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Review

Cholesterol changes in Alzheimer's disease: methods of analysis and impact on the formation of enlarged endosomes

Jack-Christophe Cossec, Catherine Marquer, Mai Panchal, Adina N. Lazar, Charles Duyckaerts, Marie-Claude Potier *

Centre de Recherche de l'Institut du Cerveau et de la Moelle, CNRS UMR7225, INSERM UMR975 and UPMC, Hôpital de la Pitié-Salpêtrière 47, Bd de l'Hôpital 75013 Paris, France

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ABSTRACT

An increasing number of results implicating cholesterol metabolism in the pathophysiology of Alzheimer's disease (AD) suggest cholesterol as a target for treatment. Research in genetics, pathology, epidemiology, biochemistry, and cell biology, as well as in animal models, suggests that cholesterol, its transporter in the brain, apolipoprotein E, amyloid precursor protein, and amyloid-β all interact in AD pathogenesis. Surprisingly, key questions remain unanswered due to the lack of sensitive and specific methods for assessing cholesterol levels in the brain at subcellular resolution. The aims of this review are not only to discuss the various methods for measuring cholesterol and its metabolites and to catalog results obtained from AD patients but also to discuss some new data linking high plasma membrane cholesterol with modifications of the endocytic compartments. These studies are particularly relevant to AD pathology, since enlarged endosomes are believed to be the first morphological change observed in AD brains, in both sporadic cases and Down syndrome.

Several lines of evidence support a strong relationship between cholesterol and the development of Alzheimer's disease (AD). Indeed, a number of reviews on cholesterol and its metabolism in the brain have been published [1–4].

1. Genetic associations of cholesterol-related genes in AD

One of the most interesting pieces of evidence for this link is indirect and involves apolipoprotein E (ApoE), a transporter of lipids and, particularly, of cholesterol. In humans, the *APOE* gene exists as three different polymorphic alleles (ε2, ε3, and ε4). ε3 is the most frequent (0.77) allele, while ε2 is the least frequent (0.08). The ε4 allele frequency is 0.15 in the general population, but 0.4 in patients with AD [5]. The risk associated with developing AD with one ε4 allele [6] is, by some orders of magnitude, the most significant in AD (three to four times).

The *APOE* gene encodes a lipid-binding protein and is critical for cholesterol transport in the central nervous system. Therefore, researchers hypothesized that the disturbance of cholesterol transport was the cause of increased AD risk in *APOE*ε4 allele carriers. More recently, *APOJ* (or clusterin), another major brain apolipoprotein, has been suspected, with two genome-wide association studies linking some isoforms of this protein with an 8.9% increased risk for AD [7,8]. Despite its role as a cholesterol transporter in the brain, ApoE has been shown to promote the proteolytic degradation of amyloid-β (Aβ) within microglia [9]. This effect depends on the level of lipidation of *ApoE*, which is regulated by liver X receptors. Further, cholesterol metabolism is regulated by APP and ApoE [10,11]. Cholesterol is increased in the periphery, but not in the brains, of ApoE4 knock-in mice [12,13]. However, in the exofacial leaflet of synaptic plasma membranes, cholesterol is higher in ApoE4 knock-in mice [12,14]. These findings suggest that APP, Aβ, ApoE, and cholesterol interact and compete with one another, for better or worse. Recently, studies have even suggested that APP may function as a cholesterol sensor and Aβ may also regulate cholesterol metabolism [4,15].

Additional genetic studies have shown associations between AD and polymorphisms in the genes encoding cholesterol 25-hydroxylase and CYP46, two cholesterol metabolizing enzymes, ABCA1, a cholesterol transporter, and LRP1, a lipoprotein receptor [16]. Meta-analysis of genetic data revealed that *SOAT1*, the gene encoding ACAT1, could also be associated with the risk of AD [17]. A more detailed explanation of ACAT function is provided later in this review. Additionally, a common polymorphism of *SOAT1* is associated with a lower prevalence of AD in ethnically distinct populations. On the molecular scale, patients carrying this protective genotype display both low brain amyloid load and low CSF levels of cholesterol [18].

* Corresponding author. Tel.: +33 142162141; fax: +33 142162146.
E-mail address: marie-claude.potier@upmc.fr (M.-C. Potier).

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2. Methods for measuring cholesterol in the brain of AD patients

Initial cholesterol staining techniques on tissues were based on chemical reactions such as the Schultz reaction, Lieberman-Burchard reaction, or reaction with fluoric–chloride–sulphuric acid and perchloric acid. Current colorimetric and fluorescent techniques permit the visualization of cholesterol in tissues. Filipin is the most widely used fluorescent probe for visualizing cholesterol accumulation because it binds with high affinity to the 3β-hydroxyl group of cholesterol [19]. Filipin staining of AD brains shows a high level of diffusible cholesterol in tangle-bearing cells, with respect to adjacent cells [20]. An accumulation of cholesterol has also been demonstrated in mature senile plaques, but not in diffuse or immature plaques, using filipin and cholesterol oxidase [21,22]. The cholesterol oxidase method relies on the production of hydrogen peroxide by oxidation of cholesterol in the presence of horseradish peroxidase, resulting in oxidation of 10-acetyl-3,7-dihydroxyphenoxazine (AmplexRed) into the highly fluorescent compound resorufin. However, these methods are limited by the rapid bleaching of filipin under confocal microscopy and the lack of specificity of both fillipin and cholesterol oxidase staining [23].

