LIPID METABOLISM AND TRANSPORT IN CNS HEALTH AND DISEASE

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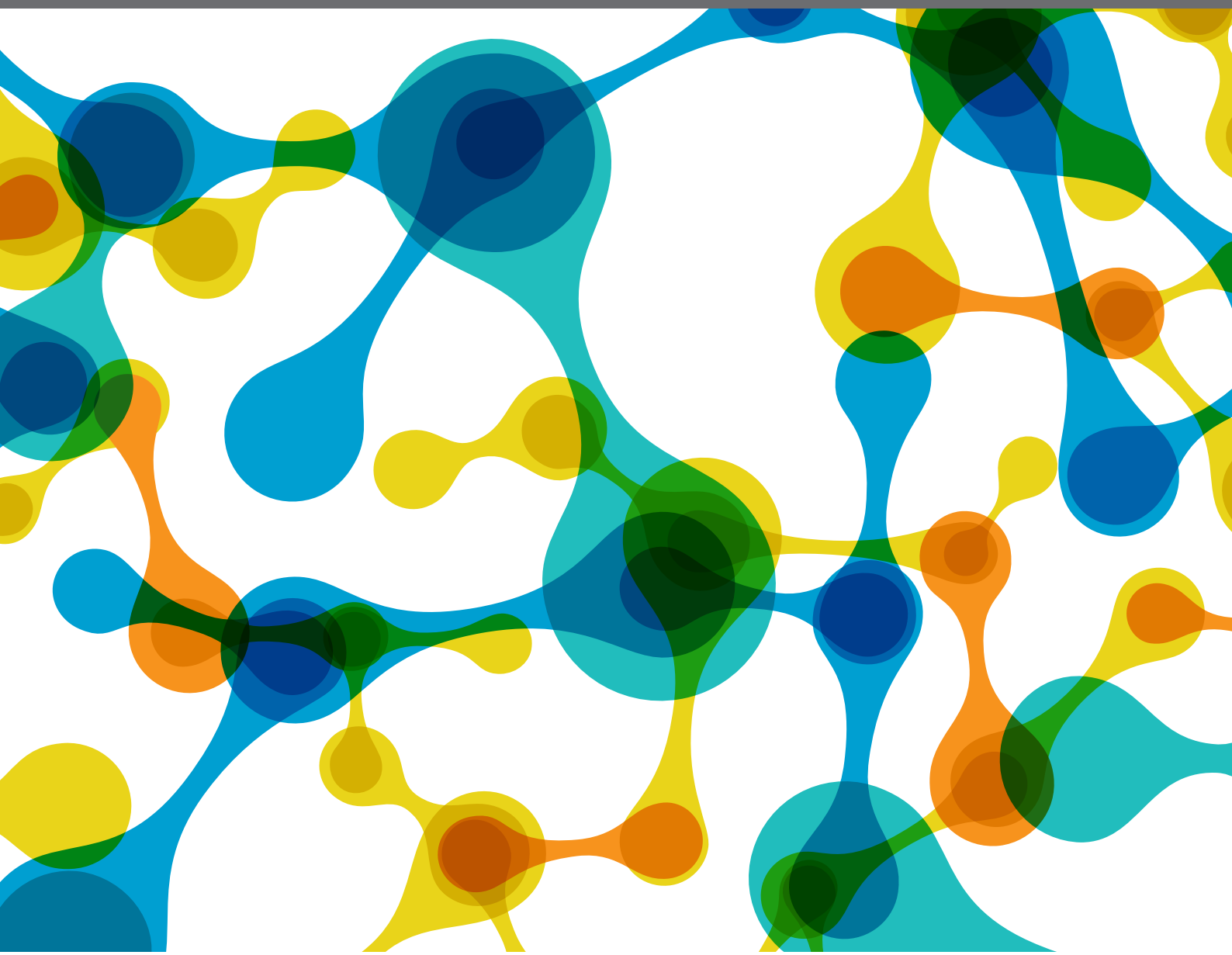

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LIPID METABOLISM AND TRANSPORT IN CNS HEALTH AND DISEASEV

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EDITORIAL
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Editorial: Lipid Metabolism and Transport in CNS Health and Disease

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Editorial on the Research Topic

Lipid Metabolism and Transport in CNS Health and Disease

The brain is mostly composed of lipids, yet lipid metabolism and processing in the brain are incompletely understood. With the advancement of higher-resolution technologies, we are becoming increasingly aware that even slight changes in lipid and lipoprotein processing in the brain can precede or even predict disease. This Research Topic gathers contributions that highlight how lipids are transported; across the blood-brain barrier (BBB), by microglia, stored as lipid droplets (LDs), or transported in the form of APOE-containing high-density lipoprotein particles, and the importance of lipid composition in predicting disease. The compiled group of articles provides a better understanding of these processes, which may lead to the development of more targeted strategies to diagnose and treat neurodegenerative disorders (NDs).

In the first article, Huguenard et al., describe the long-standing need for biomarkers that can diagnose mild Traumatic Brain Injury (mTBI) and Post-Traumatic Stress Disorder (PTSD). Building on previous work detecting chronically altered brain-associated phospholipids in the blood of subjects with mTBI, the authors reason that the analysis of peripheral lipids may also identify patients with mTBI and PTSD. Using liquid chromatography-mass spectrometry (LC-MS) to examine lipids in the blood of active-duty soldiers, they identified elevated Triglycerides (TG) and Diglycerides (DG) in subjects with mTBI + PTSD. Whereas, *APOE* ε4 carriers with mTBI showed elevated DGs (Huguenard et al.). This study suggests that examining peripheral TGs and DGs may be a useful biomarker for mTBI and PTSD, but ε4 status should be considered.

Changes in lipid composition may also be a useful predictor of Alzheimer's Disease progression (AD). Fonteh, Cipolla, et al., measured polyunsaturated fatty acids (PUFAs) in the cerebrospinal fluid (CSF) of participants that were either cognitively healthy with normal $A\beta_{42}$/T-tau (established biomarkers of AD), cognitively healthy with pathological $A\beta_{42}$/T-tau, or were diagnosed with AD. When normalized for the number of particles in the CSF, higher PUFA levels were observed in cognitively healthy individuals compared to subjects with AD, suggesting that higher PUFA levels may enhance cognitive resilience in the pre-symptomatic AD population (Fonteh, Cipolla, et al.). These findings warrant further studies that examine whether PUFA composition can predict the transition from pre-symptomatic AD to AD and whether PUFA supplementation may prevent AD progression.

In a follow-up study, Fonteh, Chiang, et al., revealed further differences in the lipid composition of the CSF from cognitively healthy individuals, with either normal or pathological levels of $A\beta_{42}$ and Tau or subjects with AD dementia. Cognitively healthy subjects with abnormal $A\beta_{42}$/Tau showed elevated phosphatidylcholine glycerophospholipids (GPs) and higher sphingomyelin levels than those with AD, suggesting higher lipid levels in pre-symptomatic AD and lipolysis with AD progression (Fonteh, Chiang, et al.). In addition, the authors show that Phospholipase A_2 (PLA_2), which

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hydrolyzes membrane lipids, is increased in patients with dementia (Fonteh, Chiang, et al.). More work is needed to determine whether this is causative to the AD lipidome and whether altering PLA_2 activity is a plausible strategy for treating AD.

