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Cholesterol as a Causative Factor in Alzheimer Disease: A Debatable Hypothesis

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Abstract

High serum/plasma cholesterol levels have been suggested as a risk factor for Alzheimer disease (AD). Some reports, mostly retrospective epidemiological studies, have observed a decreased prevalence of AD in patients taking the cholesterol lowering drugs, statins. The strongest evidence causally linking cholesterol to AD is provided by experimental studies showing that adding/reducing cholesterol alters amyloid precursor protein (APP) and amyloid beta-protein (Aβ) levels. However, there are problems with the cholesterol-AD hypothesis. Cholesterol levels in serum/plasma and brain of AD patients do not support cholesterol as a causative factor in AD. Prospective studies on statins and AD have largely failed to show efficacy. Even the experimental data are open to interpretation given that it is well-established that modification of cholesterol levels has effects on multiple proteins, not only APP and Aβ. The purpose of this review, therefore, is to examine the above-mentioned issues and discuss the pros and cons of the cholesterol-AD hypothesis, and the involvement of other lipids in the mevalonate pathway, such as isoprenoids and oxysterols, in AD.

Keywords

Alzheimer disease; amyloid beta-protein; apolipoprotein E; cholesterol; isoprenoids; oxysterols; statins

Twenty three years ago the late Larry Sparks and his colleagues made the intriguing observation that brains of patients with advanced coronary heart disease had senile plaques similar to those seen in Alzheimer patients (Sparks *et al.* 1990). In a subsequent paper, they reported that feeding rabbits a diet high in cholesterol induced plaques in brain tissue of treated animals (Sparks *et al.* 1994). Those initial observations stimulated a large body of research ranging from retrospective and prospective epidemiological studies on the

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cholesterol lowering drugs statins and Alzheimer disease (AD) to animal, cellular and molecular studies on cholesterol and amyloid beta-protein (Aβ). Elevated serum/plasma cholesterol levels have been proposed to be a risk factor for developing AD (Pappolla *et al.* 2003) but there are issues with that hypothesis (Wood *et al.* 2005;Daviglus *et al.* 2010). A modification of that hypothesis is the notion that high serum cholesterol levels during middle age are associated with an increased risk of AD (Solomon *et al.* 2009a). Effectiveness of statins in treating or preventing AD is controversial particularly with respect to prospective studies (McGuinness *et al.* 2013). The strongest support for a role of cholesterol in AD comes from studies in animal models of AD and *in vitro* studies using primary and immortalized cells. A majority of the animal and cell culture studies have reported that increasing cholesterol levels increases Aβ abundance whereas the opposite effects are noted when cholesterol levels are reduced and that work has been extensively reviewed (Wood *et al.* 2003;Posse de Chaves 2012;Maulik *et al.* 2013;Burns and Rebeck 2010;Ong *et al.* 2013).

Data from human studies of cholesterol levels whether in serum/plasma or brain are not consistent in supporting a role of cholesterol in AD, and this conundrum will be examined in this review. This lack of consistently in the human literature differs in comparison with results of most animal and cell culture studies. What appears to be a paradox between the human and animal/cell culture studies will be critically examined from the perspective of the multiple roles of cholesterol in addition to the cholesterol-mediated actions in AD. Cholesterol is not the only important lipid produced in the mevalonate pathway as noted in Figure 1, and this review will examine whether a case can be made for linking other mevalonate-derived lipids (farnesyl pyrophosphate, geranylgeranyl pyrophosphate, 24S-hydroxycholesterol) to AD. Emphasis of this review will be on the role of cholesterol as factor in the development of AD. However, we will examine the alternative hypothesis that AD and specifically Aβ perturb cholesterol homeostasis.

Serum/Plasma Cholesterol Levels and AD

Elevated serum cholesterol levels have been proposed to increase the risk of developing AD (Pappolla *et al.* 2003). An early study on cholesterol and AD reported that apoE genotype and AD were dependent on total cholesterol levels, age and gender (Jarvik *et al.* 1995). The authors concluded that total serum cholesterol may accelerate development of AD. However, that conclusion is not supported by their data, which showed that mean serum cholesterol levels did not significantly differ between AD patients and controls. In another study, total serum cholesterol levels were not significantly different between AD and control subjects, but it was observed that low-density lipoprotein cholesterol (LDL-C) was significantly higher and high-density lipoprotein cholesterol (HDL-C) was significantly lower in AD patients than control subjects (Kuo *et al.* 1998). In contrast, LDL-C levels were reported to not be significantly different in AD patients compared with control subjects (Romas *et al.* 1999), but total cholesterol levels were significantly lower in AD patients than control subjects. Although the mean total cholesterol levels were significantly different, the physiological effects of such small changes (3.9% difference) are unclear. A recent study found that plasma cholesterol levels were approximately 10% higher in AD patients as compared with control subjects and that this difference was significant (Popp *et al.* 2013).

An earlier study by the same group reported that plasma cholesterol levels of AD and control individuals were not significantly different (Popp *et al.* 2012).

The argument has been made that elevated serum cholesterol levels at midlife may be predictive of developing AD, and that longitudinal studies may be more revealing as compared with cross-sectional studies (Kivipelto *et al.* 2001). In a longitudinal study at the first exam, cholesterol levels were significantly higher in AD patients compared with control subjects but were not significantly different at the second exam ranging from 11-26 years after the first exam. Odds ratio analysis using different models indicated that midlife cholesterol levels were a significant risk for developing AD. However, there are problems with that interpretation. The mean cholesterol levels at the first exam were 278 mg/dl for the AD group and 259 mg/dl for the control group. At the second exam, cholesterol levels were 232 and 224 mg/dl for the AD and control groups. Normal cholesterol levels are considered to be under 200 mg/dl. Both AD and control groups were hypercholesterolemic at midlife but only 48 individuals were eventually diagnosed with AD compared with 1,352 subjects not receiving an AD diagnosis. Missing from the study was a groups of individuals with normal cholesterol levels both at midlife and late-life with or without a subsequent diagnosis of AD. Another study reported that high cholesterol levels at midlife were associated with the risk of developing late-life dementia (Whitmer *et al.* 2005). In that study there were 2844 individuals with cholesterol levels above 240 mg/dl and out of that group 266 or 9% were identified with dementia using medical records. Based on a Cox proportional hazards model, the authors concluded that high midlife cholesterol levels were associated with an increased risk of developing AD. Similar conclusions were reached in a later study using Cox proportional hazards models of cholesterol levels and development of AD (Solomon *et al.* 2009a). There were 9,844 subjects whose cholesterol levels were determined when in their early 40’s. Less than 5% were later diagnosed in their late 60's with AD using electronic records. Approximately 2% were diagnosed with vascular dementia. Mean cholesterol levels were similar for all three groups at midlife: no dementia, 224 mg/dl; AD, 228mg/dl; vascular dementia, 226 mg/dl. A prudent interpretation of those results is that elevated cholesterol levels at midlife is a risk factor for developing AD for a very small number of individuals but other causative factors are at play.

