normal elderly participants.

## 3.2 METHODS

*Population*

The Ginkgo Evaluation of Memory (GEM) study (2000-2008) was a multi-site, placebo-controlled, double-blind, randomized clinical trial of daily use of *Ginkgo biloba* in 3069 community-dwelling participants 72-96 years old. Exclusion criteria were reported in detail elsewhere (*unpublished data, Snitz and Klunk*) but included prevalent dementia, present use of cholinesterase inhibitors or other medication with significant psychotropic effects, recent hospitalization for depression, diagnosis of Parkinson disease, abnormal blood/serum tests at screening, or disease-limited life expectancy of <5 years. Median follow-up time from randomization was 6.1 years. Study visits occurred at 6-month intervals; a wide array of cognitive, genetic, functional, proxy-reported and medical history variables were collected (DeKosky, 2006).

In 2009, approximately one year following the GEM study closeout, n=197 participants from the Pittsburgh site were recruited into the GEM Imaging Sub-Study. Sub-study participants went on to receive MR imaging and PiB-PET. The inclusion criterion was completion of the GEM Study protocol. Exclusion criteria were: 1) dementia at GEM Study close-out in 2008, and 2) contraindications for neuroimaging (e.g., pacemaker). Comparisons to the 671 participants at

the Pittsburgh site have been detailed (*unpublished data, Snitz and Klunk*). Compared to all 671 Pittsburgh site participants who completed the GEM Study protocol and did not reach a dementia endpoint, the Imaging Sub-Study participants were younger (mean±SD, 84.0±2.8 vs. 84.4±3.0 years at close-out, p=0.03) but comparable in sex, race, education, ApoE4 status, estimated premorbid IQ and estimated income by zip code (p>0.05). Compared to all 966 Pittsburgh site participants at GEM Study baseline, they were younger, had higher estimated premorbid IQ (p<0.05) but did not differ by sex, race, education, ApoE4 status or estimated income by zip code (p>0.05) (*unpublished data, Snitz and Klunk).*

*Imaging Study*

MRI scanning used a Siemens 12-channel head coil and was performed on a 1.5T Siemens MR scanner at the MR Research Center of the University of Pittsburgh. Four series of MRI images were acquired on the MR scanner, these included magnetization-prepared rapid gradient echo (MPRAGE) T1-weighted images, fluid-attenuated inversion recovery (FLAIR) images, diffusion weighted images (DTI, b-values=0 and 1000 s/mm; 12 diffusion directions with four repeats), and two series of sagittal scans (with and without the off-resonance saturation pulse). A radiologist checked the MR images used in this study and excluded any unexpected findings from the study.

*Image Processing and Analysis*

Brain tissue volumes (gray matter, white matter, and cerebrospinal fluid), were calculated by segmenting the skull-stripped T1-weighted image in native anatomical space using the FAST - FMRIB's Automated Segmentation Tool (Zhang, Brady et al. 2001). The total gray matter

volume, white matter volume, and cerebrospinal fluid volume were estimated in cubic millimeters by summing all voxels classified as these tissue types. Total intracranial volume (ICV) was computed as the volume contained within the 'inner skull' using the brain extraction tool (BET) with an advanced option (−A)(Jenkinson, Pechaud et al. 2005). White matter hyperintensity (WMH) were visualized from T2-weighted FLAIR image using an automated method for quantification and localization of WMH. The WMH quantification was done using a fuzzy connected algorithm with automated seed selection (Wu, Rosano et al. 2006). Total WMH volume was estimated by summing all the voxels classified as WMH and then normalized by brain volume. Segmented volumes of the gray matter (GM), white matter (WM), white matter hyperintensities (WMH) and cerebrospinal fluid (CSF) are shown as a ratio, adjusted for the total ICV.

*GEM Visit Assessments*

Sub-Study participants underwent a shortened visit which included an abbreviated neuropsychological (NP) battery, 10-question CES-D for depression, timed walk, and inventory of the participant's prescription and over-the-counter medications. Statin use was assessed at each visit. For the purpose of this analysis participants were categorized as ever using statins and current statin use at their last recorded medication assessment.

*Cognitive Status*

Cognitive adjudication was completed blind to neuroimaging results by the GEM study Cognitive Diagnostic Center (DeKosky, 2006), taking into account historical serial cognitive assessments from the parent GEM Study. Criteria for mild cognitive impairment (MCI) included

1 - 3 tests impaired at cutoffs of 1.5 SD below age-education adjusted means. MCI was further subtyped according to Winblad et al. (2004). Four or more impaired tests resulted in a cognitive classification of 'dementia-level impairment'.

*Blood Draws*

All GEMS subjects who returned to the clinics for their final evaluation between October 2007 and March 31, 2008 were requested to have blood drawn at their last visit. Of the 197 participant in the GEM Imaging Sub-Study, 183 had plasma samples available at study close.

*Assay of Oxysterols and Cholesterol*

Single tubes for analysis of total plasma lipids and oxysterols were thawed once for each assay. Oxysterols (24S-hydroxycholesterol and 27-hydroxycholesterol) were assayed by isotope dilution using gas chromatography/mass spectroscopy (GC/MS)(Dzeletovic and Breuer 1995). Reproducibility and reliability of oxysterol measures were determined using a random selection of 14 samples in this analysis repeated one month apart. We obtained direct measures of lipids (total cholesterol, HDLc, triglycerides) and calculated LDLc from the same sample tubes.

*Statistical Analysis*

We investigated participant characteristics as potential covariates and determinants of oxysterol levels using ordinary least squares regression to obtain least squares means (LSmeans) and standard errors (SE) for each potential covariate. Potential neuroimaging determinants of oxysterols assessed in this study were: (1) PiB-status (positive or negative) using PiB-PET scan and (2) brain volumes on MRI (gray matter (GM), white matter (WM), white matter

hyperintensities (WMH) and total brain volume). Significance of differences of participant characteristics between PiB-status were assessed using t-tests and chi-squared tests. Correlations are shown as Spearman correlation coefficients. Multivariable logistic regression was used to estimate the association between PiB-positive versus PiB-negative participants in levels of 24-OHC and 27-OHC. Covariates included in analyses were age and gender (model 1) plus current statin use at last visit (model 2). Because certain statins are known to affect oxysterol and lipid levels in the plasma, potential effect modification by statin use was assessed with interaction term (PiB status*statin use) in estimating mean oxysterol and lipid levels.