AD treatment is particularly challenging since multiple biological, genetic, and lifestyle factors contribute to its neuropathogenesis. This issue is reviewed by Chew et al., in this Research Topic, where the authors highlight that altered lipid transport and metabolism is a common denominator driving the mechanisms leading to increased AD risk, such as blood-brain barrier function, amyloid precursor protein (APP) processing, myelination, membrane remodeling, receptor signaling, inflammation, oxidation, cardiovascular disease and energy balance. This comprehensive assessment of the current literature galvanizes the importance of interrogating the interaction between lipid metabolism and each facet of AD to help develop potential therapies that target lipid pathways (Chew et al.).

While changes in lipid composition may help diagnose and even treat NDs, the cellular processing of these lipids remains elusive, hindering the development of lipids as therapeutics. In recent years, microglia, the key immune effector cells of the CNS, have been critical to developing NDs. In a timely review, Loving and Bruce evaluate emerging evidence supporting the role of microglial subpopulations, defined by enhanced expression of lipoprotein processing machinery and increased phagocytosis, as potentially protective during development, damage, and disease. This review highlights the need for further studies that determine whether brain-derived lipoproteins and microglial lipoprotein receptors may be novel targets for treating NDs, such as AD and beyond.

Emerging evidence suggests that the cells of the CNS, including microglia, accumulate LDs during disease and aging. While LD biology has been well-characterized in peripheral metabolic tissues, the biology and significance of LDs within neurons and glia are relatively understudied. A review article in this Research Topic critically evaluates new research showing that LDs have diverse roles in the CNS, such as cellular fuel stores, markers of inflammation, signaling hubs, waste reservoirs, products of lysosomal degradation, and as hallmarks of aging (Farmer et al.). In light of these diverse roles, it is not surprising that LDs have been attributed to neurodegeneration and aberrant cerebral metabolism (Farmer et al.). However, further work is needed using improved imaging techniques (e.g., matrix-assisted laser desorption/ionization imaging mass spectrometry [MALDI-IMS]) to resolve region and cell-specific LD composition to further understand the contribution of LDs to NDs (Farmer et al.).

Many studies, including those outlined in this topic, support the hypothesis that supplementation with specific lipids (e.g., PUFAs) may improve cognitive function during aging and prevent the development of ND. However, we do not yet know how lipids are transferred from the blood to the brain. Thus, inconsistent outcomes following PUFA supplementation in AD patients likely relate to our incomplete understanding of lipid transport. In this current topic, Pifferi et al. carefully review our current understanding of lipid transport into and out of the CNS, paying particular attention to the differences between the blood-brain barrier (BBB) and the blood-cerebrospinal fluid (CSF) barrier (BCSFB). In addition, the authors highlight the understudied role of the choroid plexus (CP) within the BCSFB, a secretory tissue within the brain ventricles, as potentially critical to the maintenance of lipid and cholesterol transport (Pifferi et al.). Given the importance of CSF turnover and composition to the development of AD, an in-depth study of lipid transport mechanisms within the BCSFB is much needed (Pifferi et al.).

One transport mechanism that is critical for AD development yet also remains critically understudied is high-density lipoprotein (HDL) transport into the brain. Here, Van Valkeburgh et al. critically evaluate recent findings regarding the transport mechanisms of HDL particles (HDL-P), which are synthesized in both the brain and the periphery. HDL-associated apolipoproteins are some of the most important determinants of Alzheimer's disease (AD) pathology and vascular dementia. However, the extent to which HLD-P can exchange their protein and lipid components between the central nervous system and the systemic circulation remains unclear. Here, the authors delineate the structure and functions of HDL's apolipoproteins that influence brain amyloid metabolism and atherosclerosis; and explore how HDL can be modified to enhance its brain delivery with potential for treating brain neurodegenerative and vascular diseases (Van Valkenburgh et al.). Small HDL-P appear to possess properties that can favor their brain delivery, but further research is needed to understand their transport mechanisms (Van Valkenburgh et al.).

In conclusion, the Research Topic "*Lipid Metabolism and Transport in CNS Health and Disease*" is a collection of manuscripts highlighting the importance of lipid processing in brain structure and function. These manuscripts underscore what is known, what needs to be studied, and the benefits of lipid analyses in CNS diseases; to understand disease mechanisms, identify biomarkers, and discover novel treatments.

AUTHOR CONTRIBUTIONS

KB, HY, and AF wrote and edited the manuscript. All authors contributed to the article and approved the submitted version.

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Plasma Lipidomic Analyses in Cohorts With mTBI and/or PTSD Reveal Lipids Differentially Associated With Diagnosis and *APOE* ε4 Carrier Status

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The differential diagnosis between mild Traumatic Brain Injury (mTBI) sequelae and Post-Traumatic Stress Disorder (PTSD) is challenging due to their symptomatic overlap and co-morbidity. As such, there is a need to develop biomarkers which can help with differential diagnosis of these two conditions. Studies from our group and others suggest that blood and brain lipids are chronically altered in both mTBI and PTSD. Therefore, examining blood lipids presents a minimally invasive and cost-effective approach to identify promising biomarkers of these conditions. Using liquid chromatography-mass spectrometry (LC-MS) we examined hundreds of lipid species in the blood of healthy active duty soldiers (n = 52) and soldiers with mTBI (n = 21), PTSD (n = 34) as well as co-morbid mTBI and PTSD (n = 13) to test whether lipid levels were differentially altered with each. We also examined if the apolipoprotein E (*APOE*) ε4 allele can affect the association between diagnosis and peripheral lipid levels in this cohort. We show that several lipid classes are altered with diagnosis and that there is an interaction between diagnosis and the ε4 carrier status on these lipids. Indeed, total lipid levels as well as both the degree of unsaturation and chain lengths are differentially altered with diagnosis and ε4 status, specifically long chain unsaturated triglycerides (TG) and both saturated and mono-unsaturated diglycerides (DG). Additionally, an examination of lipid species reveals distinct profiles in each diagnostic group stratified by ε4 status, mainly in TG, saturated DG species and polyunsaturated phosphatidylserines. In summary, we show that peripheral lipids are promising biomarker candidates to assist with the differential diagnosis of mTBI and PTSD. Further, ε4 carrier status alone and in interaction with diagnosis has a strong influence on peripheral lipid levels. Therefore, examining ε4 status along with peripheral lipid levels could help with differential diagnosis of mTBI and PTSD.