Data supporting elevated cholesterol levels as a risk factor for AD are not robust. Further weakening the cholesterol-AD hypothesis are studies showing that serum cholesterol levels do not differ between AD and control individuals. A meta-analysis of 10 studies published between 1986 and 1999 found that cholesterol levels were actually significantly lower in AD patients than in control subjects (Knittweis and McMullen 2000). The well-recognized Framingham study found that total serum cholesterol levels were not associated with the risk for developing AD (Tan *et al.* 2003). Serum cholesterol levels were determined at baseline and across 15 biennial cycles as well as neurological and neuropsychological examinations for dementia. Serum cholesterol levels in normal individuals 65 years of age or older were not associated with the risk of subsequently developing dementia or AD (Li *et al.* 2005). The Honolulu-Asia Aging study found that serum cholesterol levels at midlife were not associated with later development of dementia or AD as assessed by a neurological and neuropsychological exams and neuroimaging (Stewart *et al.* 2007). The observation was

made in that study that cholesterol levels in individuals with dementia and AD had declined over at least 15 years. It was suggested that the decline in cholesterol levels might actually contribute to the development of dementia (Stewart *et al.* 2007).

Elevated total serum cholesterol levels whether at midlife or later do not have a major role in the development of AD. This conclusion is not surprising based on the following data: 1) the estimated number of US adults with cholesterol levels of 200 mg/dl and higher is 98.9 million and of that number 31.9 million have cholesterol levels of 240 mg/dl and higher (Go *et al.* 2013); and 2) 4.7 million individuals are estimated to have AD (Hebert *et al.* 2013). If high serum cholesterol levels were a risk factor for AD then the incidence and prevalence of AD would be higher. Another issue to consider is the biological rationale for examining serum cholesterol levels in AD patients. Serum cholesterol and brain cholesterol levels are not in equilibrium (Kabara 1973). Normally, changes in serum cholesterol do not affect brain cholesterol homeostasis, and no relevant cholesterol flux from the periphery into brain seems to take place (Dietschy and Turley 2004). This line of evidence is supported by data from our laboratories on apoE-knockout mice and cholesterol-fed rats. ApoE-knockout mice have peripheral atherosclerotic lesions and show highly elevated serum and liver cholesterol values, but brain cholesterol levels of these animals do not differ compared with age-matched wild-type mice (Kirsch *et al.* 2003a). In the same study, treatment of young Wistar rats for at least 6 months with 2% cholesterol had no effect on brain cholesterol levels of synaptic plasma membranes (SPM) compared with control animals. Plasma and liver cholesterol levels were significantly increased in the animals on the 2% cholesterol diet.

Brain Cholesterol Levels and AD

Elevated serum and plasma cholesterol levels do not appear to be a risk factor for AD. Serum and plasma cholesterol levels are not in equilibrium with brain cholesterol as discussed above. There is evidence that the blood-brain barrier may be dysfunctional in AD (Erickson and Banks 2013) which could alter brain cholesterol homeostasis. If high serum/plasma cholesterol levels were a factor in the development of AD then it follows that cholesterol should be in greater abundance in brain tissue of AD patients as compared with normal individuals. Cholesterol levels have been determined in different brain regions and the cerebrospinal fluid (CSF) of AD patients compared with control subjects. There have been reports of reduced cholesterol levels, increased cholesterol levels, and no changes in cholesterol levels in AD patients versus control subjects (Wood *et al.* 2005). Cholesterol levels were lower in the temporal gyrus of autopsied brains of AD patients than control subjects (Mason *et al.* 1992). Cholesterol levels in the frontal cortex gray matter of AD patients was modestly but significantly higher (2.65 ± 0.14 mg/g wet tissue weight) with the apoE4 genotype compared with apoE4 control subjects (2.04 ± 0.18) (Sparks 1997). Cholesterol levels were similar in the cerebral cortex of AD and control individuals (Heverin *et al.* 2004) but a small but significant increase (approx 15 μg vs 12 μg/mg brain tissue) in cholesterol levels was noted in the basal ganglia of AD patients compared with control subjects. Cholesterol levels did not differ in hippocampal tissue of AD patients compared with control subjects (Eckert *et al.* 2000). We reported that cholesterol levels and HMG-CoA reductase gene expression were similar in post-mortem human brain frontal cortex of AD and control individuals (Eckert *et al.* 2009). However, in two recent studies it was

reported that CSF cholesterol levels were significantly lower (approximately 12%) in AD patients than controls (Popp *et al.* 2012;Popp *et al.* 2013).

