Neutral Lipid Determination in Peripheral Blood Mononuclear Cells: A Useful Tool for Diagnostic and Therapeutic Interventions in Dementia

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Abstract

The objective of this review was to focus on recent studies indicating how deregulation of lipid metabolism may be of particular importance for central nervous system (CNS) injuries and neurodegenerative disorders. Furthermore, since an accumulation of neutral lipids (NLs), mainly cholesterol esters (CEs) in the form of cytoplasmic lipid droplets was previously found by our group in peripheral blood mononuclear cells (PBMCs) of Alzheimer (AD) patients and their first degree relatives (AD-FDR), we reviewed current data providing evidence that altered lipid metabolism in brain can also affect cholesterol metabolism in the systemic circulation. Using data from literature we proposed a mechanistic model that helps us to explain why subjects with neurological disorders often accumulate NLs in their PBMCs. If validated by future research, it should provide a rationale for NL-PBMCs determination by Oil Red O (ORO) staining method as a useful tool for diagnostic and therapeutic interventions in AD and possibly in other forms of dementia occurring in childhood as well as in elderly.

Keywords: Brain; Dementia; Alzheimer; Autism; Cholesterol homeostasis; Cholesterol esterification

Abbreviations: ACAT: acyl-coenzyme A (CoA):cholesterol acyltransferases; AD: Alzheimer Disease; AD-FDR: AD First Degree Relatives; APP: Amyloid Precursor Protein; ADHD: Attention Deficit Hyperactivity Disorders; ASD: Autistic Spectrum Disorders; BBB: Blood-Brain Barrier; CNS: Central Nervous System; CSF: Cerebrospinal Fluid; CEs: Cholesterol Esters; FC: Free Cholesterol; LCAT: Lecithin-Cholesterol Acyltransferase; 24S-OHC: 24(S)-hydroxycholesterol; NLs: Neutral Lipids; ORO: Oil Red O; PBMCs: Peripheral Blood Mononuclear Cells

Background

The term dementia in its broadest sense refers to a group of different conditions and diseases that share some similar neuropsychological and behavioral abnormalities pertaining to the human brain wherein cumulative pathological insults produce progressive loss of memory or cognitive functions further complicated by non-cognitive symptoms leading to an explosion in the number of scientific papers being published since an accumulation of neutral lipids (NLs), mainly cholesterol esters (CEs) in the form of cytoplasmic lipid droplets was previously found by our group in peripheral blood mononuclear cells (PBMCs) of Alzheimer (AD) patients and their first degree relatives (AD-FDR), we reviewed current data providing evidence that altered lipid metabolism in brain can also affect cholesterol metabolism in the systemic circulation. Using data from literature we proposed a mechanistic model that helps us to explain why subjects with neurological disorders often accumulate NLs in their PBMCs. If validated by future research, it should provide a rationale for NL-PBMCs determination by Oil Red O (ORO) staining method as a useful tool for diagnostic and therapeutic interventions in AD and possibly in other forms of dementia occurring in childhood as well as in elderly.

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The brain contains more cholesterol than any other organ, where it comprises roughly a quarter of the total FC in the human body [7]. CEs are much less polar than FC and do not contribute to membranes but are packed into lipid particles [1,8]. In the blood compartment, lecithin-cholesterol acyltransferase (LCAT, also called phosphatidylcholine-sterol O-acyltransferase), acting on surface area of lipoprotein particles, converts FC into CEs [9]. CEs are then sequestered into the core of lipoproteins thus generating the main forms by which cholesterol is transported through the body. In tissues, the membrane-bound enzymes acyl-coenzyme A (CoA):cholesterol acyltransferases (ACAT) also known as sterol O-acyltransferase (SOAT) utilizes long-chain fatty acyl-CoA and cholesterol originates from in situ neo-synthesis or