Resulting individual p-values were considered significant if p-value <0.05. Interaction terms were considered significant at the p-value <0.10. The analytic sample size consisted of 183 participants who received PiB-PET imaging, blood draw in 2009, and had viable measures of oxysterols and lipids.

## 3.3 RESULTS

At the time of blood draw and neuroimaging, participants had a mean age of 85 years, 74 (40%) were women, 31 (17%) were ApoE-4 allele carriers, and 34 (18%) demonstrated mild cognitive impairment (MCI). The differences in characteristics between the imaging sub-study participants and the entire cohort have been described previously (Klunk and Snitz, *unpublished data*). Lipid measurements from stored blood samples in 2009 were available on 176 (96%) participants.

Oxysterols were measured in 182 participants with mean(±SD) levels of 38(±12) ng/mL and 170(±48) ng/mL for 24-OHC and 27-OHC, respectively. Table 4 details the association

between participant characteristics and oxysterol levels. Measures of plasma oxysterols were not associated with age, ApoE-4 carrier status, MMSE, and MCI diagnosis. Plasma oxysterols were significantly associated with gender. Women in this study had higher levels of 24-OHC ($p=0.003$) and lower levels of 27-OHC ($p=0.03$). Plasma levels of 24-OHC were significantly correlated with plasma concentrations of total cholesterol ($r=0.22$) and HDLc ($r=0.18$), all $p<0.05$. Levels of 24-OHC were only marginally associated with LDLc ($r=0.14$, $p=0.06$) and not associated with triglycerides ($r=0.005$, $p=0.89$). Plasma levels of 27-OHC were not associated with cholesterol, HDLc, LDLc, triglycerides in the blood.

Plasma concentrations of oxysterols were not significantly different between participants with MCI and those who were cognitively normal in 2009 ($p=0.55$); additionally levels of 24-OHC did not differ by MMSE score ($p=0.16$). Participants using statins at their last visit had significantly lower levels of 27-OHC ($p<0.01$) and marginally lower 24-OHC ($p=0.08$). The interaction term between cognitive status and statin use predicting 24-OHC was significant ($p=0.02$) indicating that levels of 24-OHC are lowest in cognitively normal statin users and highest in statin users with MCI ($p<0.01$).

**Table 4: Cross-sectional associations between participant characteristics and oxysterol levels.**

| | | | 24-OHC | | 27-OHC | |
|---|---|---|---|---|---|---|
| | | n | LSmeans | p-value | LSmeans | p-value |
| Age | | | | | | |
| | <85 | 52 | 37.9 | 0.6479 | 176.2 | 0.6057 |
| | 85-86 | 53 | 35.0 | 0.0995 | 167.4 | 0.7118 |
| | 86-87 | 36 | 39.5 | 0.8503 | 165.3 | 0.5984 |
| | >87 | 41 | 39.0 | *ref* | 171.0 | *Ref* |
| Gender | | | | | | |
| | Men | 108 | 35.5 | 0.003 | 176.4 | 0.0347 |
| | Women | 74 | 40.7 | *ref* | 161.3 | *Ref* |
| ApoE | | | | | | |
| | No e-4 | 121 | 37.5 | 0.2109 | 172.9 | 0.6857 |
| | e-4 | 33 | 40.4 | *ref* | 169.1 | *Ref* |
| MMSE_7 | | | | | | |
| | <28 | 106 | 38.2 | 0.1646 | 169.4 | 0.8077 |
| | ≥28 | 66 | 35.7 | *ref* | 171.2 | *Ref* |
| Diagnosis | | | | | | |
| | Normal | 148 | 37.4 | 0.5501 | 171.1 | 0.6373 |
| | MCI | 34 | 38.0 | *ref* | 166.8 | *Ref* |
| Treatment group | | | | | | |
| | Pla□eb□ | 91 | 37.6 | 0.9718 | 175.9 | 0.0975 |
| | Active | 91 | 37.7 | *ref* | 164.2 | *Ref* |
| Statins | | | | | | |
| | No statin | 95 | 39.1 | *ref* | 179.6 | *Ref* |
| | Ever | 87 | 36.1 | 0.0879 | 164.3 | 0.1198 |
| | Current | 69 | 35.7 | 0.0789 | 154.5 | 0.0004 |

*p-values unadjusted

The associations between plasma oxysterols and segmented brain volumes are presented in Table 5. In linear models, volume of WMH/ICV was marginally correlated with 24-OHC (p=0.09); gray matter, white matter and total brain volume showed no suggestion of association with 24-OHC in linear models. Expressed as quartiles, brain volumes were not associated with oxysterols. The relationship between plasma 24-OHC and quartiles of WMH/ICV appears to be U-shaped, with lower levels of 24-OHC in the bottom and upper quartiles and higher in the middle two quartiles. The lowest category of WM/ICV volumes was marginally associated with 24-OHC (p=0.07). Despite not approaching significance, monotonic declines in mean levels of oxysterols were visible across categories of WM/ICV volumes. Segmented brain volumes were not associated with levels of 27-OHC or the ratio 0f 24-OHC/27-OHC.