Simply put, brain cholesterol levels of AD patients are highly variable, and the data do not support the hypothesis that total brain cholesterol abundance is a causative factor in AD. Contributing to the variability amongst the studies obviously are issues pertaining to sample selection, preparation and assays methods. Another consideration is that total cholesterol levels are at best a gross estimate of the role of cholesterol in any biological process. Cholesterol exists for example in different domains within membranes (Wood *et al.* 2007;Schroeder *et al.* 2010;Sonnino and Prinetti 2013;Wood *et al.* 2011) . Data from animal and cell culture studies have shown that large changes can occur in membrane cholesterol domains that take place in the absence or minimal changes in total cholesterol levels. We have shown for example that the distribution of cholesterol between the two membrane leaflets of SPM was altered in animal models of aging, alcoholism (Igbavboa *et al.* 1996;Wood *et al.* 1989), statin administration (Kirsch *et al.* 2003b;Burns *et al.* 2006;Eckert *et al.* 2013), and apoE genotype (Hayashi *et al.* 2002;Igbavboa *et al.* 1997). Another type of cholesterol domain attracting great interest albeit with some controversy is lipid rafts (Sonnino and Prinetti 2013;Head *et al.* 2013). Lipid rafts are thought to be scaffolds for interactions of proteins and lipids. These cholesterol enriched membrane domains have been implicated in certain neurodegenerative diseases such Smith-Lemli-Opitz syndrome, Huntington's, Niemann-Pick Type C and AD (Korade and Kenworthy 2008). A study in lipid rafts from human AD brain samples reported changes in fatty acid composition between AD and control samples but that cholesterol content was similar in the two groups (Martin *et al.* 2010). A different approach to lipid analysis is time-of-flight secondary ion mass spectrometry (ToF-SIMS) which is now gaining acceptance as a tool for imaging lipids in biological samples (Passarelli and Winograd 2013). ToF-SIMS was used in a recent study to determine cholesterol distribution in cortical layers of white and grey matter in AD patients and controls, and it was found that the cholesterol signal in the lower half of the cerebral cortex was higher in AD patients than controls (Lazar *et al.* 2013). The novel findings reported by Lazar et al., provide the first indication that cholesterol distribution within a specific brain region may differ in AD as compared with control samples. It would have been helpful in that study if total levels of cholesterol in the cerebral cortex were determined using other methodology. Such data would have provided insight as to whether the observed changes were due to differences in the total amount of cholesterol as compared with a change in how cholesterol was distributed. Faulty cholesterol distribution plays a key role in lysosomal storage diseases such as Niemann-Pick Type C (NPC) and it has been suggested that lysosomal dysfunction may be present in AD (Nixon 2004). Hydroxypropyl-β-cyclodextrin which binds cholesterol and has been used in NPC animal models and patients (Matsuo *et al.* 2013) improved behavior in a mouse model of AD (Yao *et al.* 2012). Aβ levels also were reduced and Aβ clearance increased. Certainly investigating lipid domains whether at subcellular levels or within brain regions gives a micro versus macro view of the potential role of cholesterol in AD. What is not forthcoming from such an approach is that it does not provide insight as to whether cholesterol is a causative factor in AD or is this crucial lipid a casualty of the disease (Wood *et al.* 2003;Posse de Chaves 2012). That notion is discussed later in this review.

Farnesyl pyrophosphate and geranylgeranyl pyrophosphate in AD

Support is lacking for elevated serum or brain cholesterol levels as causative factors in AD. Cholesterol is one of many molecules synthesized within the mevalonate pathway (Fig. 1). The two upstream isoprenoids, farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP) have attracted much attention in diseases and conditions as diverse as cancer(Wiemer *et al.* 2009), osteoporosis(Mo *et al.* 2012), cardiovascular disease(Liao and Laufs 2004), premature and normal aging(Reddy and Comai 2012;Hooff *et al.* 2012) and neurodegenerative diseases including AD(Weber *et al.* 2005;Cole and Vassar 2006;Hooff *et al.* 2010;Li *et al.* 2012). FPP is a key branch point in the mevalonate pathway, and it is a precursor of GGPP and cholesterol. Both FPP and GGPP are required for the post-translational processing of small GTPases. Progress on understanding how regulation of FPP and GGPP levels affects the function of small GTPases in normal brain and neurodegenerative diseases such as AD has been impeded because of analytical difficulties in FPP and GGPP isolation and detection. We have recently overcome these technical obstacles and reported for the first time FPP and GGPP levels in human brain tissue of normal individuals and AD patients (Eckert *et al.* 2009). FPP and GGPP levels of AD patients were significantly higher 36% and 56% respectively, when compared with age-matched controls. Total cholesterol levels were similar in brain tissue of AD and control samples even though FPP is a precursor of cholesterol; FPP is also the precursor of GGPP. Consistent with elevation of FPP and GGPP levels, we showed that gene expression of FPP synthase and GGPP synthase (protein products catalyze FPP and GGPP production) were also elevated in AD brain tissue. HMG-CoA reductase expression levels were unchanged. This study suggests a specific targeting of FPP and GGPP regulation in AD. However, these results need to be confirmed using a larger sample size. It remains to be determined if increases in FPP and GGPP in AD brain are specific to AD or are a manifestation of neurodegeneration which might occur in other diseases.

The consequences of elevated FPP and GGPP levels on protein prenylation require investigation. GTPases must be prenylated either by FPP or GGPP in order to be inserted into membranes and to become active (McTaggart 2006). The majority of small Rho-GTPases are prenylated by GGPP involving geranylgeranyltransferase-I (GGTase-I), which catalyzes the covalent attachment of GGPP via thioether linkage to the CAAX-motif of those proteins. FPP normally prenylates for example Ras proteins (HRAS, KRAS, NRAS) through actions of farnesyl transferase (FTase) but when FTase is inhibited FPP can become a substrate for GGTase-I and prenylation of KRAS and NRAS but not HRAS Ras can occur (Roskoski 2003;Downward 2003)

The Rho family of GTPases such as Rac1, RhoA, and Cdc42 are major regulators of synaptic plasticity, acting on dendrite morphogenesis and stability, growth cone motility and collapse (Linseman and Loucks 2008;Sekino *et al.* 2007;Ramakers 2002) and affecting neuronal architecture and synaptic connectivity (Pilpel and Segal 2004). There are data indicating that certain small Rho family GTPases such as Rac1, RhoA, and Cdc42 contribute to AD pathogenesis (Désiré *et al.* 2005;Mendoza-Naranjo *et al.* 2007;Ma *et al.* 2008;Wang *et al.* 2009;Oh *et al.* 2010;Huesa *et al.* 2010). In addition, the level of Ras in the brain is increased in the early stage of AD (Gärtner *et al.* 1999;Gärtner *et al.* 1995). Rho and Ras

proteins are prenylated by GGPP and FPP, respectively and AD may have a global effect on protein prenylation. Reducing protein prenylation by decreasing levels of FPP and GGPP and inhibiting prenyltransferases have been strategies used to counteract neuronal dysfunction in animal and cell models of AD (Cole and Vassar 2006;Li *et al.* 2012). However, it has been reported that administration of simvastatin which reduces FPP, GGPP and cholesterol stimulated long-term potentiation (LTP) in the CA1 region of the hippocampus in slices from wild-type C57BL/6 mice which was due to depletion of FPP and not GGPP (Mans *et al.* 2012). Geranylgeraniol (GGOH), the dephosphorylated derivative of GGPP rescued impairment of hippocampal long-term potentiation induced by statins and suppression of cholesterol 24-hydroxylase (Kotti *et al.* 2006;Kotti *et al.* 2008). Protein prenylation is required for synaptic plasticity (Tolias *et al.* 2011), and the long-term consequences of reducing prenylation in brain are not known. Effects of altering isoprenoid levels on brain function is not without controversy and requires much more investigation.