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circuiting lipoprotein particles as substrates to form CEs [8]. With the exception of intestinal epithelial cells and hepatocytes in which CEs serve as the cholesterol reservoir for producing chylomicrons and very low-density lipoproteins (VLDL), respectively, and steroidogenic tissues, such as adrenals, for producing steroid hormones, only trace of CEs are present in adult tissues, including brain [8]. A significant greater portion (up to 5%) of total cholesterol is found to be esterified in developing tissues [10]. Indeed, in pathologic tissues, typically, atherosclerotic plaques, a large amount of CEs are sequestered as neutral lipid droplets within the cytoplasm of the cells [11]. Although, a substantial number of studies, reviews, and opinions in the medical literature have assessed the involvement of cholesterol and other lipid moieties in the risk for and progression of dementia related disorders, the role of FC and of lipoprotein metabolism in the pathogenesis of dementia is still under intense debate. More recently a number of researches pointed to the intracellular esterification process as relevant target for dementia [12-18]. Pronounced accumulation of CEs has been associated with brain dysfunctions; children with inherited lipid storage diseases, in which neutral lipids (NLs) accumulate in various cells and tissues, develop neurologic disturbances including dementia [19]. The block of cholesterol esterification by ACAT1 inhibitors has been shown to improve cognitive function and brain efficiency in an AD-mouse model [13]. Inhibition of ACAT1 may induce a series of changes in lipid metabolism, any one of which could potentially compromise cognitive functions of the human brain; therefore, the challenge now is to understand the possible mechanisms by which changes in cholesterol esterification pathways may facilitate cognitive disorders. In the current review, we focus on recent reports indicating how deregulated lipid metabolism may be of particular importance for CNS injuries and neurodegenerative disorders. Furthermore, since an accumulation of NLs, mainly CEs in the form of cytoplasmic lipid droplets was previously found by our group in peripheral blood mononuclear cells (PBMCs) of AD patients and their first degree relatives (AD-FDR) [18] we also review current data providing evidence that altered lipid metabolism in brain can affect cholesterol metabolism in the systemic circulation.

Roles of Cholesterol in the CNS

Cholesterol is required for brain growth and myelination of axons in the developing brain and for continued axon growth and synapse remodeling in the mature brain [20]. It is perhaps for this reason the human CNS contains over 25% of the total body cholesterol while accounting for only 2% of the total body mass. Most of this cholesterol (about 70%) in FC form is in myelin membranes. Studies on mutant mice that lack cholesterol synthesis clearly showed that cholesterol incorporation is an essential and rate-limiting factor for myelin membrane growth and thus it must be constantly replaced by active turnover among neuronal and glial cells [21]. A demyelinated axon, as occurs following neuronal injury, has two possible fates: 1) functional recovery (remyelination) with the support of new oligodendrocyte generation; 2) progressive decline with degeneration and death [22].

Another important role of cholesterol in the CNS is in the synapses: it has been recently shown that, the fusion of synaptic vesicles with the neuron plasma membrane, a crucial step in the release of neurotransmitter, flops, if cholesterol levels in neurons are below the normal limits (Figure 1) [23]. Therefore, changes into one or more of the integrated sets of homeostatic mechanisms that finely regulate FC, by altering myelination and synapses could adversely affect the normal brain functions including cognition, emotion, and memory.

In this context it is also important to mention the role of cholesterol in the synthesis of neurosteroids. These compounds act as allosteric modulators of neurotransmitter receptors and are synthesized especially in hippocampal neurons, which are involved in learning and memory processes, from cholesterol or steroidal precursors [24-26]. They include 3β-hydroxy-A5 derivatives, such as pregnenolone and dehydroepiandrosterone, their sulfates, and reduced metabolites such as the tetrahydro derivative of progesterone 3a-hydroxy-5a-pregnane-20-one. Last but not least, cholesterol in the CNS has an important role in the construction of special parts of neuron membranes called lipid rafts. Lipid rafts are made up of high amounts of FC and special kinds of lipids called sphingolipids. These rafts allow some sections of the membrane to be distinct from other areas. Some lipid rafts are needed in order to export proteins out of the cell, others are used to anchor specific proteins in the membrane and keep protein clusters together [27]. Reduction of cellular cholesterol leads to disruption of raft functions [28]. It has repeatedly shown that trafficking and proteolytic processing of amyloid precursor protein (APP) by β- and γ-secretases occur and critically dependent on the integrity of lipid rafts [29]. APP, β-secretase and γ-secretase complex, are present in the lipid rafts. A leading role of APP in the pathogenesis of AD is well-established; however, the physiological functions of APP and its proteolytic fragments in the CNS are still poorly understood. APP is a member of an evolutionary highly conserved gene family that includes in mammals the APP-like proteins APLP1 and APLP2 [30-32]. These proteins exhibit a high degree of sequence homology and are proteolytically processed by the same set of enzymes (α, β and γ secretases). It has been shown that APLP2 and APP are synergistically required to mediate neuromuscular transmission, spatial learning and synapse formation and function, plasticity, learning and memory [33]. Altogether, these data suggest a possible connection between low cholesterol levels in neuronal raft microdomains and memory loss and favor the view of high brain cholesterol as essential for memory, learning and other mental functions.