Keywords: traumatic brain injury, post-traumatic stress disorder, biomarkers, lipids, apolipoprotein ε4, mass spectrometry

INTRODUCTION

Mild traumatic brain injury (mTBI) and post-traumatic stress disorder (PTSD) are considered signature injuries among United States soldiers returning from recent conflicts in Iraq and Afghanistan (Tanielian and Jaycox, 2008; Carlson et al., 2013). In fact, nearly 20% of soldiers report sustaining a TBI, whilst 5–15% report having PTSD (Tanielian and Jaycox, 2008). Currently, mTBI is undetectable using conventional neuroimaging techniques in clinical settings (Dretsch, 2010). As a consequence, there is reliance on self-report of injury characteristics and symptoms (e.g., alteration in consciousness), and cognitive disturbances, in order to diagnose mTBI (Kim et al., 2017; Dretsch et al., 2019). Diagnosing PTSD also requires self-reporting of symptoms in order to detect emotional, behavioral, and cognitive problems which emerge in patients after traumatic events (American Psychiatric Association, 2013). However, there is considerable homogeneity in symptoms reported by both patients with PTSD and those experiencing persistent symptoms after mTBI, making it difficult to accurately diagnose and differentiate these conditions (Dash et al., 2010; Laskowitz and Grant, 2016; National Center for PTSD, 2018). To further complicate matters, mTBI and PTSD are often comorbid in soldiers returning from combat, where severe psychological trauma occurs either within the context of mTBI or emerges as a consequence of stress following trauma (Stein and McAllister, 2009; Bryant, 2011; Dretsch et al., 2016; Stein et al., 2016; Avallone et al., 2018; Vasterling et al., 2018). Hence, differential diagnosis of mTBI and PTSD presents a challenge. Therefore, investigations of blood biomarkers may advance research efforts aimed at better classifying these conditions in order to provide appropriate care, diagnosis and treatments to patients suffering from mTBI and/or PTSD.

Biomarker discovery work in TBI has identified several promising blood protein biomarkers: glial fibrillary acidic protein (GFAP), ubiquitin C-terminal hydrolase-L1, neuron specific enolase and S100β (Wang et al., 2018). While these proteins may be useful biomarkers of acute and severe TBI, their use in reliably detecting milder forms of TBI remains to be fully investigated (Adrian et al., 2016; Joseph et al., 2018). At present, there are no blood biomarkers available for PTSD, and only broad inflammatory markers and cerebrospinal fluid (CSF) cortisone levels have been thoroughly examined (Schmidt et al., 2013; Michopoulos et al., 2015). Additionally, long-term consequences of mTBI and PTSD may predispose these vulnerable patient populations to the risk of developing neurodegenerative diseases later in life (Perry et al., 2016; Wilson et al., 2017; He et al., 2019) and are known to lead to worse outcomes when comorbid with other disorders (Lippa et al., 2015; Ahmadian et al., 2019). In that regard, several studies have focused on examining blood amyloid-β (Aβ), neurofilaments and tau levels in individuals with TBI (Bogoslovsky et al., 2017; Kim et al., 2018). Among these, elevated blood Aβ40 and Aβ42 fragments have been detected acutely and chronically after mTBI (Lejbman et al., 2016; Bogoslovsky et al., 2017). However, blood Aβ levels appear to lack specificity for TBI (Posti et al., 2019). As such, in both mTBI and PTSD, there is a current need for biomarkers to help characterize biological changes differentially associated with each disorder in order to improve clinical detection and monitoring of long-term consequences of mTBI and PTSD.

Blood lipids are emerging as candidate biomarkers of neurological disorders given their important role in the maintenance of brain health through their contributions to metabolic, inflammatory and neuro-signaling processes (Piomelli et al., 2007). Recent studies suggest that blood and brain lipids are chronically disturbed in both mTBI and PTSD (Hauer et al., 2013; Emmerich et al., 2016). Further, we and others have shown that major phospholipid (PL) classes involved in providing both structural and functional support to the brain are chronically altered in both brain and blood of mouse models of severe and mTBI (Abdullah et al., 2014; Ojo et al., 2018; Thau-Zuchman et al., 2019), in the blood of soldiers with mTBI (Emmerich et al., 2016) and in CSF of civilians after severe TBI (Pasvogel et al., 2010). Many of the post-TBI changes in lipids that have been described involve alterations in blood and brain polyunsaturated fatty acids (PUFA)-containing PLs required for maintaining membrane integrity and synaptogenesis (Liu et al., 2015; Emmerich et al., 2017; Knobloch, 2017; Fiandaca et al., 2018). Lipid associated factors such as cholesterol may be peripherally altered in TBI as, under normal physiological conditions, brain cholesterol is compartmentalized and restricted to the brain by the presence of the blood brain barrier (BBB) (Cartocci et al., 2016); however, BBB disturbances after TBI (Sulhan et al., 2018) may alter the brain's cholesterol content by allowing an exchange between peripheral and CNS cholesterol pools which may in turn adversely affect neuronal function. Studies have shown that CSF cholesterol increases acutely after TBI (Kay et al., 2003). Although cholesterol ester (CE) levels are thought to increase after excitotoxic insults to the brain (Malgrange et al., 2015), whether cholesterol and CE levels are chronically altered after TBI and PTSD remains to be investigated. Additionally, other lipids such as diacylglycerides (DG) and ceramides (Cer) play a known role in inflammation (Reisenberg et al., 2012; Grösch et al., 2018) and may be reflective of ongoing neuroinflammatory processes in TBI and PTSD. As such, blood lipids that are altered in response to neuropathological changes following mTBI and PTSD could be useful biomarkers of these conditions and their evolving pathologies.

Apolipoprotein E (ApoE, encoded by the *APOE* gene) is a constituent of lipoprotein particles responsible for transporting lipids from the bloodstream to various tissues including the brain (Jonas and Phillips, 2008; Tudorache et al., 2017). Among the three major *APOE* polymorphisms, the ε4 allele is known to have impaired lipid transport to the brain and is also associated with worse cognitive outcomes in both TBI and PTSD (Mota et al., 2017). ApoE/lipoprotein complexes facilitate lipid transport to the BBB where lipids are processed and subsequently transported into the brain by fatty acid binding proteins (FABP) as well as by other transporters (Mitchell and Hatch, 2011; Sepe et al., 2018). Given the role of ApoE in brain injury and lipid transport, it is possible that different ApoE isoforms may affect blood lipid levels in interaction with injury.

Based on the known role of lipids in response to injury and the potential interaction with *APOE* genotype, we hypothesized that blood lipid levels would be affected both by diagnosis and the *APOE* ε4 allele. Using LC/MS, we examined several major blood lipid classes in a cross-sectional military cohort of soldiers with a diagnosis of mTBI, PTSD or both, as well as healthy controls. Further, we investigated the protein biomarkers FABP3, GFAP, Aβ38, Aβ40, Aβ42 as well as the ratio of Aβ42 to Aβ40, which has been shown to be altered in TBI (Lejbman et al., 2016), to compare lipid changes to protein biomarkers. This study will help determine whether peripheral lipids may be promising biomarkers to eventually help clinicians with the differential diagnosis and prognosis of mTBI sequelae and PTSD.