**Table 5: Associations between oxysterols and segmented brain volumes (n=158)**

| | 24-OHC | | | 27-OHC | | | ratio 24/27-OHC | | |
|---|---|---|---|---|---|---|---|---|---|
| | LSmeans | SE | P-VALUE | LSmeans | SE | P-VALUE | LSmeans | SE | P-VALUE |
| White matter / ICV | | | | | | | | | |
| Q1 | 35.3 | 1.8 | 0.083 | 164.8 | 7.8 | 0.574 | 0.23 | 0.01 | 0.484 |
| Q2 | 38.9 | 1.8 | 0.671 | 167.1 | 7.7 | 0.702 | 0.25 | 0.01 | 0.758 |
| Q3 | 38.6 | 1.8 | 0.592 | 169.1 | 7.7 | 0.845 | 0.24 | 0.01 | 0.708 |
| Q4 | 40.0 | 1.9 | *ref* | 171.3 | 8.2 | *ref* | 0.24 | 0.01 | *ref* |
| *trend* | | 0.334 | | | 0.949 | | | 0.7552 | |
| Interaction with statins** | | | 0.064 | | | 0.52 | | | 0.007 |
| Gray matter / ICV | | | | | | | | | |
| Q1 | 37.6 | 1.8 | 0.793 | 177.2 | 7.7 | 0.507 | 0.23 | 0.01 | 0.769 |
| Q2 | 38.3 | 1.8 | 0.994 | 167.3 | 7.7 | 0.826 | 0.24 | 0.01 | 0.742 |
| Q3 | 38.2 | 1.9 | 0.965 | 156.3 | 7.9 | 0.216 | 0.26 | 0.01 | 0.154 |
| Q4 | 38.3 | 1.9 | *ref* | 169.7 | 7.9 | *ref* | 0.23 | 0.01 | *ref* |
| *trend* | | 0.992 | | | 0.31 | | | 0.3504 | |
| Interaction with statins | | | 0.034 | | | 0.017 | | | 0.002 |
| CSF / ICV | | | | | | | | | |
| Q1 | 35.0 | 1.8 | 0.175 | 171.9 | 7.7 | 0.473 | 0.22 | 0.01 | 0.155 |
| Q2 | 40.9 | 1.8 | 0.356 | 174.5 | 7.6 | 0.343 | 0.25 | 0.01 | 0.994 |
| Q3 | 37.7 | 1.8 | 0.728 | 160.0 | 7.8 | 0.717 | 0.24 | 0.01 | 0.776 |
| Q4 | 38.5 | 1.9 | *ref* | 163.9 | 8.0 | *ref* | 0.25 | 0.01 | *ref* |
| *trend* | | 0.136 | | | 0.5285 | | | 0.4179 | |
| Interaction with statins | | | 0.298 | | | 0.754 | | | 0.437 |

Table 5
(continued)

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| WMH / ICV | | | | | | | | | |
| Q1 | 35.9 | 1.9 | 0.520 | 160.8 | 7.9 | 0.510 | 0.24 | 0.01 | 0.559 |
| Q2 | 39.3 | 1.8 | 0.525 | 181.3 | 7.6 | 0.238 | 0.23 | 0.01 | 0.970 |
| Q3 | 39.3 | 1.8 | 0.524 | 161.3 | 7.5 | 0.523 | 0.25 | 0.01 | 0.233 |
| Q4 | 37.6 | 1.9 | *Ref* | 168.3 | 7.8 | *ref* | 0.23 | 0.01 | *Ref* |
| *trend* | | 0.490 | | | 0.187 | | | 0.581 | |
| Interaction with statins | | | 0.600 | | | 0.236 | | | 0.399 |
| WMH / WM | | | | | | | | | |
| Q1 | 36.2 | 1.9 | 0.540 | 165.8 | 7.7 | 0.664 | 0.24 | 0.01 | 0.796 |
| Q2 | 40.8 | 1.8 | 0.272 | 181.1 | 7.5 | 0.336 | 0.24 | 0.01 | 0.561 |
| Q3 | 38.1 | 1.8 | 0.901 | 159.5 | 7.3 | 0.304 | 0.25 | 0.01 | 0.385 |
| Q4 | 37.8 | 1.9 | *Ref* | 170.7 | 7.7 | *ref* | 0.23 | 0.01 | *Ref* |
| *trend* | | 0.347 | | | 0.207 | | | 0.8302 | |
| Interaction with statins | | | 0.576 | | | 0.167 | | | 0.513 |

*Adjusted for age and gender

** Model 2: adjusted for age, gender, statin use, interaction statin use and volume

Cerbrospinal fluid (CSF), intracranial volume (ICV)

Table 6 presents the sample characteristics of the n=182 participants in the analytic sample by PiB-Status. In non-demented participants included in this study, 101 (55%) were PiB-positive. Participants PiB-positive were more likely have taken *gingko biloba* during the trial (p=0.05), ApoE-genotype (p<0.01) and to have mild cognitive impairment (MCI) (p<0.01).

**Table 6: Cross-sectional associations between participant characteristics and PiB-Status.**

| | | **Total** (n=182) | | **PiB-negative** (n=81) | | **PiB-positive** (n=101) | | p-value |
|---|---|---|---|---|---|---|---|---|
| | | *n* | *%* | *n* | *%* | *n* | *%* | unadjusted |
| Age | | | | | | | | |
| | <85 | 52 | 28.6 | 25 | 30.9 | 27 | 26.7 | 0.534 |
| | 85-86 | 53 | 29.1 | 23 | 28.4 | 30 | 29.7 | 0.889 |
| | 86-87 | 36 | 19.8 | 15 | 18.5 | 21 | 20.8 | 0.710 |
| | >87 | 41 | 22.5 | 18 | 22.2 | 23 | 22.8 | *ref* |
| Women | | 74 | 40.7 | 30 | 37.0 | 44 | 43.6 | 0.373 |
| ApoE | | | | | | | | 0.006 |
| | e22 | 1 | 0.5 | 1 | 1.2 | 0 | 0.0 | |
| | e23 | 19 | 10.4 | 13 | 16.0 | 6 | 5.9 | |
| | e24 | 1 | 0.5 | 0 | 0.0 | 1 | 1.0 | |
| | e33 | 101 | 55.5 | 48 | 59.3 | 53 | 52.5 | |
| | e34 | 31 | 17.0 | 4 | 4.9 | 27 | 26.7 | |
| MMSE | | | | | | | | |
| | <27 | 44 | 24.2 | 14 | 17.3 | 30 | 29.7 | 0.054 |
| | 27-28 | 62 | 34.1 | 27 | 33.3 | 35 | 34.7 | 0.877 |
| | 28-30 | 36 | 19.8 | 18 | 22.2 | 18 | 17.8 | 0.437 |
| | >30 | 30 | 16.5 | 16 | 19.8 | 14 | 13.9 | *ref* |
| Diagnosis | | | | | | | | |
| | Normal | 148 | 81.3 | 73 | 90.1 | 75 | 74.3 | 0.008 |
| | MCI | 34 | 18.7 | 8 | 9.9 | 26 | 25.7 | |
| Treatment group | | | | | | | | |
| | Active | 91 | 50.0 | 34 | 42.0 | 57 | 56.4 | 0.053 |
| Statins | | | | | | | | |
| | Ever statin | 87 | 47.8 | 49 | 60.5 | 38 | 37.6 | 0.830 |
| | Current statin | 69 | 37.9 | 37 | 45.7 | 32 | 31.7 | 0.692 |

Table 7 presents the levels of plasma oxysterols and lipid by PiB-status. Plasma oxysterols and lipids levels were not associated with PiB-status. However the lower levels of oxysterol levels in the plasma of statin users resulted in a significant interaction term between PiB-status and statin use predicting plasma oxysterols.