Brain Cholesterol Homeostasis

Brain does not have direct access to cholesterol carried by the plasma lipoproteins and synthesizes most of its own cholesterol in glial cells with only a small amount of cholesterol synthesized in neurons. In glial cells neo-synthesized cholesterol is package into apoprotein E (ApoE)–containing lipoprotein particles, and secreted into the cerebrospinal fluid (CSF) through the ATP-binding cassette
transporter 1 (ABCA1). ApoE-containing lipoproteins are then taken up by neurons, delivered to a lysosome and degraded; FC released is then utilized for the above-mentioned functions (Figure 1) [20,34-36]. The cells of the CNS keep constant their cholesterol concentrations through the same homeostatic mechanisms regulating the intracellular cholesterol metabolism in peripheral tissues: cholesterol is synthesized in the ER from acetyl-CoA by the mevalonic acid pathway, the rate limiting enzyme being 3-hydroxy-3-methyl-glutaryl coenzyme A reductase (HMG-CoAR). In adults, however, the rate of synthesis exceeds the need for new structural sterol, so despite the efficiency of the cholesterol recycling machinery in the brain, to maintain steady state, there is a persistent necessity to export the excess of cholesterol into the circulation [34]. Since cholesterol cannot pass the blood brain barrier (BBB), during normal turnover the excess of cholesterol is catabolized by neurons largely in the form of its polar metabolite 24(S)-hydroxycholesterol (24S-OHC) [37].

Cholesterol in CNS Disorders

The following is a list and a brief description of the most common neurological diseases that have received a significant amount of media attention in recent years to be characterized by aberrations in brain cholesterol homeostasis.

Alzheimer’s disease (AD)

Among the various neurodegenerative disorders in which alterations of brain cholesterol homeostasis have been described, AD is undoubtedly the most popular. The major advances made in understanding its pathogenesis have also served as a model for studying other neurological disorders characterized by dementia. AD is a severe disorder characterized by loss of memory and cognitive decline that at a cellular level, exhibits several histopathological markers including beta-amyloid (Aβ) plaques, formed after sequential cleavage by β (BACE1) and γ secretases of the APP, neurofibrillary tangles within neurons, and the loss of synaptic connections manifested as brain atrophy [38]. The prevalence of AD is expected to rise dramatically, therefore, in the last few years extensive research has been done to identify reliable surrogate markers to diagnose and monitor the progression of this devastating disease. Unfortunately, the development of these biomarkers was limited not only by the individuality of brain function and the heterogeneity of the clinical symptoms, but especially by the inability to obtain neural cells from the brains of living patients. The first evidence for a role of cholesterol in AD pathogenesis was the finding that sporadic AD was significantly associated with the ε4 allele [39]. Afterwards, cholesterol levels has been found to be higher in the brains of patients with AD than in brains of age-matched control subjects [40] and in vitro studies indicated that the level of cholesterol could affect the localization of APP and its cleavage enzymes at the plasma membrane [41]. Increased brain cholesterol levels have also been found to stimulate the activity of the BACE1 and consequently the amount of Aβ peptides [42]. More recently, it has been demonstrated that cholesterol loading increases Aβ production by altering the accessibility of BACE1 to its substrate, APP [43]. Other reports, however, suggested that Aβ peptides inhibit the HMG-CoAR activity [44], and therefore reduce the levels of FC in the brain. In addition, in CHO cells and various neuron-like cells grown in culture, the decrease of CEs either by genetic inactivation of ACAT1 or by pharmacological inhibition of this enzyme, was associated with a decrease of Aβ secretion and substantially with a reduction of amyloid plaque density [12-15]. Based on these studies, it has been suggested that the ratio between FC and total cholesterol (TC) is a primary regulator of the APP processing, and that ACAT1 may be considered as a drug target for therapeutic intervention against certain form(s) of AD [12-15]. In this regard, the ablation of ACAT1 in AD-mice was able to reduce more than 60% the full-length APP protein as well as its proteolytic fragments, and to ameliorate cognitive deficits [15]. Because of the extremely high ratio between FC and CE in the adult human brain, at the present it is difficult to understand how ACAT modulates APP processing in vivo, however, the aforementioned studies address altered cholesterol homeostasis as a central component of neurodegenerative cascades, able to influence multiple aspects of AD process. Unfortunately, despite the large body of evidence that cholesterol is implicated in the pathogenesis of AD, the inaccessibility to brain samples and the notion that brain cholesterol is independent of changes in circulating cholesterol, have until now limited the use of pathways regulating cholesterol homeostasis as biomarkers and diagnostic tools for neurological disorders. Peripheral tissues that have been utilized for providing useful molecular markers for AD include skin fibroblasts, platelets and PBMCs [45]. Our studies showing a significant higher concentration of neutral lipids (NLs), mainly CE in PBMCs from AD patients and in some of their first degree relatives (FDR) [18] have indicated that PBMCs could be useful to perform studies on lipid-related molecular targets involved in AD. In these studies we analyzed almost 400 patients diagnosed with possible or probable AD and almost 200 of their FDR enrolled at the Alzheimer Center, USL 8, Cagliari, and at the Geriatric Unit of the University Hospital, Cagliari (Italy). Additionally, 170 individuals: 57 volunteers aged between 66-87 years, with no cognitive impairment, as established by clinical interview and by tests used to evaluate cognitive function, and 113 blood donors, aged between 20-66 years, with no personal or family history of neurological or psychiatric disorders, served as controls. Considering that CE cycle is altered in AD brains and that comparison of various analytic methods to quantify NLs in peripheral cells revealed no statistically significant differences [16-18], we proposed the determination of NL-PBMCs by Oil Red O (ORO), which was, among the utilized, the less invasive, and the cheaper, easier and faster procedure, as peripheral biomarker of AD as well as an index of brain cholesterol dysfunction. PBMCs isolated from 100-200 µl of whole blood by density centrifugation were sufficient to adequately assess ORO staining technique. The isolated cells were washed with PBS, fixed by soaking in 10% formalin and treated with isopropyl alcohol (60%), re-washed and stained with ORO. This is a