MATERIALS AND METHODS

Cohort Characteristics and Measurements

The recruitment details of these military cohorts have been previously described in Emmerich et al. (2016), where basic demographics as well as deployment related history, psychological health questionnaires and neurobehavioral symptoms data were collected from two cohorts of 120 active duty male soldiers, pre-deployment to the Middle East for Operation Iraqi Freedom/Operation Enduring Freedom, who participated on a voluntary basis under IRB approved consent. For the Army, a non-deployable status in relation to a psychiatric condition requires a clinician diagnosis in their medical record. Due to the nature of our study design, we did not scrub medical records of soldiers from the respective brigade to maintain their anonymity. Similar to a psychiatric condition, a non-deployable status in relation to a mTBI requires three or more documented injuries in their medical record. Hence all subjects in this study were deemed medically fit for deployment after physical and psychiatric assessments through deployment medical screening. Our diagnostic categories for participants were determined by screening instruments at pre-deployment. All participants were screened for mild TBI (mTBI) and PTSD using the Defense and Veterans Brain Injury Center Brief Traumatic Brain Injury Screen (BTBIS, Schwab et al., 2006) and the PTSD Checklist Military Version where the PCL-M, with a score $\geq$ 35 was considered positive in order to provide a provisional diagnosis of PTSD. We chose a cut-score of 35 which is suggested when screening in general population samples that have an estimated prevalence of PTSD below 16%. Diagnosis was then assigned by a trained neuropsychologist. Participants were also screened for both depression and alcohol consumption levels, using the Zung Depression Scale (Zung, 1965) and the Alcohol Use Dependency Identification Test (Lundin et al., 2015), respectively. Additionally, level of anxiety was assessed using the Zung Anxiety Scale (Zung, 1971, 1974) and self-perceived stress level using the Perceived Stress Scale (Cohen et al., 1983). Sleep quality was assessed using the Pittsburgh Sleep Quality Index (Buysse et al., 1989) and daytime sleepiness was assessed using the Epworth Sleep Scale (Johns, 1991). Finally, post-concussive symptoms were assessed using the Neurobehavioral Symptom Inventory (NSI, Cicerone and Kalmar, 1995). The numbers per diagnostic groups were the following: 52 controls, 21 mTBI, 34 PTSD, 13 mTBI + PTSD. Additionally, neurocognitive battery, Central Nervous System – Vital Signs test (CNS-VS, Gualtieri and Johnson, 2006) was administered to participants at the time of sampling, CNS-VS includes multiple subtests to assess verbal memory, information processing speed, complex attention, cognitive flexibility, reaction time, and executive function domains.

Non-fasting blood samples were collected throughout the day at phlebotomy stations by staff blinded to the diagnosis status of the study participants using previously established standard operating procedures. Briefly, blood was drawn into EDTA tubes for preparing plasma and DNA genotyping. Samples were coded at the time of collection and remained coded for all subsequent processing and data generation. For preparing plasma and to remove platelets and other blood cells, whole blood was centrifuged at room temperature on site at high speed of 1380 × *g* for 5 min, following separation the clarified plasma was immediately transferred to a 15 mL conical tube supplemented with a 20× protease inhibitor cocktail (PIC) (Roche) containing 0.5M EDTA to a final concentration of 1X PIC and vortexed. Samples were stored in dry ice before transport to a −80°C medical specimen freezer at the end of each day. Additionally, samples from whole blood aliquots were *APOE* genotyped in our laboratory, by purifying the DNA (Gentra Puregene Blood Kit, Gentra Systems), then using standard polymerase chain reaction as described by Emi et al. (1988) followed by electrophoresis with experimenters blinded to participants' group membership and other group characteristics (all procedures described in more details in Emmerich et al., 2016). Due to low numbers when stratifying by specific genotype in addition to diagnosis (six possible allele combination for four different diagnostic groups) and since most participants had either an ε3/ε3 or ε3/ε4 genotype, participants were grouped into ε4− and ε4+ groups where the ε4− group was composed of participants who did not have any ApoE ε4 allele and the ε4+ group of participants who had one or two ApoE ε4 alleles. The final numbers per subgroups were; 37 ε4−controls, 15 ε4+controls, 16 ε4−mTBI, 5 ε4+mTBI, 24 ε4−PTSD, 10 ε4+PTSD, 8 ε4−mTBI + PTSD, and 5 ε4+mTBI+PTSD.

Lipidomics Analysis

Plasma from this cohort was analyzed following a randomized block group design with experimenters performing the sample preparation, lipid extraction, qualitative and quantitative mass spectrometry analyses blinded to participants' group membership and other group characteristics. The extraction procedure and nano-LC/MS conditions were previously published by our laboratory (Joshi et al., 2018). Plasma (10 μL) was spiked with 5 μl of a mix of SPLASH LipidoMIX stable isotope and Cer/Sph Mixture I (Avanti, Polar Lipids, Inc.) diluted 1:10 in SPLASH internal standard (IS) mix. All solvents were HPLC grade purchased from ThermoFisher Scientific. Samples were extracted using a modified Folch method. Methanol (80 μL) was added to the samples followed by vortexing for 1 min before adding 120 μL of chloroform and vortexing again for 1 min. Samples were then

centrifuged at 4°C at 20,000 relative centrifugal force (RCF) for 10 min. The supernatant was then transferred to a low retention Eppendorf microtube, and 40 μL of 0.88% potassium chloride was added. Then samples were vortexed for 1 min. Samples were centrifuged as before, and the lower phase was transferred to another low retention Eppendorf microtube and evaporated by vacuum centrifugation. For the cleanup procedure, non-sterile micro-centrifugal filters (Thermo Scientific) were prepared by applying 200 μL of 1:1 chloroform:methanol to the filters and centrifuging at 4°C, 10,000 RCF for 5 min. The flow-through was discarded, and 1:1 chloroform:methanol was added to the samples which were vortexed and then applied to the filters and centrifuged as before. After, the filters were discarded and the flow-through transferred to auto-sampler vials with inserts and dried under vacuum and re-suspended in 50 μL of 70:30 mobile phase A–B, with mobile phase A comprised of 27% isopropanol, 42% water, 31% acetonitrile, 10 mM ammonium formate with the addition of 0.1% formic Acid. Mobile phase B was comprised of 90% isopropanol, 10% acetonitrile, 10 mM ammonium formate, and 0.1% formic acid.