24S-hydroxycholesterol in AD

The cholesterol metabolite 24S-hydroxycholesterol (24S-OHC) is an oxysterol (Fig. 1) proposed to be a marker of brain cholesterol metabolism (Lütjohann and von Bergmann 2003). Cholesterol 24-hydroxylase catalyzes the synthesis of 24S-OHC and 24-hydroxylase has been identified in specific regions of neurons (endoplasmic reticulum, cell body, dendrites) and brain areas (e.g., hippocampal CA1 pyramidal cells, hippocampal and cerebellar interneurons) (Lund *et al.* 2003;Ramirez *et al.* 2008). There are reports that 24S-OHC levels are altered in CSF and plasma of AD patients as compared with control subjects (Papassotiropoulos *et al.* 2002;Popp *et al.* 2012;Popp *et al.* 2013). Significantly elevated 24S-OHC was observed in CSF of AD patients (approximately 2.1 ng/mL) in contrast to control subjects (approximately 1.3 ng/mL) (Papassotiropoulos *et al.* 2002). Plasma 24S-OHC levels were similar for the AD and control samples. Opposite results were found in another study looking at CSF and plasma 24S-OHC in AD and control samples (Popp *et al.* 2012). CSF levels were 2.99 ng/mL and 2.68 ng/mL for AD patients and controls respectively, which were not significantly different. Plasma 24S-OHC levels were significantly higher (78.82 ng/mL) for AD patients than control samples (62.28 ng/mL). Similar findings were reported recently showing that AD patients had higher levels of plasma 24S-OHC as compared with control subjects but that CSF 24S-OHC levels were comparable (Popp *et al.* 2013). However, significantly lower 24S-OHC plasma levels were found in AD patients as compared to patients with mild cognitive impairment and individuals with subjective cognitive complaints (Solomon *et al.* 2009b).

The data on 24S-OHC and AD are equivocal. Another concern is that reductions in serum and plasma 24S-OHC levels have been reported recently in patients with multiple sclerosis and Huntington disease (van de Kraats *et al.* 2013;Leoni *et al.* 2013). Changes in 24S-OHC levels may simply reflect neuronal dysfunction common to certain neurodegenerative diseases.

Animal and Cell Culture AD Models

What would appear to be the strongest evidence supporting cholesterol as a factor in AD comes from animal and cell culture studies. There have been several recent reviews covering *in vivo* and *in vitro* studies that examined perturbation of the mevalonate pathway and effects on amyloid precursor protein (APP) and Aβ (Eckert *et al.* 2010;Williamson and Sutherland 2011;Li *et al.* 2012;Posse de Chaves 2012;Gamba *et al.* 2012;Maulik *et al.* 2013). Generally, reducing cholesterol levels decrease Aβ abundance and are neuroprotective whereas increasing cholesterol levels have opposite effects. Applying those findings to what occurs in AD patients is challenging due to the multifaceted roles of cholesterol in cells. Reducing or increasing cholesterol can have numerous structural and functional effects in addition to effects on AD associated proteins. One study reported that reducing brain cholesterol synthesis by crossing cholesterol 24-hydroxylase knockout mice with an AD mouse model (B6.Cg-Tg(APPswe, PSEN1E9)85Dbo/J) had a slight effect on Aβ levels but increased lifespan of the AD mice (Halford and Russel 2009)Basic studies on cholesterol, membrane structure and function have been reviewed in detail previously (Schroeder *et al.* 2001;Wood *et al.* 2002;Vance *et al.* 2005;Wood *et al.* 2007;Lee 2011). Removing cholesterol increases fluidity of biological membranes. Cholesterol alters lipid packing, curvature and interdigitation of the membrane leaflets. Modifying cholesterol levels in SPM and synaptosomes altered sodium-dependent Ɣ-aminobutyric acid (GABA) (North and Fleischer 1983). Reducing cholesterol in membranes produced a loss in GABA uptake, and the uptake was restored by the addition of cholesterol. Activity of Ca^{2+} + Mg^{2+}-ATPase but not Na^{+},K^{+}-ATPase was reduced by oxidation of cholesterol (Wood *et al.* 1995). Increasing membrane cholesterol in erythrocytes reduced Na^{+},K^{+}-ATPase activity whereas opposite effects were observed when cholesterol was increased (Yeagle 1983). The conventional wisdom regarding an explanation for cholesterol-induced changes in protein function has been alterations in membranes properties such as fluidity, lipid packing, curvature and changes in interdigitation or overlapping of the membrane leaflets. However, it is becoming recognized that certain proteins may have specific cholesterol binding sites (Lee 2011). A cholesterol binding domain was identified in the nicotinic acetylcholine receptor (AChR) (Corbin *et al.* 1998). Recently, it was reported that cholesterol depletion accelerates the internalization of the AChR (Borroni and Barrantes 2011). A specific cholesterol binding site was shown for the human β_2-adrenergic receptor (Hanson *et al.* 2008).

The multifaceted effects of cholesterol pose a problem for explaining how either reducing or increasing cholesterol modifies APP and Aβ. A specific mechanism has not been forthcoming. Several years ago (Avdulov *et al.* 1997) we found that the binding affinity of cholesterol to $A\beta_{40}$ polymers (K_D of 3.24×10^{-9} *M*) was strikingly higher as compared with other lipids (phosphatidylcholine K_D of 7.07×10^{-7} *M*; stearic acid K_D of 9.42×10^{-8} *M*). Cholesterol also was reported to bind to Aβ at the α-secretase cleavage site (Yao and Papadopoulos 2002). Just recently, a cholesterol-binding domain was identified within an APP transmembrane domain (Barrett *et al.* 2012),and a cholesterol binding site was reported for the Aβ 22-35 amino acid fragment (Di Scala *et al.* 2013). Both Aβ and APP contain lipophilic domains, which would enhance cholesterol binding. Aβ 25-35 favors the