Figure 2: NLs determination by ORO staining method in PBMCs from AD, FDR-AD and corresponding controls. A. Representative images for each subject (AD, FDR-AD and controls) and group (Control aged 60 yr, Central aged 70 yr, AD- FDR aged 60 yr). B. Statistical results of NLs amount in each age group and group.
lipid-soluble dye which stains NLs, including CE, but not FC which appear as bright red spots in the cytoplasm (Figure 2A). After staining, cells were imaged using an inverted phase microscope fitted with a digital camera. The red intensity was scored on a semi-quantitative scale (from 0 to 5) by two blinded observers: 0 indicated no staining; 1, rare positive cells or staining barely visible at low power (≤200); 2, focal staining or faint diffuse staining clearly visible at low power; 3, multifocal staining or moderate diffuse staining; and 4.5, intense diffuse staining. For NL quantization we also determine, the area fraction (% of area) of ORO staining images, by using the color threshold plugin for Image J software (NIH). Percentage of area was obtained by measuring areas in the image above a given level of intensity (threshold value) (Figure 2B).

To give support to our proposal, changes in expressions of a number of genes responsible for cholesterol homeostasis and APP processing were also examined in PBMCs from AD patients and compared with two groups of controls (aged <60 years and >70 years).

Blood samples were obtained from 50 subjects randomly selected from the 400 AD patients enrolled in the above-mentioned studies [14]. We evaluated the expression of genes involved in a) cholesterol uptake: LDLR b) cholesterol neosynthesis and regulation: HMG-CoAR, SREBP2, a transcription factor regarded as the main regulator of cholesterol homeostasis; c) cholesterol trafficking: caveolin-1 (Cav1) and ABCA1; d) cholesterol ester cycle: ACAT1 and neutral cholesterol ester hydrolase (nCEH); e) APP and BACE1 and f) Aβ degradation: nepilysin. Analysis of variance (ANOVA) showed that with the exception of ACAT1 and ABCA1, the mRNA levels of all other genes involved in cholesterol homeostasis decreased significantly in PBMCs from old man compared to that from middle-aged subjects. Despite of this, PBMCs from old subjects maintained the mechanisms responsible for regulation of cholesterol homeostasis [38]. The age-related decline in PMBC cholesterol metabolism was closely associated with an age-related decrease in the expression of genes normally involved in APP processing [38]. These results fit well with the notion that the rate of de novo cholesterol synthesis and turnover, relatively high in developing CNS, strongly declines to a very low level in the aging brain [20,34] and that at the molecular level, intracellular cholesterol regulates APP processing and Aβ production [20,34]. In the same study, we also found that LDLR and APP mRNAs were most abundant in AD compared to old controls, whereas SREBP2 and particularly nCEH were present at much lower RNA levels in AD-PBMCs [38] further supporting the concept that at least some of physiologic cholesterol homeostatic mechanisms are impaired in AD patients. In addition, our results provide indirect evidence that besides ACAT1, nCEH, the enzyme responsible for hydrolysis of CE, plays a major role in NL accumulation observed in AD-PBMCs [38]. In the light of these findings we assumed that the determination of NL-PBMC by ORO staining, and of expressions of genes related to cholesterol homeostasis and APP processing, could represent potential adjunctive tools to evaluate AD risk. In addition they could also help to development of therapeutic strategies, as well as to prediction of clinical outcome of AD and possibly of other neurodegenerative disorders (Figures 3A and 3B).