An Easy-nanoLC 1000 instrument was used in combination with a nanoflex ESI source and a Thermo QE/Orbitrap mass spectrometer. Samples were injected into an Acclaim PepMap 100, 75 μm × 2 cm nanoViper C18, 3 μm, 100Å trapping column and Acclaim PepMap RSLC, 75 μm × 15 cm nanoViper C18, 2 μm, 100Å analytical column for lipid chromatographic separation, running the following gradient at a constant flow rate of 250 nL/min. The starting conditions were 30% B, then from 1 to 50 min program from 50 to 98% B, then switch to 30% B from 50 to 65 min. All samples were run in triplicate in batches of 8 along with a blank and quality control (QC) sample. Full-scan MS data was acquired in both positive and negative ion modes, with a mass range of m/z 130–2,000 in the positive ion mode, m/z 220–2,000 in the negative ion mode, at a resolution of 30,000 for both. The heated capillary was maintained at 200°C, with a spray voltage of 1,500 V. A maximum inject time of 200 ms was used with 13 microscans/acquired scan. Peak areas were integrated using the Tracefinder™ software using a target compound list of lipids of interests containing m/z and retention time for each target lipid and IS for that specific lipid class. For each lipid class, the concentration of lipid species was calculated using the spiked IS corresponding to that class (except for hexosylceramides where the closest eluting IS was used, see **Supplementary Table S8**), by dividing the target compound area by the IS area and multiplying by the known IS concentration spiked in. Each species in a sample run that had a coefficient of variance (CV) > 25% was excluded from further analysis as considered not to have been measured reliably. Each analytical batch was normalized using its QC ([sample] × [batch QC/normalizing QC]).

Immunoassays

Amyloid-Beta

A V-PLEX Aß Peptide Panel (Meso Scale Discovery) was used to quantify Aβ38, Aβ40 and Aβ42 in the plasma samples. All procedures were performed as per the manufacturer's instructions with samples run in duplicate. Concentrations were obtained in $pg.ml^{-1}$, with a stated dynamic range of 16.7–8475 $pg.ml^{-1}$ for Aβ38, 9.97–7000 $pg.ml^{-1}$ for Aβ40, and 0.368–1271 $pg.ml^{-1}$ for Aβ42.

GFAP

A R-PLEX Human GFAP Antibody Set (Meso Scale Discovery) was used to measure GFAP in the plasma samples. As GFAP was at first not detected in the samples following standard instructions, the assay was repeated with samples run neat (without dilution or addition of assay buffer) and left to incubate overnight at 4°C with the primary antibody. The experiment was otherwise conducted as per the manufacturer's instructions. Samples were run in duplicate. Concentrations were obtained in $pg.ml^{-1}$, with a stated dynamic range of 63–500,000 $pg.ml^{-1}$.

FABP3

A FABP-3 Human ELISA Kit (Invitrogen, Thermo Fisher Scientific) was used to measure FABP3 in the plasma samples. As FABP3 was not detected in all samples following standard instructions, the assay was repeated running the samples neat (without dilution or addition of assay buffer). The experiment was otherwise conducted as per the manufacturer's instructions. Samples were run in duplicate. Concentrations were obtained in $pg.ml^{-1}$, with a stated dynamic range of 156.3–5000 $pg.ml^{-1}$ and an inter-assay coefficient of variance (CV) of 6.2% and intra-assay CV of 3.9%.

For all immunoassays, duplicates with CV > 25% were removed from further analysis. Data from each immunoassay was analyzed separately in SPSS.

Statistical Analysis

As these were exploratory studies, *post hoc* power calculations were conducted using the G-power software which showed a greater than 97% power at $\alpha = 0.05$ for total content of specific lipid class, with the observed effect size $f = 0.98$ for total TG, $f = 1.7$ for total DG, $f = 1.2$ for total Cer, and $f = 1.02$ for HexCer for the n per groups utilized in this study. Differences in age, genotype and sex were assessed between diagnostic groups by first running a Brown–Forsythe test to examine the equality of variance between groups as group numbers varied. No significant differences in variance were detected so a One-Way ANOVA was performed on each variable. The Fisher's exact test was performed on each medication therapeutic category to determine whether the distribution of medication use significantly differed between groups. If this was found to be the case main effect and interaction effect with diagnosis and *APOE* genotype were tested using the Mixed Linear Model (MLM) function. For triglycerides (TG), diglycerides (DG), cholesterol, cholesterol esters (CE), phosphatidylserines (PS), ceramides (Cer), and hexosylceramides (Hexcer) triplicate data was uploaded to SPSS for lipidomic analysis and the mean of duplicate data for the immunoassays. The percentage of included/excluded values of each lipid species was reviewed, and those with < 75% of values included were removed from further analysis, as the sample size was considered too small.

For lipids, totals were calculated by adding up all lipid species belonging to that class. Saturated fatty acids (SFA) were calculated

by adding up all lipid species with no double bonds, mono-unsaturated fatty acids (MUFA) by adding up lipid species with one double bond and polyunsaturated fatty acids (PUFA) by adding up lipid species with more than one double bond. For grouping lipids by chain length, a principal component analysis (PCA) was performed on individual species which were grouped by chain length based on their correlation coefficients, as some chain lengths were found to be highly correlated. Normality of the data was assessed by plotting histograms. Non-normally distributed data was normalized using the natural log function. The main effect of diagnosis and *APOE* genotype as well as their interaction effect was tested using the MLM function for triplicate data and general linear model (GLM) function for immunoassay data. Based on these findings, a MLM was run for lipidomic data or One way ANOVA for immunoassay data examining factors previously found to be significant. This was followed by multiple comparisons to test for significant differences between groups using least significant difference (LSD), chosen as a less stringent *post hoc* analysis for these exploratory data. This was done for totals as well as for saturation, chain length groups and lipid species. For the heatmap, fold-changes against ε4−controls were calculated *B/A*. For all analyses, a p-value < 0.05 was considered significant to minimize the risk of false positives. For the individual lipid species, as many comparisons were made, a Benjamini–Hochberg (*B–H*) correction was performed to control for false positives, with a false discovery rate Q set to 0.1. For *B–H* correction p-values were sorted in ascending order and assigned with a rank then each individual p-value's *B–H* critical value was calculated $(i/m)Q$ (with i = rank, m = total number of tests performed, Q = the false discovery rate). The largest p-value than its critical p-value and all other ranked above were retained as significant (Simes, 1986). All comparisons were made against control, ε4− or ε4+ control groups as stated. GraphPad Prism was used to graph all data. Although statistical analyses were performed on normalized data, raw data were graphed for all immunoassays. As each lipid class was quantified using one IS corresponding to that class, the quantification is relative, and data are presented as percent of control in order to be more informative. However, the raw quantified data is available in **Supplementary Tables S3–S8**. For DG MUFA as well as CE of chain length 22 few lipid species (n = 2) were detected and/or passed quality control, for these the data presented may be more representative of changes in few individual lipids rather than class-wide changes in saturation states or chain lengths ($n \geq 4$ for all others). The ε4 + mTBI + PTSD group was removed from total Cer after data cleanup as its number was too low (n = 1).