**Table 7: Cross-sectional associations between lipid components and PiB-status**

| | | n | Total n=182 | | PiB-negative n = 81 | | PiB-positive n = 101 | | p-value |
|---|---|---|---|---|---|---|---|---|---|
| | | | *mean* | *std dev* | *mean* | *std dev* | *mean* | *std dev* | |
| 24-OHC | (ng/mL) | 182 | 37.6 | 11.7 | 37.2 | 11.1 | 38.1 | 12.2 | 0.651 |
| 27-OHC | (ng/mL) | 182 | 170.0 | 47.6 | 173.1 | 45.9 | 168.0 | 49.0 | 0.475 |
| Ratio_24/27 | | 182 | 0.23 | 0.09 | 0.23 | 0.09 | 0.24 | 0.10 | 0.272 |
| Cholesterol | (mg/dL) | 176 | 163.1 | 32.1 | 163.1 | 33.1 | 162.6 | 31.2 | 0.918 |
| LDL | (mg/dL) | 176 | 87.9 | 27.6 | 88.5 | 29.6 | 87.1 | 25.9 | 0.733 |
| HDL | (mg/dL) | 176 | 49.2 | 13.4 | 47.6 | 12.6 | 50.3 | 13.9 | 0.179 |
| Triglycerides | (mg/dL) | 176 | 131.3 | 62.3 | 135.0 | 60.5 | 128.3 | 64.3 | 0.481 |

Table 8 shows effect of statin use on the levels of oxysterol and lipids by PiB status. Participants using statins at their last visit had significantly lower levels of 27-OHC regardless of PiB-status ($p<0.001$). A significant trend in the levels of 27-OHC were found across the interaction groups. Levels of 27-OHC were highest in PiB-positive/no statin, followed by PiB-negative/no statin, and lowest in PiB-negative/statin groups. Participants using statins at last visit and were PiB-negative at PET scan had significantly lower levels of 24-OHC than participants not taking statins($p<0.05$). Figure 5 shows the levels of 24-OHC among statins users was

significantly affected by PiB-status. PiB-negative participants taking statins had significantly lower ($p<0.05$) levels of 24-OHC than PiB-positive participants taking statins.

**Table 8: Associations between oxysterols and PiB-status stratified by current statin use**

| | **No Statin Use** (n=113) | | | | | | **Statin Use** (n=69) | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **PiB-negative** (n=49) | | | **PiB-positive** (n=64) | | | **PiB-negative** (n=32) | | | **PiB-positive** (n=37) | | | |
| | LSmeans | SE | p-value | LSmeans | SE | p-value | LSmeans | SE | p-value | LSmeans | SE | p-value | Overall p-value |
| 24-OHC (ng/mL) | | | | | | | | | | | | | |
| Model 1 | 39.8 | 1.7 | *ref* | 38.1 | 1.5 | 0.429 | 33.2 | 2.1 | 0.013 | 37.9 | 1.9 | 0.437 | 0.092 |
| Model 2 | 40.7 | 1.6 | *ref* | 38.3 | 1.4 | 0.285 | 33.7 | 2.0 | 0.008 | 38.4 | 1.9 | 0.355 | 0.082 |
| 27-OHC (ng/mL) | | | | | | | | | | | | | |
| Model 1 | 187.2 | 6.6 | *ref* | 173.9 | 5.8 | 0.129 | 151.5 | 8.1 | 0.001 | 158.0 | 7.6 | 0.004 | 0.003 |
| Model 2 | 185.0 | 6.6 | *ref* | 173.1 | 5.7 | 0.173 | 150.4 | 8.1 | 0.001 | 156.4 | 7.6 | 0.005 | 0.003 |
| Ratio 24/27-OHC | | | | | | | | | | | | | |
| Model 1 | 0.22 | 0.01 | *ref* | 0.23 | 0.01 | 0.476 | 0.23 | 0.02 | 0.629 | 0.25 | 0.02 | 0.129 | 0.505 |
| Model 2 | 0.23 | 0.01 | *ref* | 0.24 | 0.01 | 0.7 | 0.24 | 0.02 | 0.744 | 0.26 | 0.01 | 0.155 | 0.532 |
| Total cholesterol (mg/dL) | | | | | | | | | | | | | |
| Model 1 | 160.7 | 4.6 | *ref* | 167.7 | 4.1 | 0.257 | 166.6 | 5.7 | 0.420 | 153.8 | 5.3 | 0.331 | 0.177 |
| Model 2 | 163.9 | 4.4 | *ref* | 168.6 | 3.8 | 0.424 | 168.2 | 5.4 | 0.537 | 156.6 | 5.0 | 0.278 | 0.259 |
| LDLc (mg/dL) | | | | | | | | | | | | | |
| Model 1 | 86.4 | 4 | *ref* | 89.6 | 3.5 | 0.549 | 91.7 | 5.0 | 0.404 | 82.8 | 4.6 | 0.562 | 0.538 |
| Model 2 | 88.3 | 4 | *ref* | 90.0 | 3.5 | 0.746 | 92.9 | 4.9 | 0.469 | 84.3 | 4.5 | 0.511 | 0.617 |