Nieman-Pick type C1 (NPC1) disease

NPC1 is a rare, non curable autosomal recessive lipid storage disorder [46–49], which leads to progressive brain damage responsible for disability and premature death beyond early childhood. The neurological symptoms include ataxia, dysarthria, dysphagia, tremor, and epilepsy. In the terminal stages there is also severe dementia. Since NPC1 is a disorder characterized by altered cholesterol metabolism, it has been extensively utilized for better understanding cholesterol trafficking in peripheral cells and in the CNS [46–49]. The disorder is caused by a mutation in the gene encoding NPC1, a protein necessary for the movement of FC from the lysosomal compartment of cells to the metabolically active pool in the cytosol [50]. As a consequence, nearly every cell in the body accumulates FC that is derived from the LDL receptor-mediated endocytosis [46–49]. This accumulation of sterol in the late endosomal/lysosomal compartment of cells leads to pulmonary failure, liver dysfunction, and neurological damage [51]. Although it has been reported that NPC1 protein contains a conserved sterol-sensing domain (SSD), similarly to HMG-CoAR, SCAP and SREBP2, all of which are well known regulators of cholesterol homeostasis [52], until now, neither the functional role of NPC1 protein nor the mechanisms by which NPC1 patients develop neurological symptoms are known. Studies have indicated that the expression of NPC1 mRNA and protein are regulated by liver X receptors (LXRs) [53]. These are ligand-activated transcription factors that are members of the nuclear receptor superfamily that preferentially bind with their heterodimeric partner, retinoid X receptor (RXR), to LXR response elements (two hexanucleotide repeats separated by four nucleotides) to activate gene expression [54]. Oxysterols such as 24S-OHC are thought to be responsible for the LXR activation in vivo [55]. In this connection, it is of interest that treatment with LXR agonists increased the lifespan of NPC1 null mice [56]. Accumulation of FC in tissues including the brain and high levels of 24S-OHC in the plasma has been reported in NPC1 patients [57,58]. It should be noted that the extremely long half-life of the majority of brain cholesterol has obscured the direct identification of cholesterol accumulation using analytical biochemical methods. However, using filipin microfluorodensitometry, Treiber-Held et al. [57] demonstrated a significant accumulation of FC in the brains of NPC1 null mice, with the cholesterol accumulating before the onset of the disease phenotype. A consequence of this accumulation may be a loss of neuronal membrane cholesterol which contributes to excessive amyloidogenesis and neurodegeneration. Accordingly, NPC1 patients often have eczematous derrite formation and neurofibillary tangles similar to those seen in AD [58]. These findings help to explain why NPC1 patients suffer of progressive neurological symptoms.

Smith-Lemli-Opitz syndrome (SLOS)

SLOS is the most common disease due to inborn errors of cholesterol synthesis. It is caused by a deficiency of 7-dehydrocholesterol reductase (7-DHC-R) that catalyzes the reduction of 7-DHC to cholesterol in the
final reaction of cholesterol synthesis. Even if an immediate precursor of cholesterol, 7-DHC is not able to substitute for cholesterol as a component of cell membranes [59]. Affected individuals usually have low plasma cholesterol levels and invariably have elevated levels of cholesterol precursors, including 7-DHC. In fact, whereas control populations show plasma cholesterol levels of ~180 mg/dl and 7-DHC of ~0.005-0.05 mg/dl, SLOS patients can have plasma cholesterol levels of ~85 mg/dl or lower and 7-DHC levels as high as ~25 mg/dl [60,61]. The clinical spectrum is wide and includes both pre- and post-natal growth retardation, mild to severe mental retardation, multiple congenital malformations (both major and minor), and characteristic face. It is common for SLOS children to exhibit sensory hyper-reactivity, irritability, language impairment, sleep cycle disturbance, self-injurious behavior, and autism spectrum behaviors. In spite of the fact that the disease is clearly due to a deficiency of 7-DHC-R, SLOS pathogenesis is not yet well understood. There is some evidence that increased levels of 7-DHC impair lipid raft stability of the neuronal plasma-membranes, providing a rational for symptoms of neurodegeneration in SLOS [27].

Dietary cholesterol supplementation is the most commonly proposed potential treatment for SLOS [62]. As synthesis of sterols, including 7-DHC, is positively controlled by HMG-CoAR [63,64], combined HMG-CoAR inhibitor, simvastatin (able to cross the BBB) and dietary cholesterol supplementation therapy has been also recommended [65,66]. Intriguing, given the high frequency of autistic symptoms in SLOS patients, a similar treatment has been also mentioned for autism spectrum disorders (ASD) [65,66].