RESULTS

Basic Demographics, Psychological Health, Neurobehavioral and Cognitive Data of the Study Population

Cohorts characteristics are presented in **Supplementary Tables S1**, **S2**. Briefly, there were no significant differences in allelic distribution, age, race, mother's education, previous number of deployment or paygrade (related to rank) between diagnostic groups with or without ε4 stratification. When examining potential confounding factors for medication use, only anti-depressants were found to be a potential confounder. However, no main effect or interaction with diagnosis was observed for antidepressants on the lipid classes examined (**Supplementary Table S1**), except for CE where antidepressant use was found to be a potential confounder, however, removing participants on anti-depressants did not alter our results. Therefore, in the absence of any confounding by medication use, analyses were performed with the whole cohort. In **Supplementary Table S2** we present psychological health, neurobehavioral data and neurocognitive assessment measures. We observe higher NSI scores in the mTBI, PTSD and mTBI + PTSD group compared to controls, indicating endorsement of more post-concussive symptoms as well as higher severity of these symptoms. We also report both poorer sleep quality, increased daytime sleepiness and higher anxiety in our PTSD diagnosis group compared to controls, our mTBI and mTBI + PTSD groups also reported poorer sleep quality and higher anxiety. We also found a higher prevalence of depression in our PTSD group. In these cohorts we did not find any significant differences between controls and diagnostic groups in alcohol abuse, stress and neurocognitive scores (**Supplementary Table S2**).

APOE ε4 Influences the Association of Neutral Lipids and Sphingolipids With mTBI and PTSD Diagnosis

Total plasma levels of TG, DG, CE, cholesterol, Cer, Hexcer, and PS were examined for their association with mTBI, PTSD and mTBI + PTSD diagnoses (**Figure 1A**). There was an interaction of diagnosis and ε4 status for TG ($F_{(3,345.742)} = 3.054$, $p = 0.029$), DG ($F_{(3,311.703)} = 6.582$, $p = 0.000252$), Cer ($F_{(3,107.561)} = 3.918$, $p = 0.01$) and Hexcer ($F_{(3,243.025)} = 4.836$, $p = 0.003$) lipids. There was a significant main effect of diagnosis and the ε4 carrier status and an interaction between them for total DG levels ($p \leq 0.05$). There was no effect of diagnosis or the ε4 status on total cholesterol and PS levels. *Post hoc* comparisons of controls showed that Hexcer were significantly decreased among ε4 + controls compared to ε4− controls ($p \leq 0.05$, **Figure 1B**). Among ε4− participants, total TG and DG were significantly elevated in the mTBI + PTSD group compared to control and other diagnostic groups ($p \leq 0.01$, **Figure 1B**). In ε4+ groups, total DG levels were also increased in mTBI compared to controls and PTSD groups ($p \leq 0.01$, **Figure 1B**). Additionally, in ε4− groups total Cer levels were elevated in the mTBI + PTSD compared to controls and PTSD groups ($p \leq 0.01$, **Figure 1B**). In ε4+ groups, total Cer levels were elevated in the PTSD group compared to the mTBI group ($p \leq 0.01$, **Figure 1B**). Compared to ε4− controls, total Hexcer levels were lower in ε4 + controls and in ε4− mTBI + PTSD participants ($p \leq 0.01$, **Figure 1B**). Among ε4 + participants, total Hexcer levels were increased in participants with mTBI + PTSD compared to controls and PTSD ($p \leq 0.01$, **Figure 1B**).

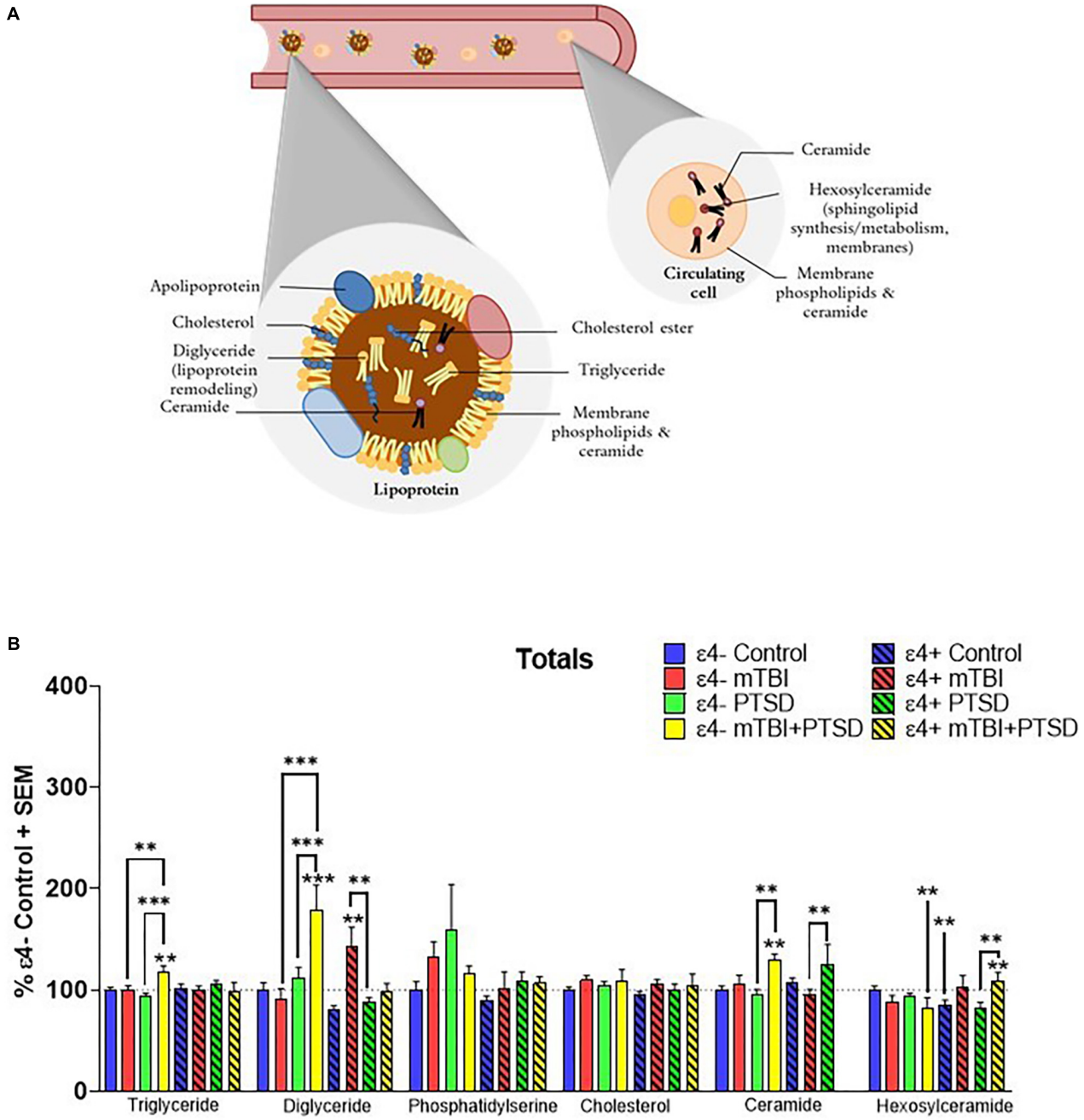

FIGURE 1 | Examination of total peripheral levels of several lipid classes in mTBI, PTSD and mTBI + PTSD in individuals with and without the APOE ε4 allele using an LC-MS assay. **(A)** Schematic illustration of possible main contributors to the signals observed. **(B)** There was an interaction of diagnosis and ε4 status for TG ($p = 0.029$), DG ($p = 0.000252$), Cer ($p = 0.01$), and Hexcer ($p = 0.003$) lipids. Statistical analyses; MLM with LSD *post hoc*, $p \leq 0.05$ cut-off, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$. TG, Triglycerides; DG, Diglycerides; Cer: Ceramides; HexCer, Hexosylceramides.