membrane hydrophobic interior (Mason *et al.* 1996) which would facilitate interaction. These novel findings provide a potential mechanism for APP, Aβ cholesterol interactions. One speculative prediction is that cholesterol binding to APP could provide an optimal physico-chemical environment for the activity of secretases and stimulate Aβ production. What conditions facilitate and inhibit binding need to be examined. The distribution for example of cholesterol in the two membrane leaflets could be altered which may impact cholesterol binding to APP/Aβ. Such changes in distribution can occur in the absence of changes in the total amount of brain cholesterol. Cholesterol in the SPM exofacial leaflet of mice expressing human apoE4 was two-fold greater as compared with the exofacial leaflet of apoE 3 mice but total SPM cholesterol did not differ between the two groups (Hayashi *et al.* 2002). The larger amount of cholesterol in the SPM exofacial leaflet of the apoE4 mice could enhance binding of Aβ to the membrane resulting in perturbation of the membrane with consequences on lipid rafts, ion transport, endocytosis and exocytosis which would be inimical to cells. We have reported that the SPM exofacial leaflet of 24-25 mo old mice contained twice as much cholesterol as compared with the exofacial leaflet of 3-4 mo old mice (Igbavboa *et al.* 1996). Lipid raft protein and lipid composition differ in SPM of apoE4 mice as compared with apoE3 mice and the apoE4 lipid raft composition was similar to aged wild-type mice (Igbavboa *et al.* 2005). Support for membrane cholesterol facilitating actions of Aβ was shown in an *in vitro* study of hippocampal neurons (Nicholson and Ferreira 2009). Mature neurons cultured for 21 days showed greater Aβ-induced toxicity and had higher membrane cholesterol content than neurons cultured for shorter time periods. Just recently it was shown in model membranes with cholesterol asymmetry of the two leaflets similar to that of aged and apoE4 SPM that the C-terminus of $A\beta_{42}$ resided in the exofacial leaflet and such repositioning may promote Aβ oligomerization or oxidative reactivity (Liguori *et al.* 2013). Taken together, the results of the cholesterol studies on hippocampal neurons (Nicholson and Ferreira 2009), leaflet model membranes (Liguori *et al.* 2013) and SPM leaflets (Igbavboa *et al.* 1996;Hayashi *et al.* 2002) suggest that neuronal membranes of aged individuals, and those with the apoE4 allele would be more susceptible to Aβ perturbation than either younger individuals or those carrying the apoE2 or apoE3 alleles as illustrated in the membrane leaflet model in Figure 2.

This section focused on cholesterol as a causative factor in the development/progression of AD particularly with respect to APP and Aβ. However, there is a smaller body of work indicating that Aβ impacts cholesterol homeostasis (Wood *et al.* 2002;Wood *et al.* 2003;Posse de Chaves 2012). $A\beta_{40}$ and $A\beta_{28}$ inhibited cholesterol esterification in plasma (Koudinov *et al.* 1996). Free and esterified cholesterol synthesis was reduced by $A\beta_{40}$ in HepG2 cells (Koudinova *et al.* 1996). Cholesterol esterification was inhibited by $A\beta_{40}$ in rat primary neurons (Liu *et al.* 1998). $A\beta_{42}$ inhibited protein prenylation and disrupted protease cleavage of sterol regulatory element-binding protein-2 (SREBP-2) (Mohamed *et al.* 2012). Cleaved SREBP-2 localizes to the nucleus where it stimulates transcription by binding to nonpalindromic sterol response elements (SRE) in the promoters of several genes including: *HMG-COA reductase*, *FPP synthase*, and *squalene synthase* (Horton *et al.* 2002). SREBP-2 is the primary transcription factor of the SREBP family for regulation of cholesterol but it was reported that SREBP-1, SREBP-2 and HMGCR mRNA levels were reduced as was cholesterol in rat cortical neurons expressing APP (Pierrot *et al.* 2013). The authors

concluded that APP may act to regulate cholesterol turnover. Cholesterol trafficking into and out of cells was altered by Aβ (Liu *et al.* 1998;Michikawa *et al.* 2001). Aβ stimulated cholesterol containing MTT formazan-transporting vesicles from B12 cells and increased cholesterol esterification in rat primary neurons (Liu *et al.* 1998). Oligomeric $A\beta_{40}$ but not monomers or fibrils induced release of cholesterol, phospholipids and GM1 from rat primary neurons and astrocytes (Michikawa *et al.* 2001). The Golgi complex plays an important role in cholesterol trafficking (Mendez 1995;Heino *et al.* 2000). We had the idea that Aβ might target cholesterol homeostasis in the Golgi complex (Igbavboa *et al.* 2003). $A\beta_{42}$ caused a redistribution (movement from the *cis* to the *trans* region) of cholesterol within the Golgi complex and also induced translocation of plasma membrane cholesterol to the Golgi complex in astrocytes. This Aβ-induced trafficking of cholesterol from the plasma membrane to the Golgi complex was due to retrograde movement of the cholesterol transport protein, caveolin-1 (Igbavboa *et al.* 2009).

Cholesterol transport proteins have attracted notice in the AD field. There has been for example growing interest in the role of the ABC family of proteins and their involvement in Aβ trafficking (Hirsch-Reinshagen and Wellington 2007;Wolf *et al.* 2012). Certainly, apoE has received the most attention of the cholesterol transport proteins in AD. The majority of those studies have focused on the apoE4 allele and risk of AD and functional studies of the apoE isoforms and Aβ dynamics; topics that have been covered previously in several excellent reviews (Kim *et al.* 2009;Holtzman *et al.* 2012;Liu *et al.* 2013). For the late onset sporadic AD (> 95% cases), the strongest genetic risk factor is one's genotype for apolipoprotein E (*APOE*). There are three common *APOE* alleles in humans: *APOE*-ε2, *APOE*-ε3, and *APOE*-ε4, with an allele frequency of 7, 78, and 15%, respectively (Strittmatter and Roses 1996). While the *APOE*-ε2 allele confers some protection against AD (Corder *et al.* 1994), the *APOE*-ε4 allele is associated with an increased risk of AD (Corder *et al.* 1993;Poirier *et al.* 1993). One *APOE*-ε4 allele increases the risk of AD by three times (heterozygotes) and two *APOE*-ε4 alleles increase the risk of AD by 15 times (homozygotes) (Farrer *et al.* 1997). However, the question of how the *APOE*-ε4 allele promotes AD has not been answered. The protein product of the *APOE* gene, apoE, is a 299-amino acid glycoprotein in humans. The three *APOE* alleles lead to the production of three isoforms of apoE with different amino acid residues at position 112 and 158: apoE2 (Cys112, Cys158), apoE3 (Cys112, Arg158), and apoE4 (Arg112, Arg158), resulting in differences in structure and function of apoE isoforms (Zhong and Weisgraber 2009). ApoE is expressed by several cell types but it is primarily expressed in the liver and in the brain (Mahley 1988). In the circulation, apoE is associated with different classes of lipoprotein particles. In the brain, apoE is predominantly produced by astrocytes and associated with high-density lipoprotein (HDL)-like particles. ApoE interacts with multiple lipoprotein receptors and plays an important role in cholesterol transport and lipid metabolism. ApoE4 is less efficient than the other apoE isoforms in recycling of membrane lipids and neuronal repair (Poirier 1994). Several other studies also indicate apoE isoforms function differently in brain cholesterol metabolism (Michikawa *et al.* 2000;Gong *et al.* 2002;Rapp *et al.* 2006), potentially due to the structural differences among apoE isoforms that determine their lipid- and receptor-binding properties. ApoE has been implicated as a chaperone that modulates Aβ aggregation and deposition or clearance (Verghese *et al.* 2011). Expression of human