**Autism spectrum disorders (ASD)**

ASD describe a group of disorders with early childhood onset, characterized by persistent deficits in socialization, language, and stereotypic and repetitive behavior [67]. It is well established that ASD have a strong genetic component; however, for at least 70% of cases, the underlying causes are unknown (sporadic ASD). The list of well-defined genetic disorders with ASD continues to expand, with commonly studied examples including fragile X syndrome, tuberous sclerosis, untreated phenylketonuria, Rett syndrome, and SLOS. Thus, studies involving such genetic disorders have begun to reveal the neurobiologic features of behavioral phenotypes of children with sporadic ASD [67]. Evidence supporting a role for cholesterol in the pathogenesis of these disorders is, in fact, based on studies regarding children with inborn errors of cholesterol synthesis, chiefly SLOS [68]. Three mechanisms working in concert have been hypothesized to explain how low cholesterol levels may contribute to sporadic ASD: a) impaired sonic hedgehog (SHH) signaling molecules which are involved in the regulation of organogenesis including the organization of the brain. During embryonic development, SHH is covalently modified with both palmitate and cholesterol and secreted as part of a lipoprotein complex that regulates brain morphogenesis through the patched/smoothened signaling system [62]; b) alterations in membrane lipid raft structure and protein function resulting in abnormal synaptic plasticity, and c) impaired neurosteroid synthesis [62,68]. Therefore, multiple mechanisms are likely to arise as etiologies of the ASD phenotype and autism research involving sterols and other metabolites continues to gain popularity.

Starting from these considerations, and from the notion that many scientists consider AD “nothing more than autism in the elderly”, we thought it might be interesting to determine NLs by ORO staining method in PBMCs from children with sporadic autism. In addition, since, many studies have outlined the dimensionality of autism in regard to its comorbidity with other neurodevelopmental disorders such as ADHD we also determined NL levels in PBMCs from children affected by ADHD [69]. This is the most common psychiatric disorder in children, affecting about 3 to 5 percent of children globally [70,71] and is characterized primarily by “the co-existence of attention problems and hyperactivity, with symptoms starting before seven years of age [70,71]. In about 30 to 50 percent of those individuals diagnosed in childhood, symptoms persist into adulthood [72,73]. The diagnosis is established by satisfying specific criteria and may be associated with other neurological, significant behavioral, and/or developmental/learning disabilities. Although many theories have been proposed, the cause of ADHD remains a mystery and adequate therapies do not exist. Among the candidate causes, a nutritional deficit has a prominent place. Lower amounts of specific polar lipid fatty acids in plasma (20:4n-6, 20:5n-3, and 22:6n-3), and lower concentrations of total fatty acids in RBCs were found in subjects with ADHD [74].

As shown in figures 4A and 4B, in a manner similar to that observed in the AD patients, both ASD and ADHD children unveiled higher NLs levels in cytoplasm of their PBMCs compared to that of age-matched control children. Lower plasma cholesterol levels were also found in ASD and ADHD children (unpublished results). Although obtained in a small number of children (10 with autism and 20 with ADHD aged 8-15 years and 10 age-matched controls), these data further supported the attractive hypothesis of NLs determination in PBMCs as a useful tool for diagnostic and therapeutic interventions in different type of dementia and possibly as an index of metabolic derangements and alterations in brain lipids.