Model 1: unadjusted

Model 2: adjusted for age and gender

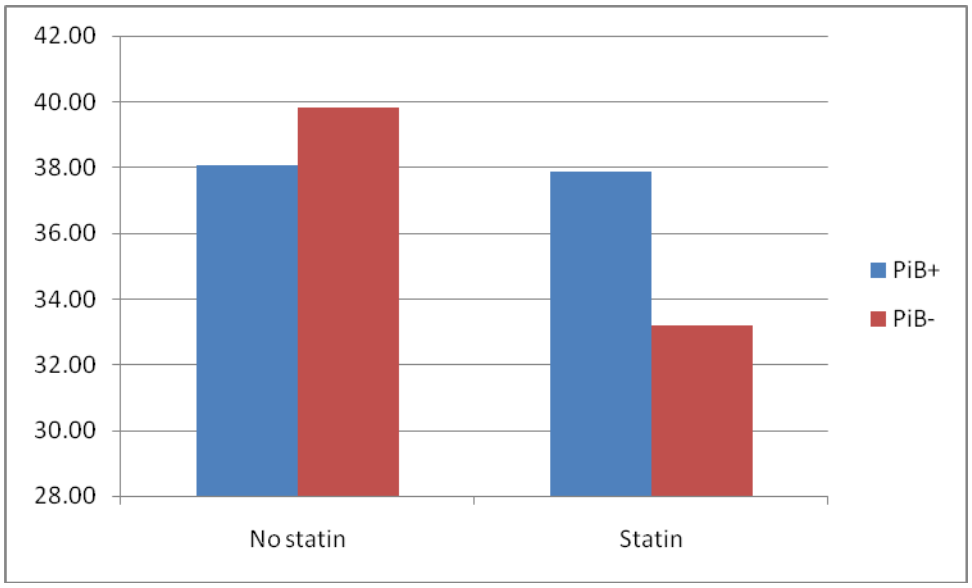


**Figure 5: Concentrations of plasma 24S-hydroxycholesterol (ng/mL) by PiB-Status and current statin use.**

## 3.4 DISCUSSION

In this population of non-demented elderly participants, the levels of plasma oxysterols, 24-OHC and 27-OHC, were not significantly associated with having PiB-positive PET scans. Any relationship between PiB status and oxysterols was highly dependent on the concurrent use of statins. Participants using statins tended to have lower levels of 24-OHC and significantly lower levels of 27-OHC in the plasma. Statin use modified the relationship between 24-OHC and PiB-status. Specifically, the levels of 24-OHC were significantly lower in participants who were PiB-negative and taking statins. Interestingly participants taking statins but were PiB-positive had approximately the same levels of 24-OHC as those not taking statins. In this study plasma, oxysterol levels were not significantly different between normal and MCI participants. This

differs from our earlier findings in CHS-CS and the findings of other studies in the literature that report that levels of plasma 24-OHC tend to be higher in MCI participants compared to normal controls(Lutjohann, Papasotiropoulus et al. 2000; Papassotiropoulus and Lutjohann 2000). Few studies adjust for the effects of statin use on plasma oxysterol levels in studies of AD. Statins are thought to suppress oxysterol production through inhibition of its cholesterol precursors. This is evident by the significantly lower levels of 27-OHC in statin users regardless of PiB-status.

A limitation of this study is that the specific statin used by each participant was not taken into account. All statins were considered as a single group. Statins are shown to suppress oxysterol levels in the plasma. It is believed that statin able to cross the BBB can suppress cholesterol synthesis in the brain. Potent statins, simvastatin (Locatelli and Lutjohann 2002), lovastatin(Vega and Weiner 2007) have been shown to cross the blood brain barrier and suppress cholesterol synthesis in the brain and lower levels of 24-OHC in the plasma. Not all statins have the ability to cross the blood brain barrier due to their relative lipophilicity. Other statins, such as pravastatin, have do not decrease plasma 24-OHC levels(Thelen and Laaksonen 2006). Despite the documented effects of statins on oxysterol levels, few studies account for these effects by adjusting, stratifying or restricting analysis based on statin use.

*Conclusions*

Plasma oxysterols, 24-OHC and 27-OHC, are non-invasive markers of enzymatic cholesterol metabolism in the brain and the periphery, respectively. Oxysterols can be measured in fresh and stored plasma or serum samples. Cross-sectional associations between 24-OHC and PiB-PET indicate a limited potential relationship between cholesterol metabolism in the brain and Aβ deposition as plaque in elderly participants. Statin use likely modifies the relationship

between PiB-status and oxysterol levels, indirectly through the suppression of cholesterol synthesis. Longitudinal measures of oxysterols over nine years (baseline and 2009 of GEMS) will provide insight into the potential changes of cholesterol and cholesterol metabolism occurring in the brain in cognitively normal elderly participants.

# 4.0 DISSERTATION DISCUSSION

## 4.1 OVERALL SUMMARY OF FINDINGS

Our review of the literature indicates inconsistent associations between brain-derived plasma oxysterols and AD. Epidemiological design and methodological limitations may explain these conflicting results. Potential methodological issues include: residual confounding, lack of temporal associations, and inconsistent direction of associations resulting from stage of the disease at which oxysterols were measured. Few studies of oxysterols and cognitive impairment seek to identify potential confounders and adjust for them in analyses. A wide survey of the literature focused on oxysterols and neurodegenerative disease indicates that 24-OHC is associated with: age, global cognition scores, severity of disease, gender, statin use and ApoE-4 carrier status. Many of these characteristics are also established risk factors for cognitive impairment; however, associations between oxysterols and cognitive impairment reported in the literature do not attempt to adjust for these potential confounders. These studies mostly rely on case-control design and often select participants from hospitals and memory clinics. These cross-sectional studies lack crucial information about temporality of these associations. It remains uncertain whether the relative differences in plasma concentrations of oxysterols between cases and controls occurs prior to cognitive impairment or as a result of these neurodegenerative processes. Studies of oxysterols and longitudinal evaluation of cognition suggest that plasma

oxysterols are associated with baseline speed of processing but unrelated to concurrent cognitive tests or changes in cognition over six years of follow-up. Furthermore, studies of oxysterols and cognitive impairment have yet to report the trajectory of oxysterols over the dementia process.

Studies of oxysterols and other neurodegenerative diseases suggest that plasma levels of 24-OHC increase early in the disease process. Careful review of the literature shows that 24-OHC tends to be higher in individuals with cognitive impairment than cognitively normal controls when measured early in the disease process. Plasma 24-OHC is negatively correlated with global scores and severity of disease; therefore the levels of 24-OHC are lower in participants with a long duration of dementia. Despite these inconsistencies of direction seen in the literature, studies of oxysterols that measure both dementia and MCI outcomes have good internal validity. These studies show that plasma 24-OHC measured in patients with AD, VaD and MCI are always in the same direction of association compared to controls.