Apo E2, E3 and E4 in mice leads to isoform-specific differences in amyloid load, with E4 > E3 > E2 (Holtzman *et al.* 2000). Further, the clearance of soluble Aβ in the brain interstitial fluid (ISF) has been shown to depend on the isoform of human apoE expressed (E4 < E3 ≤ E2) (Castellano *et al.* 2011). However, a recent study demonstrates that there is little direct interaction between soluble Aβ and apoE in the extracellular fluids and proposes that apoE isoforms affect Aβ metabolism by interfering with the interactions between Aβ and its receptors/transporters (Verghese *et al.* 2013). Thus, the puzzle how apoE4 increases the risk of AD has not been solved.

ApoE not only affects Aβ but Aβ acts on apoE regulation. Aβ increases cellular apoE protein abundance (Hu *et al.* 1998;Igbavboa *et al.* 2003;Igbavboa *et al.* 2006;LaDu *et al.* 2000;LaDu *et al.* 2001). The mechanism for that increase was not established until recently. $A\beta_{42}$ stimulated apoE mRNA and protein expression in astrocytes (Rossello *et al.* 2012). In the same study it was reported that upregulation of apoE by Aβ involved the β_2 -adrenergic receptor (β_2AR) and the transcription factor AP-2β. Aβ directly binds to the β_2AR (Wang *et al.* 2010). Cells expressing the AP-2 transcription factor showed $A\beta_{42}$-induced activation of a co-expressed luciferase reporter gene construct under the control of an apoE promoter fragment containing AP-2 binding sites in contrast to cells not expressing AP-2 (Rossello *et al.* 2012).

The functional significance of the Aβ-stimulated increase in apoE and retrograde transport of cholesterol by caveolin-1 is not known. A model is proposed which is summarized in Figure 3. Aβ increases apoE synthesis via the β_2AR. Newly synthesized apoE in astrocytes migrates through the Golgi complex (Dekroon and Armati 2001). A functional effect of this increase in apoE abundance is that more apoE containing cholesterol is removed from the *cis* region and transported to the *trans* region of the Golgi as illustrated in Figure 3, panel A. We know that Aβ reduces cholesterol in the *cis* region and increases it in the *trans* region (Igbavboa *et al.* 2003). Consequences of this increase in cholesterol in the *trans* region could have a negative impact on Golgi mediated protein and lipid trafficking. Cholesterol loading of the Golgi impedes vesicular transport from the trans Golgi network (Ying *et al.* 2003). A portion of the increased cholesterol abundance in the *trans* region is accounted for by Aβ stimulating caveolin-1 transport of plasma membrane cholesterol (Fig. 3, panel B) (Igbavboa *et al.* 2009). Aβ disruption of caveolae may alter normal function of this membrane structure which is thought to be involved in signaling, lipid storage, endocytosis and remodeling of the extracellular environment (Parton and del Pozo 2013). We are proposing that Aβ stimulation of apoE synthesis is harmful to cell function. However, both apoE and β-adrenergic receptors have been reported to facilitate neuroprotection afforded by astrocytes (Junker *et al.* 2002;Laureys *et al.* 2010;Rebeck *et al.* 2002). ApoE mimetics are neuroprotective in models of AD and neuronal injury (Christensen *et al.* 2011;Li *et al.* 2010;Wang *et al.* 2007). β-adrenergic receptor agonists increase apoE protein expression (Cedazo-Mínguez *et al.* 2001;Igbavboa *et al.* 2006). One possibility is that the Aβ stimulation of apoE synthesis in astrocytes may have pathological consequences on Golgi structure and function but have protective effects when secreted by astrocytes and taken up by neuronal receptors of the low density lipoprotein receptor family.

Summary

When Michael Brown and Joseph Goldstein were awarded the Nobel Prize in Physiology or Medicine for their work on regulation of cholesterol they said in their Nobel lecture that "cholesterol has exerted an almost hypnotic fascination for scientists from the most diverse areas of science and medicine" (Brown and Goldstein 1985). That statement resonates with those of us studying cholesterol in the central nervous system and those who have focused on cholesterol in AD. Cholesterol dysregulation has been proposed as a factor in several divergent diseases such as suicide and depression (Zhang 2011), inborn errors of cholesterol metabolism (Herman and Kratz 2012), Huntington disease (Karasinska and Hayden 2011), and Parkinson disease (de Lau *et al.* 2006). The pioneering studies of Larry Sparks and his colleagues on cholesterol and AD initiated a field of research that continues today. An intriguing characteristic of the cholesterol-AD interaction is that several different properties/functions of cholesterol are associated with AD and in particular Aβ, which raises several major questions: 1) is cholesterol a causative factor in AD; 2) the incongruity of findings between human and animal/cell culture studies; 3) are other lipids in the mevalonate pathway linked to AD; and 4) does AD impact cholesterol homeostasis? The purpose of this review was to examine those questions.