APOE ε4 Influences the Association Between the Degree of Unsaturation and Chain Lengths of TG, DG, and CE in mTBI and PTSD Diagnoses

We stratified lipid species by their degree of unsaturation and carbon chain lengths since these parameters relate to metabolic lipid processes and PCA showed high correlation coefficients between chain groups shown here.

A significant main effect of diagnosis was observed for MUFA containing TG species ($F_{(3,351.393)} = 2.567$, $p = 0.054$) with *post hoc* analyses showing that their levels were elevated in mTBI + PTSD participants compared to other groups ($p \leq 0.05$, **Figure 2A**). There was an interaction between diagnosis and

ε4 for MUFA ($F_{(3,351.393)}$ = 4.444, p = 0.004) and PUFA TG species ($F_{(3,345.366)}$ = 2.776, p = 0.041). Among ε4− participants, MUFA- and PUFA-containing TG species were elevated in the mTBI + PTSD group compared to other groups ($p \leq 0.05$, **Figure 2C**). Compared to ε4− controls, MUFA TG were decreased in ε4− PTSD participants ($p \leq 0.05$, **Figure 2C**). Additionally, MUFA TG were decreased in the ε4 + mTBI group compared to the ε4 + PTSD group ($p \leq 0.05$, **Figure 2C**). With respect to the examination of chain-lengths in TG species, we observed an effect of diagnosis on TG chain-length 58 ($F_{(3,350.303)} = 3.136$, $p = 0.026$) and interaction between diagnosis and the ε4 carrier status on TG species of TG ≤ 48 ($F_{(3,\ 351.029)}$ = 4.057, p = 0.007), 54–55 ($F_{(3,348.046)}$ = 3.786, p = 0.011), and 56 chain-lengths ($F_{(3,351.984)}$ = 3.6, p = 0.014). *Post hoc* analyses showed a differential effect of diagnosis for TG ≥ 54 which were elevated in the mTBI + PTSD group ($p \leq 0.05$, **Figure 2B**). Additionally, TG ≤ 48 were lower in the ε4− PTSD group compared to ε4− controls and higher in the ε4− mTBI + PTSD group compared to the ε4− PTSD group ($p \leq 0.05$, **Figure 2D**). However, TG ≤ 48 were lower in ε4 + mTBI compared to the ε4 + TBI + PTSD group ($p \leq 0.05$, **Figure 2D**). Lastly, levels for TG ≥ 54 were elevated in ε4− mTBI + PTSD group compared to all other ε4− diagnostic groups ($p \leq 0.01$, **Figure 2D**). For DG, a main effect of diagnosis was observed for all unsaturated species (SFA: $F_{(3,333.756)}$ = 6.708, p = 0.001, MUFA: $F_{(3,342.936)}$ = 3.763, p = 0.011), and PUFA: $F_{(3,321.356)}$ = 3.017, p = 0.03) and chain lengths (≤ 32: $F_{(3,329.790)}$ = 2.897, p = 0.035, 34–36: $F_{(3,332.523)}$ = 5.473, p = 0.001, and 38: $F_{(3,330.429)}$ = 6.4.893, p = 0.002) as well as a main effect of the ε4 carrier status on DG with chain lengths ≥ 34–38 (34–36: $F_{(1,332.523)}$ = 4.086, p = 0.044 and 38: $F_{(1,330.429)}$ = 7.492, p = 0.007). *Post hoc* analyses showed that, compared to controls, participants with a diagnosis of mTBI + PTSD had elevated DG levels, irrespective of the degree of unsaturation and chain-length ($p \leq 0.01$, **Figures 2E,F**). A significant interaction between diagnosis and ε4 carrier status was detected for SFA-containing DG species ($F_{(3,333.756)}$ = 6.708, p = 0.000208) and DG of all chain lengths (≤ 32: $F_{(3,329.790)}$ = 6.199, p = 0.000415, 34–36: $F_{(3,332.523)}$ = 5.713, p = 0.001, and 38: $F_{(3,330.429)}$ = 5.772, p = 0.001), where SFA and MUFA DG species were elevated in the ε4−mTBI + PTSD group compared to all other ε4− groups ($p \leq 0.001$, **Figure 2G**). In addition, SFA DG were elevated in the ε4 + mTBI group compared to all other ε4 + groups ($p \leq 0.05$, **Figure 2G**). Shorter chain length DG species (≤ 32) were decreased in the ε4− mTBI group compared to ε4− controls and the ε4− PTSD group ($p < 0.05$). There was no effect of DG chain lengths on any of the other groups, as their levels were altered in the same way irrespective of DG chain length (**Figure 2H**).

Among the remaining lipids analyzed, for CE, there was no main effect of diagnosis, only a main effect of the ε4 carrier status for species with a chain length of 20 ($p \leq 0.05$). There were no differences for the degree of unsaturation (**Figure 2I**). Cholesterol esters of chain length 20 were significantly elevated in the ε4− mTBI group compared to ε4− controls ($p \leq 0.01$, **Figure 2J**). For PS, no main effects or interactions were found to be significant. No significant differences were found in PS levels between groups with or without ε4 stratification (see **Supplementary Figure S1**).

Analyses of Lipid Species to Identify Non-overlapping Potential Biomarker Lipids for Each Diagnostic Group

We next examined how lipid species profiles differed in mTBI, PTSD and mTBI + PTSD diagnostic groups. There was a main effect of diagnosis and an interaction between diagnosis and the ε4 carrier status for various lipid species shown in **Figure 3A**. The heatmap (**Figure 3A**) shows the fold-change of lipids significantly altered in each diagnostic group compared to their respective controls (ε4− or ε4+ controls) in order to examine the directionality of the changes observed in lipid species. The greatest number of changes were seen in the ε4− mTBI + PTSD group with a total of 40 lipids significantly altered compared to ε4− controls, while the ε4 + mTBI + PTSD group had 10 lipids significantly altered compared to ε4+ controls (**Figure 3A**). Further, both the ε4− mTBI and ε4+mTBI groups exhibited significant changes in over a dozen of the lipid species investigated, each compared to their respective ε4− and ε4+ control groups (**Figure 3A**). Lastly, compared to other diagnostic groups, both the ε4− and ε4+PTSD exhibited fewer changes in lipid species, with 5 and 10 changes observed, respectively (**Figure 3A**). **Figures 3B,C** show lipid species that overlapped between the diagnostic groups. Of note are unsaturated TG, PS and CE species and a number of saturated DG species that significantly differed between mTBI, PTSD and mTBI + PTSD groups in an *APOE* ε4 dependent manner.