**Potential Therapeutic Role of Rapamycin in Treating Neurodegenerative Disorders Associated with Cognitive Defects**

For all the above, it seems that the proposed dietary cholesterol supplementation combined with an inhibitor of HMG-CoAR, rather than to improve autism symptoms, may exacerbate behavioral problems related to this disorder. In 2008, a study by Ehninger et al. [75] used the mammalian target of rapamycin (mTOR) inhibitor, rapamycin, to treat learning disabilities associated with a disease called tuberous sclerosis complex (TSC) in mice [75]. This is a rare genetic disorder that causes brain tumors, seizures, learning disabilities, skin lesions and kidney tumors. In humans, half of TSC patients are autistic. The results showed that rapamycin was able to reverse mental retardation in TSC mice raising the possibility that this drug may be effective in...
the treatment of mental disorders associated with autism [75]. In 2010, Galvan and her team published a research showing that rapamycin also improves learning and memory deficits and reduces brain lesions and Aβ levels, in a mouse model of AD, suggesting that rapamycin may have an another exciting use: to fight AD [76]. Rapamycin added to diet late in life was also able to extend lifespan in a mice model of aging [77]. If these results continue to be repeated and pilot studies demonstrate that treatment works and is safe, rapamycin, which is already approved for other indications, could be utilized - sooner than expected - to prevent behavioral symptoms in autistic children and in AD patients as well as to improve health to the end of life. At this point it will be crucial to understand how rapamycin exerts its positive effect on the brain. It has been suggested that the drug operates in preventing behavioral symptoms in autism and AD and in extending lifespan through a combination of anti-neoplastic effects and effects on cellular stress resistance and response to nutrient dynamics. In a study that has just been published, it has been shown that the levels of three major monoamines (norepinephrine, dopamine and 5-hydroxytryptamine) and their metabolites (3,4-dihydroxyphenylacetic acid, homovanillic acid, and 5-hydroxyindolacetic acid) were significantly increased in midbrain of rapamycin-treated mice compared to controls. The authors suggested that oral administration of rapamycin, enhances learning and memory in young adults, maintains memory in old C57BL/6 mice, and has concomitant anxiolytic and antidepressant-like effects, possibly by stimulating major monoamine pathways in brain [78]. In this context, studies by our laboratory have repeatedly shown that beside its role as mTOR inhibitor, rapamycin also interferes with cholesterol homeostasis being a potent inhibitor of cholesterol esterification [17,79]. When added to cultured fibroblasts obtained by skin biopsy from AD patients, a significant reduction of cholesterol ester (CE) accumulation, as determined by ORO staining method, was observed [17]. As already mentioned, starting from 2001, several papers by Kovacs et al., provided genetic, biochemical, and metabolic evidence that intracellular cholesterol distribution regulates Aβ generation [12-14]. They showed that ACAT modulates Aβ production by maintaining a delicate balance between FC and CEs. Importantly, ACAT inhibitors strongly reduced amyloid pathology and improved cognitive performance in transgenic mouse models of AD. The same Authors, more recently, tested the anti-amyloidogenic effects of CI-1011 in two age groups of hAPP transgenic mice (these mice are characterized by large deposits of Aβ). They showed that CI-1011 partially protects young mice from development of amyloid pathology and reduces amyloid burden in old animals with preexisting amyloid deposits. Intriguingly, their results suggest that by limiting further Aβ generation, ACAT inhibition may be able to reverse neuronal damage caused by earlier Aβ accumulation [15]. Since, CI-1011 has demonstrated efficacy in preclinical models of AD [80], the Authors proposed it for AD treatment. All these results strongly support the possibility that CEs and ACAT are important therapeutic targets for the treatment of AD, and lead us to hypothesize that inhibition of cholesterol esterification could be another attractive mechanism by which rapamycin improves the learning and memory impairment found in children with autistic disorders and in patients with AD. However, important challenges associated with the development of drugs for AD and autism concern their safety and their ability to cross the BBB. In addition, more researches are needed to better understand the molecular mechanisms by which the drugs ameliorate the AD and ASD symptoms. CI-1011, a [(2,4,6-tris(1-methylphenyl)phenyl) acetyl]sulfamic acid, 2,6-bis(1-methylphenyl)phenyl ester, also known as avasimibe, is suitable for clinical use because of an improved pharmacological and safety profile, however, little is known about its BBB penetrability [15]. By contrast, rapamycin not only has the advantage to be already on the market, even if for other purposes, but it is also able to cross BBB [75-78]. Therefore, it might be safe and effective in improving behavioral abnormalities found in AD and possible in ASD.

**Relationship between Peripheral and Central Cholesterol Homeostasis**

As regards lipid metabolism, one of the most interesting problems to solve is a deeper understanding of the interactions existing between cholesterol metabolism in brain and peripheral tissues. For this reason we dedicated the last part of the review to provide compelling evidence linking the metabolically active central and peripheral cholesterol pools.