Table 1 summarizes the pros and cons for the hypothesis that bulk changes in cholesterol levels are either causative factors or biomarkers of AD. That hypothesis is not supported by the data. There is large variability in the human data on cholesterol levels whether in serum, plasma and brain which argues against cholesterol as a causative factor in AD. An alternative hypothesis might be that brain cholesterol domains versus total or bulk cholesterol levels may be contributing factors in AD. To date, there is little support for that hypothesis in human brain tissue of AD patients, and studies on cholesterol domains in human brain are needed. Cholesterol is a member of the large mevalonate family, and two members of that family, FPP and GGPP, through their involvement in protein prenylation have been proposed to play roles in AD. Data on FPP and GGPP levels in human AD brain samples were reported in one study, which found that both lipids were significantly elevated as compared with control subjects. More studies are needed to confirm those results. Another product of the mevalonate pathway is the oxysterol 24S-OHC which some have proposed as a marker of CNS metabolism in AD patients. A lack of consistency however in studies on plasma and CSF levels of 24S-OHC in AD patients calls in to question the importance of that lipid in AD. Equally important is that changes in 24S-OHC have been reported in samples of patients with other neurodegenerative diseases suggesting that changes in 24S-OHC may be a non-specific marker of neuronal dysfunction and/or cell death. That interpretation may also apply to FPP and GGPP.

Though the human data pose problems for the cholesterol-AD hypothesis, the animal and cell culture studies provide the strongest support. However, interpretation of those studies must be viewed within the context of the numerous effects of cholesterol on several different proteins besides APP and Aβ. Specific mechanisms have not been identified pertaining to how manipulation of cholesterol levels modifies APP and Aβ. Lipid rafts have been proposed as a potential mechanism but a problem with that interpretation is that multiple proteins have been identified in membrane lipid raft fractions and methods to perturb lipid

rafts have non-specific effects. Aβ directly interacts with cholesterol and what remains to be established is whether such an interaction is a factor in the development of AD or a consequence of the disease. With respect to consequences, much of the support for Aβ effects on cholesterol structure and function is from *in vitro* studies in different cell types including primary neurons and astrocytes. It remains to be determined and a challenge to establish if brain cholesterol structure and functions such as membrane asymmetry, cholesterol trafficking, and sterol binding to proteins are altered in AD patients. The role of cholesterol may not be a causative factor in AD but instead a casualty of the disease, which may be a syndrome common to certain neurodegenerative diseases.

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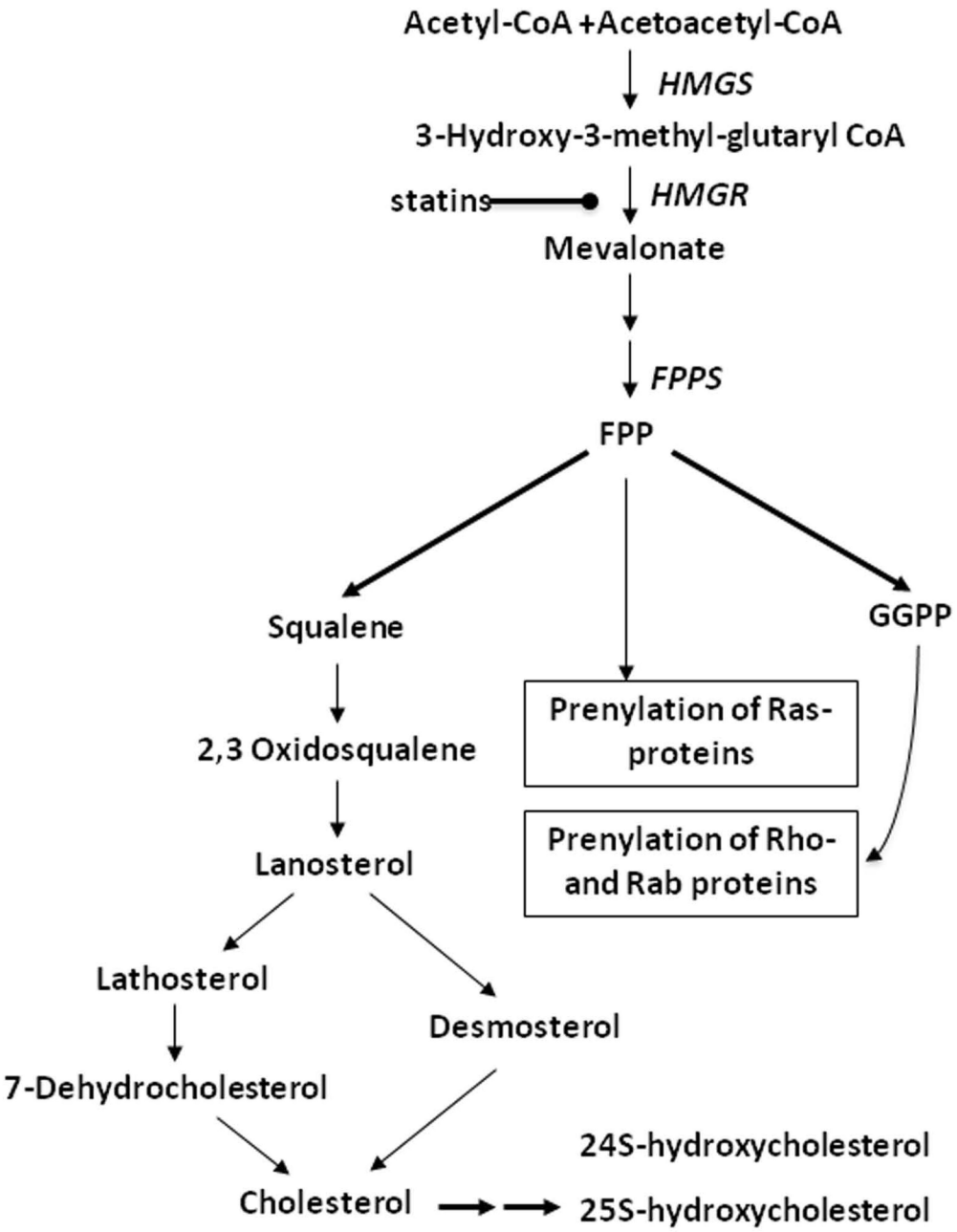

Figure 1. Mevalonate/Isoprenoid/Cholesterol synthesis pathway

Acetyl-CoA and acetoacetyl-CoA catalyzed by 3-hydroxy-3-methylglutaryl CoA synthase (HMGS) are converted to 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA), which is then converted to mevalonate through the action of HMG-CoA reductase (HMGR) and the cofactor NADPH. This reaction is the rate-limiting step for cholesterol synthesis, and statins are a substrate for HMG-CoA reductase. Through a series of reactions, mevalonate is converted to farnesyl pyrophosphate (FPP) by farnesyl pyrophosphate synthase (FPPS). FPP is a key branch point for synthesis of geranylgeranyl pyrophosphate (GGPP), cholesterol, ubiquinone, and dolichol. Both FPP and GGPP are required for prenylation of GTP-binding proteins such as Rho, Rac, and Ras, enabling those proteins to be inserted in membranes. FPP is also the precursor of squalene. Conversion of squalene to cholesterol requires over 19 reactions. Cholesterol is subsequently converted to oxysterols such as 24S-hydroxycholesterol and 25S-hydroxycholesterol.