A major methodological limitation of the literature is the scarcity of objective measures to quantify underlying structural changes occurring the aging and AD brain. Magnetic resonance imaging (MRI) and positron emission tomography (PET) are used to provide quantitative markers of underlying brain changes occurring well before the onset of the clinical disease. Our original research addresses this knowledge gap by examining the longitudinal association between oxysterols, cognition and brain imaging markers. In our first study, we found that higher levels of brain-derived oxysterols were associated with MRI markers of cerebrovascular disease and with greater risk of incident cognitive impairment over 8 years of follow-up. The levels of both 24-OHC and 27-OHC were both higher in cognitively normal older participants with the presence of higher grade white matter hyperintensities and infarcts compared to those without evidence of cerebrovascular disease. Furthermore, higher levels of 24-OHC, but not 27-OHC,

were associated with the development of incident cognitive impairment, including both the incident diagnosis of AD and MCI. These associations between oxysterols and cerebrovascular disease and incident cognitive impairment were independent of age, gender, and statin use. In the case of cerebrovascular disease, we found these associations to also be independent of concurrent total cholesterol levels. We also found that oxysterols were not associated with MRI and genetic markers of AD risk in cognitively normal elderly participants in this study. In our second study of oxysterols and underlying AD pathology, we found that oxysterol levels were not associated with the presence of significant amyloid deposition in the brains of cognitively normal and MCI elderly participants. We found that the use of lipid-lowering drugs may modify any association between plasma oxysterols levels and *in vivo* amyloid deposition in the general population freely taking statins.

## 4.2 PUBLIC HEALTH SIGNIFICANCE

The antecedents and neurodegenerative processes underlying dementia remain unclear. Cholesterol metabolism is believed to be an important factor in the initiation and progression of AD. The alteration of brain cholesterol synthesis and metabolism are current intervention targets for the prevention and treatment of dementia. Our research adds important evidence that cholesterol metabolites are more strongly associated with the presence of cerebrovascular disease than markers of AD pathology preceding cognitive impairment. Cerebrovascular disease occurring in the white matter of the brain may indicate myelin breakdown. Myelin breakdown

leads to the release of vast stores of cholesterol residing in the myelin. It is possible that higher diffusion of brain-derived metabolites of cholesterol across the BBB and into the circulation is a reaction to states of excess cholesterol in the brain. *In vitro* studies demonstrate that excess cholesterol in plasma membranes leads to Aβ production and deposition in the brain. Our study of oxysterols and *in vivo* amyloid imaging brain indicates that brain-derived oxysterols are not associated with Aβ deposition in non-demented elderly participants. We found that brain-derived oxysterols were elevated prior to the onset of cognitive impairment, particularly in those with cerebrovascular disease. Brain-derived oxysterols may be a useful marker of dementia risk, early in the dementia process; oxysterols may provide prognostic information late in the course of disease.

## 4.3 STRENGTHS AND WEAKNESSES

A particular strength of this research is that it examines the relationship between oxysterols and dementia without relying solely on the diagnosis of cognitive impairment. Our studies utilize cutting edge neuroimaging techniques to image the underlying structural changes occurring in the cognitive impairment processes. We also utilize cognitively normal participants at baseline to examine the temporal associations between oxysterols and incident cognitive impairment.

A limitation of this research is that oxysterols are only surrogate markers of cholesterol metabolism in the brain. It is unknown of elevations in oxysterols reflect innate cholesterol excesses or increased metabolism due to recent increases in cholesterol concentrations in the

brain, or other reasons that remain unknown. However, 24-OHC is the primary metabolite of cholesterol in the brain. Conversion of cholesterol to 24-OHC is suspected to increase during active myelination and repair. 24-OHC is also expected to increase cholesterol load in ApoE indirectly through the binding to LXR and up regulation of ABCA1 which in turn increases cholesterol density within ApoE. ApoE is the main transporter of cholesterol in the brain and the primary genetic risk factor for AD.

## 4.4 FUTURE RESEARCH

Our findings raise interesting questions regarding the role of cholesterol metabolism in the brain and in the dementia process. While we show that oxysterols are significantly higher in participants with evidence of cerebrovascular disease and not associated with amyloid deposition in the brain, these findings are novel and need to be replicated in larger studies with younger participants. In particular, individuals with familial hypercholesterolemia and individuals with familiar risk for AD and cerebrovascular disease would be ideal study groups. It is unknown if higher levels of oxysterols are a result of or antecedent for cerebrovascular disease. Although we did not find a relationship between oxysterol metabolites of brain cholesterol and amyloid deposition using PiB-PET, it is possible that oxysterols may be related to PiB-status in younger individuals. The mechanisms leading to amyloid deposition in the brain of exceptionally fit elderly participants is likely different from younger participants at risk for AD. Another group with particular relevance to this research area are aging individuals with multiple sclerosis. Successful treatments have extended the life-expectancy of MS patients to near normal life

expectancy of the general population. We don't yet understand the effect of chronic demyelination on the aging brain and the risk for dementia it may impose.

## 4.5 FINAL CONCLUSIONS

Brain-derived plasma oxysterols may be an important marker of cholesterol metabolism in the brain, underlying cerebrovascular disease, and an early marker of risk for the development cognitive impairment. Measuring brain cholesterol metabolites is feasible, relatively inexpensive and it could be used to assess the risk of developing cognitive impairment in a wide array of groups. Future studies investigating the relationship between oxysterols and dementia should: determine the longitudinal trajectory of oxysterol over the dementia process; obtain concurrent measures of brain structure and clinical signs of cognitive impairment, including serial MRI and PiB-PET; and access and control for confounding effects of lipid-lowering agents.

# APPENDIX

## TABLES FROM REVIEW OF CHOLESTEROL METABOLISM TO OXYSTEROLS IN THE BRAIN AND DEMENTIA

**Table A1: Genetic variability studies in of enzyme responsible for conversion of cholesterol to 24S-hydroxycholesterol**

| AUTHOR | STUDY DESIGN | SAMPLE SIZE | RESULTS | MEASURES |
|---|---|---|---|---|
| Kolsch H *et al.* (2009)(Kolsch 2009) | Case-control | Participants:<br>AD patients = 455<br>Controls = 327 | 2 of 16 SNPs in CYP46A1 associated with AD. Haplotype of carriers showed reduced CSF levels of 24-OHC and total Chol. | Oxysterols: 24-OHC & 27-OHC (CSF)<br>Genotyped: 16 SNPS in the CYP46A1 gene<br>Outocome: Case status |
| Kolsch H *et al.* (2002)(Kolsch, Lutjohann et al. 2002) | Cross-sectional, genetics | Participants:<br>Controls = 144<br>AD = 114<br>Age mean: 71±8 years | SNPs in the CYP46 gene may predispose to AD by increasing the R_24SOH ratio in the brain (CSF) | Oxysterols: 24-OHC & 27-OHC (CSF)<br>Genotyped: SNPs in the CYP46 gene<br>Outcome: Case status |