The use of fluorescent sterols or fluorescent cholesterol analogues has potential for real-time imaging. Fluorescent sterols, such as dehydroergosterol and BODIPY–cholesterol, mimic cholesterol and have been used to localize cholesterol in membranes [24]. Using these probes, Marks et al. demonstrated that cholesterol distribution in the exofacial leaflet of synaptic plasma membranes is strongly increased in ApoE- and low-density lipoprotein (LDL) receptor-deficient mice, which also exhibit a decrease in cholesterol-to-phospholipid ratio [25]. This finding indicates that ApoE and LDL receptors are important for maintaining the transbilayer distribution of cholesterol and controlling the amount of phospholipids. Other studies on mouse brain tissue using these probes demonstrated that statins reduce cholesterol levels and alter cholesterol distribution in the transbilayer. This effect highlights the possible mechanism by which statins shift APP metabolism towards a non-amyloidogenic pathway [26]. Importantly, levels of total cholesterol have been shown to be increased (between 20% and 40% depending on the method used) in the brains of AD patients (Table 1) [27].

Another method for cholesterol visualization includes BCθ, a biotinylated derivative of the protease-nicked θ-toxin originating from *Clostridium perfringens*, which binds to cholesterol with high affinity in cells and tissues. Subsequent detection with FITC–avidin allows cholesterol to be monitored with confocal microscopy or flow cytometry. This method was used to demonstrate the accumulation of cholesterol in neurons of individuals with Niemann-Pick type C disease, a rare neurodegenerative disorder [28], though it has yet to be applied in AD pathology.

The field is also being advanced by the development of improved analytical methods for lipids. For example, liquid chromatography separation of all lipids, combined with tandem mass spectrometry (MS/MS), has been used on postmortem tissues. Using MS/MS, Cutler et al. observed alterations in cholesterol metabolism during normal brain aging and in the brains of AD patients (Table 1) [29]. Further, by using ultracentrifugation to isolate crude membrane fractions from cortex grey matter, they showed that cholesterol gradually increases during progression of the disease. In a separate study, cholesterol concentrations were found to range from 15 to 30 μg/mg, depending on brain areas. Interestingly, 24*S*-hydroxycholesterol (24S-OH) and 27-hydroxycholesterol (27S-OH), which are able to cross the blood–brain barrier, were also analyzed: 24S-OH decreases and 27S-OH increases in AD brains (Table 1) [30]. More recently, total cholesterol, its oxidized forms (oxysterols), and cholesterol precursors were analyzed on lipids extract from AD cortex by gas chromatography (GC)-MS. Decreased cholesterol and increased oxysterol and cholesterol precursors concentrations correlated with heme oxygenase-1 levels, a protein that is up-regulated in AD-affected neuronal tissues, but not with cognitive impairment. Age and oxysterol levels correlated, but the correlation between age and total cholesterol levels was not significant (Table 1) [31].

3. Cholesterol in senile plaques

We quantified cholesterol in senile plaques from AD brains using laser-capture microdissection followed by LC-MS analysis and found an enrichment of free cholesterol in the senile plaque compared to the adjacent neuropil [32]. Senile plaques and the adjoining Aβ-free neuropil were microdissected from the temporal cortex after Aβ immunohistochemistry. The extracted cholesterol content was analyzed and quantified by LC-MS using a C^{13}-labeled cholesterol standard. The concentration of free cholesterol in the senile plaques was approximately twice that in the neuropil. Further, the quantity of free cholesterol per senile plaque was similar to the previously published quantity of Aβ peptide, suggesting a molar interaction between cholesterol and Aβ [33].

4. Emerging methods for high-resolution cholesterol assay in AD brains

All data presented above were obtained from AD brain homogenates or microdissected senile plaques. New methodologies need to be developed for exploring the subcellular localization and quantification of cholesterol. Time-of-flight secondary ion mass spectrometry (ToF-SIMS) imaging is a recently improved analytical method (for review, see Ref. [34]) that investigates spatial distribution of a wide range of molecules over the surface of a biological sample. ToF-SIMS is able to localize molecules, particularly lipids, on a tissue section up to m/z 1000–1500 with a resolution of 1 μm. This method has the major advantage of allowing direct and simultaneous collection of mass spectra and ion images. The analysis of individual lipid localization on the section is made under conditions that are physiologically relevant, since no treatment of the tissue sample is needed. Cholesterol and various lipids have been observed by ToF-SIMS in human striated muscle from control and Duchenne muscular dystrophy samples [35]

Table 1
Methods for measuring cholesterol in AD brains.

Methodology	Material	Cholesterol Evaluation	References
Filipin	AD brain tissue sections (occipital cortex)	Cholesterol was detected in or around β-amyloid deposits	[27]
Amplex Red	AD and control brain tissue (occipital cortex)	Cholesterol content (free cholesterol and cholesteryl esters) was significantly higher in AD brains (9.43 ± 1.41 μg/mg protein) than in non-demented brains (7.9 ± 1.11 μg/mg protein)	[27]
LC-MS	AD and control brain tissue (frontal cortex, occipital cortex, basal ganglia, pons)	Cholesterol concentration was significantly increased in the AD basal ganglia (15 μg/mg protein). No significant differences between AD and control samples for other brain areas.	[30]
	Microdissected senile plaques and neuropil from AD brain sections (temporal cortex)	Free cholesterol content was statistically increased in senile plaques versus neuropil (average of 67 ± 16 fmol of free cholesterol per plaque).	[32]
ESI-MS/MS	AD and control brain tissue (middle frontal gyrus and cerebellum)	Accumulation of free cholesterol in the middle frontal gyrus of AD patients. No change of cholesterol level in cerebellum.	[29]
GC-MS	AD brain tissue (frontal cortex)	Decrease of total cholesterol does not correlate with cognitive impairment.	[31]