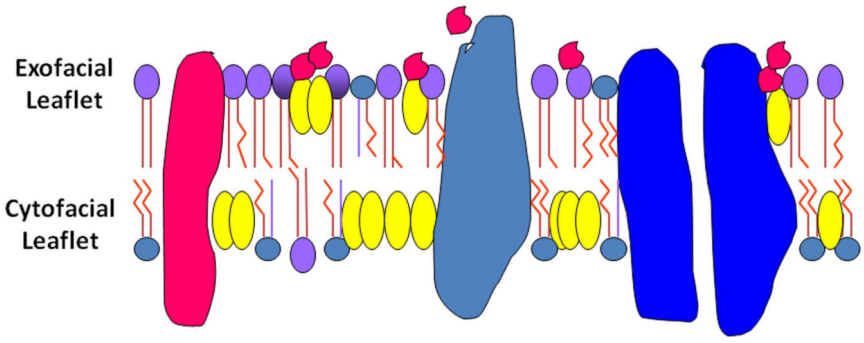

Figure 2. Model of Aβ acting on the synaptic plasma membrane exofacial leaflet

Cholesterol is asymmetrically distributed in synaptic plasma membranes (SPM) (Wood *et al.* 2011). It is predicted that conditions where the SPM exofacial leaflet has been shown to have an abnormal enrichment of cholesterol (apoE4 and aged membranes) (Hayashi *et al.* 2002;Igbavboa *et al.* 1996) those membranes would be more susceptible to Aβ (red structures) perturbation than either younger individuals or those carrying the apoE2 or apoE3 alleles. Modeling of cholesterol distribution in the two leaflets similar to aged and apoE4 SPM found that the C-terminus of $A\beta_{42}$ resided in the exofacial leaflet and such repositioning may promote Aβ oligomerization or oxidative reactivity (Liguori *et al.* 2013). Phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), sphingomyelin (SM); PC; PE, PS, PI; SM; cholesterol Globular structures represent proteins.

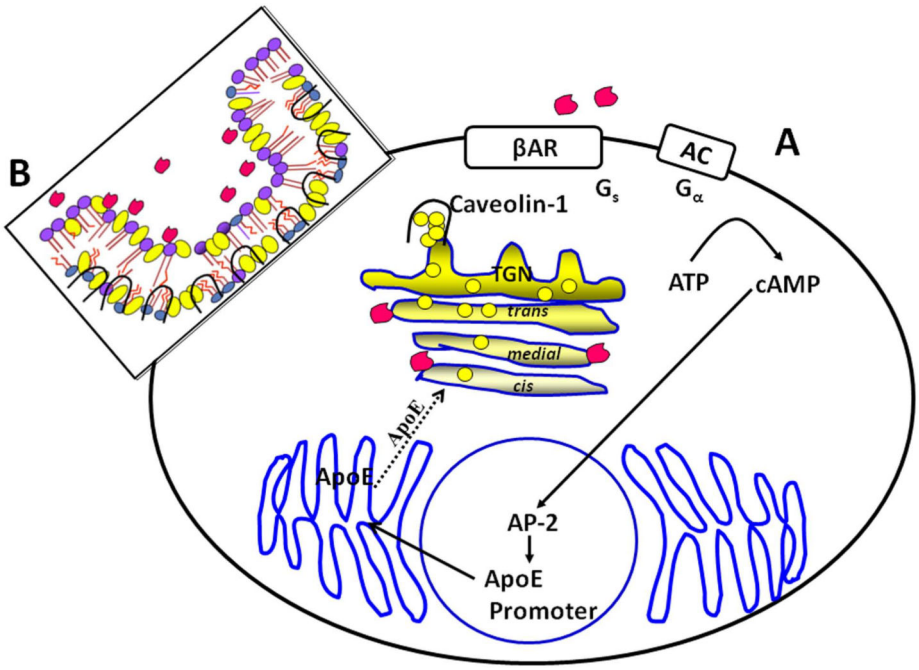

Figure 3. Model of Aβ perturbation of cholesterol homeostasis in astrocytes

Panel A shows amyloid β-protein (Aβ) (red structures) induces stimulation of apoE synthesis via the β_2 -adrenergic receptor (βAR), cAMP and the transcription factor AP-2 (Igbavboa *et al.* 2003;Igbavboa *et al.* 2006;Rossello *et al.* 2012). Newly synthesized apoE is proposed to move to the Golgi and translocate cholesterol from the *cis* to the *trans* region. Panel B is a model of Aβ acting on caveolae resulting in translocation of caveolin-1 complexed with cholesterol to the *trans*-Golgi region depicted in panel A (Igbavboa *et al.* 2009;Igbavboa *et al.* 2003). Phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), sphingomyelin (SM);

Table 1

Summary of Pros and Cons of the Hypothesis that Cholesterol is a Causative Factor in Alzheimer Disease

ENDPOINT	PROS	CONS
Serum/plasma cholesterol levels	Some studies report ↑in AD pts compared with controls.	Some studies report no differences or ↓ In studies reporting ↑, small differences between groups.
Brain/CSF cholesterol levels	Some studies report ↑in AD pts compared with controls.	Some studies report no differences or ↓in AD pts.
Brain FPP and GGPP levels	FPP and GGPP levels ↑ in AD pts compared with controls.	First study on FPP and GGPP levels in AD patients and needs to be replicated. Address question if changes are common to other neurodegenerative diseases.
Serum/CSF 24S-OHC levels	Some studies report ↑ in AD pts compared with controls.	Some studies report no differences, or ↓in AD pts. In studies reporting ↑,small differences between groups. Changes observed in other diseases.
Statins reduce AD	Retrospective epidemiological studies	Majority of prospective studies do not support statin efficacy in AD.
Animal/cell culture studies	Reducing cholesterol decreases Aβ levels, opposite effects when cholesterol is increased.	Changing cholesterol levels affects multiple proteins besides APP/Aβ.