**Table A2: Studies relating24S-hydroxycholesterol and various neurodegenerative diseases**

| AUTHOR | STUDY DESIGN | SAMPLE SIZE | RESULTS | MEASURES |
|---|---|---|---|---|
| Leoni *et al.* (2008)(Leoni and Mariotti 2008) | Case-control | Participants: 196 Total Controls = 67, HD-positive = 96, Pre-HD = 33 | Plasma 24-OHC were significantly higher in controls than HD patients. Levels of 24-OHC paralleled large decreases in caudate volumes. | Oxysterols: 24-OHC (plasma) Outcomes: Case status, Volumetric MRI of caudate |
| Leoni *et al.* (2002)(Leoni and Masterman 2002) | Case control | Participants: 301 MS patients = 118 Age matched controls = 183 | Among MS patients levels of 24-OHC were higher in younger participants and associated with higher disability scores. Increased levels of 24-OHC seen only in lesion confirmed active disease. | Oxysterols: 24-OHC (plasma and CSF) Outcomes: Case status |
| Leoni *et al.* (2004)(Leoni, Masterman et al. 2004) | Case Control stored samples | Participants: Controls = 58, MS = 88, AD = 54, Demyelinating = 21, hemorrhage = 8, viral meningitis = 12, borrelia = 14. | Patients with active demyelinating diseases had highest levels of 24-OHC in the CSF, followed by viral meningitis, MS, and AD | Oxysterols: 24-OHC and 27-OHC (plasma and CSF) Outcomes: Case status |
| Holderieder *et al.* (2004)(Holdenrieder and Lutjohann 2004) | Case-series | Participants: Occlusion of middle cerebral artery = 4 Occlusion of posterior cerebellar artery = 2 No controls | Levels of cholesterol and oxysterols did not vary significantly in the days following admission to hospital for stroke symptoms indicating that oxysterols are of limited value for assessing BBB function in acute ischemic stroke patients. | Oxysterols: 24-OHC and 27-OHC (plasma) |
| Teunissen *et al.* (2003.a)( Teunissen and Dijkstra 2003) | Case-control | Participants: total = 97 Controls = 37, MS = 60 (20 RR, 20 SP, 20 PP) | Serum R_24-OHC and cholesterol precursors were lower in MS subtypes than healthy controls | Oxysterols: 24-OHC and 27-OHC (serum) Outcomes: Case status |
| Teunissen *et al.* (2003.b)( Teunissen, Lutjohann et al. 2003) | Case-control | Participants: AD = 34 PD = 46 OCD = 46 Controls = 61 | Serum R_24-OHC found to be a significant predictor of neuro-cognitive disease. Not included in the final model of combined markers predicting neuro-cognitive disease. | Oxysterols: 24-OHC and 27-OHC (plasma) Outcomes: Combined case status (AD, PD, and OCD) |
| Bretillon L *et al.* (2000)(Bretillon and Siden 2000) | Case-control | Participants: Controls (age/sex) = 205 AD = 40; MS = 20; Guillian-Burre = 4; Meningitis = 11; Primary gliomas = 7; Brain death = 11 | Patients with brain death and AD had significantly lower plasma 24-OHC than controls; no differences were seen for MS, ischemic stroke and primary brain tumors. | Oxysterols: 24-OHC (plasma) Outcome: Case status |

**Table A3: Studies relating 24S-hydroxycholesterol to structural brain measures**

| AUTHOR | STUDY DESIGN | SAMPLE SIZE | RESULTS | MEASURES |
|---|---|---|---|---|
| Solomon A. *et al.* (2009)(Solomon, Leoni et al. 2009) | Case-control | Participants:<br>SCI = 33<br>MCI = 36<br>AD = 27<br>Mean age = 62 years | Ratio 24-OHC/Chol and 27-OHC/Chol were significantly lower in AD patients. Adjusted for age, sex, ApoE and statins, the significant relationship between R_24-OHC to grey matter volume only seen in controls. | Oxysterols: 24-OHC & 27-OHC (Plasma)<br>Outcomes: Volumetric MRI yielding fraction volumes of: CSF, gray matter, and white matter. |
| Koschak J, *et al.* (2009)(Koschak and Lutjohann 2009) | Cross-sectional | Participants<br>Cognitively normal volunteers = 69<br>Mean age = 50±13 | Participants with high levels of 24-OHC and 27-OH also had larger hippocampal volumes. 24-OHC, not 27-OH, significantly predicts hippocampal volume. | Oxysterols: 24-OHC & 27-OHC (Plasma)<br>Outcomes: Volumetric MRI (1. 5T) yielding, total, intracranial, and hippocampal volume |
| Leoni V *et al.* (2008)(Leoni and Mariotti 2008) | Case-control | Participants:<br>Controls = 67<br>HD-positive = 96<br>pre-HD = 33<br>Mean age = 46 years | Plasma levels 24-OHC significantly higher in controls than HD patients. Levels of 24-OHC paralleled large decreases in caudate volumes. | Oxysterols: 24-OHC (Plasma)<br>Outcomes: MRI of Caudate |
| Karrenbauer *et al.* (2005)(Karrenbauer and Leoni 2006) | Cross-sectional, disease severity | Participants:<br>RR = 27<br>PP = 19<br>Controls = 23<br>Ages: 23-58 years | Plasma ratio of 24-OHC/chol negatively correlated with age (in both RR and PP patients) and volume of $T_2$ lesions in RR patients (a marker of disease extent) | Oxysterols: 24-OHC (Plasma)<br>Outcomes: MRI $T_1$ and $T_2$ lesions and gadolinium positive lesions |
| Leoni V *et al.* (2002)(Leoni and Masterman 2002) | Case control | Participants:<br>MS patients = 118<br>Control = 183<br>Ages: 21-70years | Among MS patients levels of 24-OHC were higher in younger participants and were associated with higher disability scores. Increased levels of 24-OHC seen only in lesion confirmed active disease. | Oxysterols: 24-OHC (plasma and CSF)<br>Outcomes: Case status |
| Thelen *et al.* (2006)(Thelen and Falkai 2006) | Cross-sectional | Participants: n = 20<br>Decedent = 20 | Concentrations of cholesterol precursors were significantly higher in younger versus older participants. Absolute levels of 24-OHC were slightly lower in hippocampus specimans from older participants | Oxysterols: 24-OHC (CSF of brains)<br>Outcomes: post-mortem hippocampal volumes |