Peripheral and central cholesterol pools are largely distinct, the cholesterol required for brain development, brain membranes and myelination deriving almost exclusively from local synthesis, however, interactions between peripheral and central cholesterol homeostasis have been reported [20]. Experimental studies in mice have showed that the mature brain volume is reached between 13 and 26 weeks, but that, by the end of the first three weeks of life the rate of brain accretion rapidly drops [20]. Over the same time interval, there is also a rapid decrease in the rate of brain cholesterol synthesis, although this decrease is not as great as seen with the accretion rate. In human brain, the cholesterol is efficiently re-cycled and has a remarkably high half-life (up to 5 years). Cholesterol in myelin (~70%) is relatively stable, while the remaining 30%, that is present in membranes of glial and neuronal cells, is actively metabolic. As in mice, humans synthesize more brain cholesterol than the actual requirement to maintain myelin membrane growth, membrane cholesterol turnover, as well as the synthesis of neurosteroids [54]. As a result, FC pool at the endoplasmic reticulum (ER) increases. The excess ER-FC is toxic, therefore, ACAT1 located at the ER is activated, and part of it converted to CEs and stored as cytoplasmic lipid droplets. However, except for the active phase of specific pathological conditions, almost all (at least 99%) cholesterol in the nervous system is unesterified [20]; therefore to maintain brain steady-state cholesterol metabolism, part of the excess of FC must leave brain. FC, before to exit CNS, is converted into 24S-OH-CE by neurons. Cholesterol 24S-hydroxylase (CYP46A1), in fact, is expressed almost entirely in the smooth ER of neurons, including those of the hippocampus and cortex, which are important for learning and memory [81]. Once produced, 24S-OH-CE moves from neurons through CSF, cross the BBB, and is released into the systemic venous circulation. The fate of the 24S-OH-CE once it reaches the circulation has not yet been defined. It has been reported that plasma levels of 24S-OH-CE are highest during the first years of life and decrease by a factor of about four during the first two decades of life, reflecting the balance between cerebral production of cholesterol and the metabolism of circulating oxysterol [82]. An accurate method based on isotope dilution-mass spectrometry with use of individual deuterium labeled internal standards, showed that 24S-OH-CE in plasma is mainly associated with HDL and LDL [83]. In addition, it has been also reported that in the plasma, it is mainly present in esterified form (24S-OHCE), and is a substrate for lecithin: cholesterol acyltransferase (LCAT), the enzyme that esterifies FC transported in lipoproteins [84]. These findings clearly suggest that, under physiological conditions, 24S-OH-CE follows the metabolic fate of cholesterol in HDL and LDL. Changes in CNS cholesterol homeostasis have been frequently reported as occurring in association with some neurological diseases. Stage-dependent variations of brain, CSF and plasma 24S-OH-CE levels have been reported in AD patients: plasma 24S-OH-CE levels increase in the initial stages of the disease; while
Figure 5: A hypothetical model that links central cholesterol metabolism to cholesterol homeostasis in peripheral tissues during neurodegeneration.

On the basis of the above informations, we propose a mechanistic model that links central to cholesterol homeostasis in peripheral tissues during neurodegenerative process. As a consequence of neurodegeneration, a surplus of FC is released by damage neuronal membrane that may be potentially toxic for cells. To minimize this toxicity, ACAT1, located at the ER, is activated and a portion of unnecessary FC converted into CE. The increase in ER cholesterol pool also raises the substrate level for CYP46A1, leading to an increase in 24S-OHCC biosynthesis in neurons. The oxysterol is then secreted into CSF, and through the BBB, delivered into the circulation. In this fashion, high concentrations of 24S-OHC in CSF and/or plasma have been well documented that ox-LDL are avidly cleared from the circulation being recognized by scavenger receptors (SR), e.g., scavenger receptor A (SR-A) and CD36, on the surface of white blood cells. Uptake of ox-LDL leads to the development of “foam cells” that contain massive amounts of cytoplasmic CE inclusions [88] (Figure 5).

Conclusion

At present, most of neurodegenerative disorders including AD and ASD can be diagnosed on the basis of cumulative information gained from the clinical examination, brain-imaging techniques, and some of genetic markers. Owing to the complexity of neurobiology and our still limited knowledge of these disorders, specific and sensitive peripheral markers are currently not available. Among the risk factors that have been identified as affecting these disorders, variations involving different aspects of brain cholesterol homeostasis are emerging as important. Therefore, the measurement of key molecules involved in peripheral cholesterol homeostasis seems at the present a great way to prevent and/or treat specific neurodegenerative disorders. Of all the accessible potential biochemical markers for an altered brain cholesterol homeostasis, plasma concentration of 24S-OHC is the best characterized. Unfortunately, the determination of 24S-OHCC requires sophisticate procedures based on gas chromatography-mass spectrometry and high-performance liquid chromatography-mass spectrometry. In addition with few exceptions, introduction of an oxygen function in the cholesterol molecule drastically reduces the half-life of the molecule and directs it to excretion or to further oxidation to water-soluble bile acids. Therefore, another problem that limits the use of 24S-OHCC as peripheral marker for early diagnosis of neurological disorders is the risk of artifactual formation of oxysterols from the cholesterol during the workup procedures. By contrast the determination of PBMCs NLs by ORO staining procedure, which requires only small amount of whole blood and can be reproduced quickly, easily and cheaply, appears to be suitable for use in routine for diagnosis and development of therapeutic strategies as well as for personalized prediction in clinical outcome of AD and possibly other neurodegenerative disorders.

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