**Table A4: Case-control studies using diagnostic outcomes**

| AUTHOR | STUDY DESIGN | SAMPLE SIZE | RESULTS | MEASURES |
|---|---|---|---|---|
| Shafaati M *et al.* (2007)(Shafaati, Solomon et al. 2007) | Cross-sectional at diagnosis. Case-control | Participants:<br>Controls = 43<br>AD = 17<br>MCI = 20<br>Ages: 18 – 85 years | Levels of ApoE correspond to levels of 24-OHC in AD cases but not controls. These results are consistant with 24-OHC and ApoE coupling seen in neurodegeneration | Oxysterols: 24-OHC and 27-OH (CSF)<br>Stratification: Case status<br>Outcomes: ApoE levels |
| Leoni V *et al.* (2006)(Leoni, Shafaati et al. 2006) | Cross-sectional. Case-control | Participants:<br>AD = 18<br>MCI = 20<br>Controls = 35<br>Ages: 49 – 85 years | CSF levels of 27-OHC and 24-OHC were significantly higher in MCI and AD than in controls. Differences between MCI and AD were not significant | Oxysterols: 24-OHC (plasma and CSF)<br>Outcomes: Case status (AD, MCI, controls) |
| Kolsch *et al.*(2004)(Kolsch and Heun 2004) | Case-controls | Participants:<br>AD = 134<br>VaD = 24<br>MCI = 36<br>Depressed = 13<br>Controls = 43<br>Mean age: ~69 years | Plasma ratio of 24-OHC/chol and 27-OHC/chol reduced in AD, VaD and MCI compared to depressed participants and controls. Higher ratio of 24-OHC/27-OHC seen in AD, VaD and MCI compared to controls. | Oxysterols:Plasma 24-OHC and 27-OH (plasma)<br>Outcomes: Case status |
| Leoni V *et al.* (2004)(Leoni, Masterman et al. 2004) | Case Control retrospective samples | Participants:<br>Controls = 58<br>AD = 54<br>Other nerudegenerative disease = 143<br>Mean age: controls = 39<br>AD = 72 years | Patients with active demyelinating diseases had highest levels of 24-OHC in the CSF, followed by viral meningitis, MS, and AD | Oxysterols: 24-OHC and 27-OH (plasma and CSF)<br>Outcomes: Case status |
| Heverin *et al.* (2004)(Heverin and Bagdanovic 2004) | Case-control | Participants:<br>Controls = 15<br>AD = 15<br>Ages: 61 – 92 years | 24-OHC was significantly increased in AD patients, specifically in the frontal cortex. The ratio of 24- to 27-OHC was elevated in AD patients | Oxysterols: 24-OHC (CSF from brain samples)<br>Outcomes: Case status |
| Teunissen *et al.* (2003.b)(Teunissen, Lutjohann et al. 2003) | Case-control | Participants:<br>AD = 34<br>PD = 46<br>OCD = 46<br>Controls = 61<br>Ages = 42–95 years | Serum R_24-OHC found to be a significant predictor of neuro-cognitive disease. Not included in the final model of combined markers predicting neuro-cognitive disease. | Oxysterols: 24-OHC and 27-OH (plasma)<br>Outcomes: Combined case status (AD, PD, and OCD) |

**Table A4: (continued)**

| AUTHOR | STUDY DESIGN | SAMPLE SIZE | RESULTS | MEASURES |
|---|---|---|---|---|
| Schonknecht *et al.* (2002)(Schonknecht and Lutjohann 2002) | Case-control | Participants:<br>Controls = 19<br>AD = 25 | Elevated levels of 24-OHC found in the CSF, not plasma, of AD treated patients compared to controls. | Oxysterols: 24-OHC (CSF and plasma)<br>Outcomes: Case status |
| Lutjohann *et al.* (2000)(Lutjohann, Papasotiropoulus et al. 2000) | Case-control | Participants:<br>Controls = 30<br>AD = 30<br>Depressed = 18<br>Vascular AD = 12<br>Ages: 48 – 87 | Plasma levels of 24-OHC were elevated in AD and VaD participants compared to normal regardless of ApoE-4 genotype | Oxysterols: 24-OHC (plasma)<br>Outcome: Case status severity of disease using MMSE score |
| Papassotiropoulus *et al.* (2000)(Papassotiropoulus and Lutjohann 2000) | Case-series, severity of disease | Participants:<br>AD = 53(18 with mild AD)<br>Mean age: ~ 73 years | Severity of AD and inheritance of the ApoE-4 allele were independently associated with reduced plasma 24-OHC. | Oxysterols: 24-OHC (plasma)<br>Outcome: Case severity of disease using MMSE score |
| Bretillon L *et al.* (2000)(Bretillon and Siden 2000) | Case-control | Participants:<br>AD = 40 (51-80 years of age)<br>Controls = 205 (21 – 86 years of age)<br>Other brain disease = 68 (28 – 82 years of age) | Patients with brain death and AD had significantly lower plasma 24-OHC than controls; no differences were seen for MS, ischemic stroke and primary brain tumors. | Oxysterols: 24-OHC (plasma)<br>Outcome: Case status |

**Table A5: Longitudinal studies of cognition**

| AUTHOR | STUDY DESIGN | SAMPLE SIZE | RESULTS | MEASURES |
|---|---|---|---|---|
| Teunissen *et al.* (2003)(Teunissen and De Vente 2003) c | Longitudinal | Participants:<br>Baseline = 92<br>Follow-up = 116<br>Both = 65 | Higher R_24-OHC levels were related to slower speed of processing, but not other cognitive tests. Higher ratio of cholesterol precursors associated with poor cognitive performance | Oxysterols: 24-OHC and 27-OH (plasma)<br>Outcomes: Cognitive test for verbal learning and memory |
| van den Kommer et al. (2009)(van den Kommer, Dik et al. 2009) | Prospective | Participants:<br>Baseline = 1181<br>Follow-up = 1003 | Low Chol in 65 and older is an independent predictor of cognitive declines over 6 years. Ratios of 24-OHC/Chol and24-OHC/Chol significantly lower in ApoE-4 carriers. ApoE-4 potential moderator of association between ratio 24-OHC/Chol and speed of processing. ApoE-4 status, R_24-OHC and R_27-OHC showed no association to cognitive decline. Only the ratio 27-OHC/Chol inversely associated with performance on MMSE and immediate recall tests in ApoE-4 carriers | Oxysterols: 24-OHC & 27-OHC<br>Outcomes: Cognitive function (MMSE, AVLT) |

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