Immunoassay-Based Analyses Detect No Significant Changes in Plasma FABP3, GFAP, and Aβ Levels but a Significant Difference in Aβ42/Aβ40 Ratio Between PTSD and Other Diagnostic Groups

We investigated several peripheral protein biomarkers in the same cohort to compare our lipidomic approach with immunoassay platforms. We investigated plasma levels of FABP3, GFAP, and Aβ fragments Aβ38, Aβ40, Aβ42, and Aβ42/Aβ40 ratio (**Figures 4A–F**). Preliminary analyses found a significant main effect of diagnosis for Aβ38 ($F_{(3)}$ = 2.778, p = 0.045) and Aβ42/Aβ40 ($F_{(3)}$ = 3.154, p = 0.028). *Post hoc* analysis did not find Aβ38 levels to be significantly different between groups. While the ratio of Aβ42 to Aβ40 was significantly different between the PTSD group vs. mTBI and mTBI + PTSD groups ($p \leq 0.05$, **Figure 4F**).

DISCUSSION

Applications of lipidomics technology to profile plasma lipids of 120 active duty soldiers showed that several lipid classes differ by diagnostic classification in these military cohorts. We also report an influence of *APOE* ε4 carrier status on the

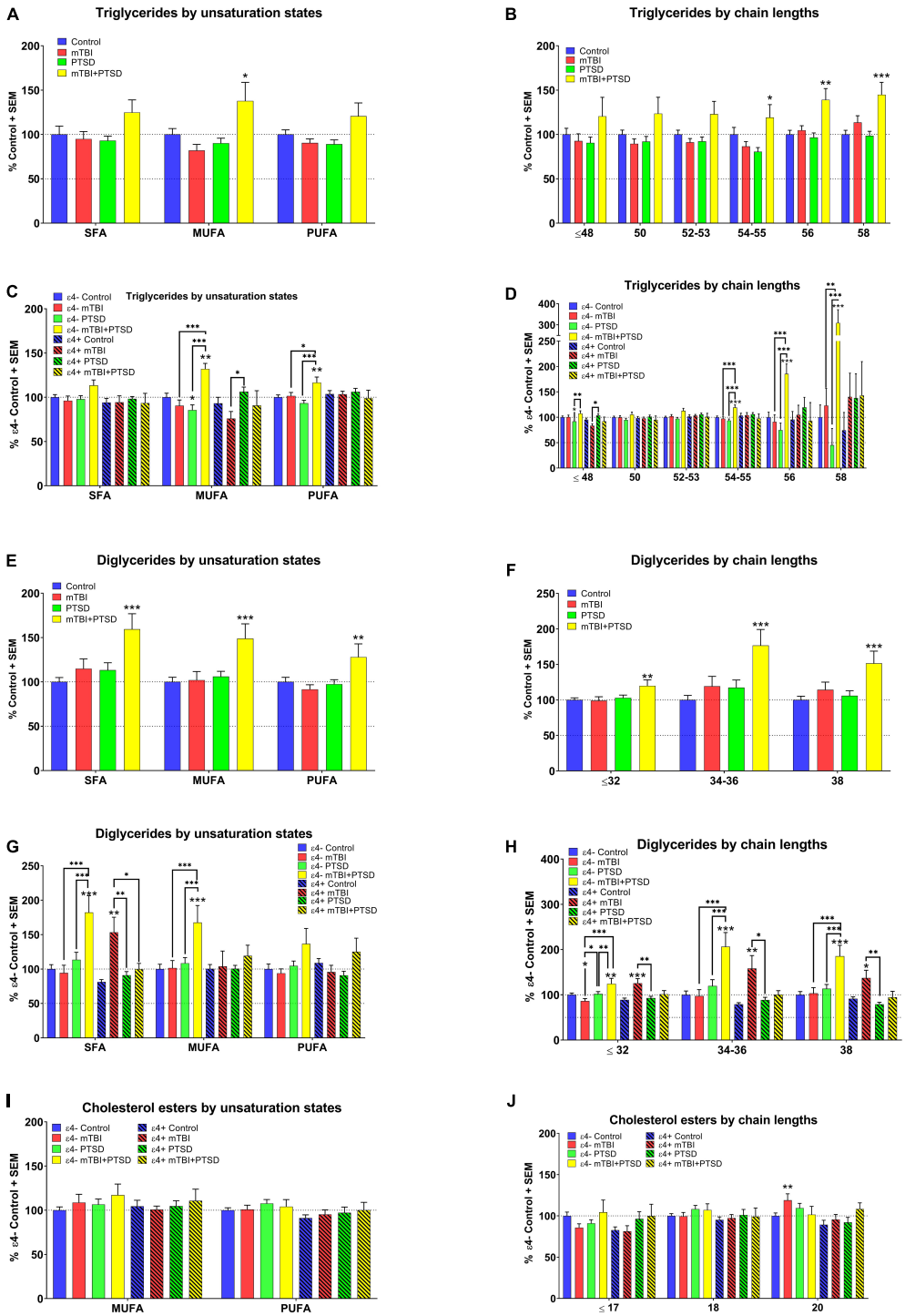

FIGURE 2 | Examination of TG, DG, and CE lipids by unsaturation and chain length status in mTBI, PTSD, and mTBI + PTSD diagnostic groups with and without ε4 stratification. **(A)** There was a main effect of diagnosis for MUFA TG ($p = 0.054$). **(B)** There was a main effect of diagnosis in TG chain length 58 ($p = 0.026$). **(C)** There was an interaction of diagnosis with ε4 status for MUFA ($p = 0.004$) and PUFA ($p = 0.041$). **(D)** There was an interaction of diagnosis with ε4 status in TG chain lengths: ≤ 48 ($p = 0.007$), 54–55 ($p = 0.011$), and 56 ($p = 0.014$). **(E)** There was a main effect of diagnosis in SFA ($p = 0.001$), MUFA ($p = 0.011$), and PUFA DG ($p = 0.03$). **(F)** There was a main effect of diagnosis in DG chain length: ≤ 32 ($p = 0.035$), 34–36 ($p = 0.001$), and 38 ($p = 0.002$). **(G)** There was an interaction of diagnosis with ε4 status in SFA DG ($p = 0.000208$). **(H)** There was an interaction of diagnosis with ε4 status for DG chain length: ≤ 32 ($p = 0.000415$), 34–36 ($p = 0.001$), and 38 ($p = 0.001$). **(I)** No main effect or interaction effect were found significant for CE unsaturation states. **(J)** No main effect or interaction effect were found significant for CE chain length. Statistical analyses; MLM with LSD *post hoc*, $p \leq 0.05$ cut-off. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$ vs. control (**A,B** and **E,F**), ε4− and ε4+ respective controls (**C,D**, **G,H**, and **I,J**). TG, Triglycerides; DG, Diglycerides; CE, Cholesterol esters; SFA, saturated fatty acid; MUFA, Mono-unsaturated fatty acid; PUFA, Poly unsaturated fatty acid.

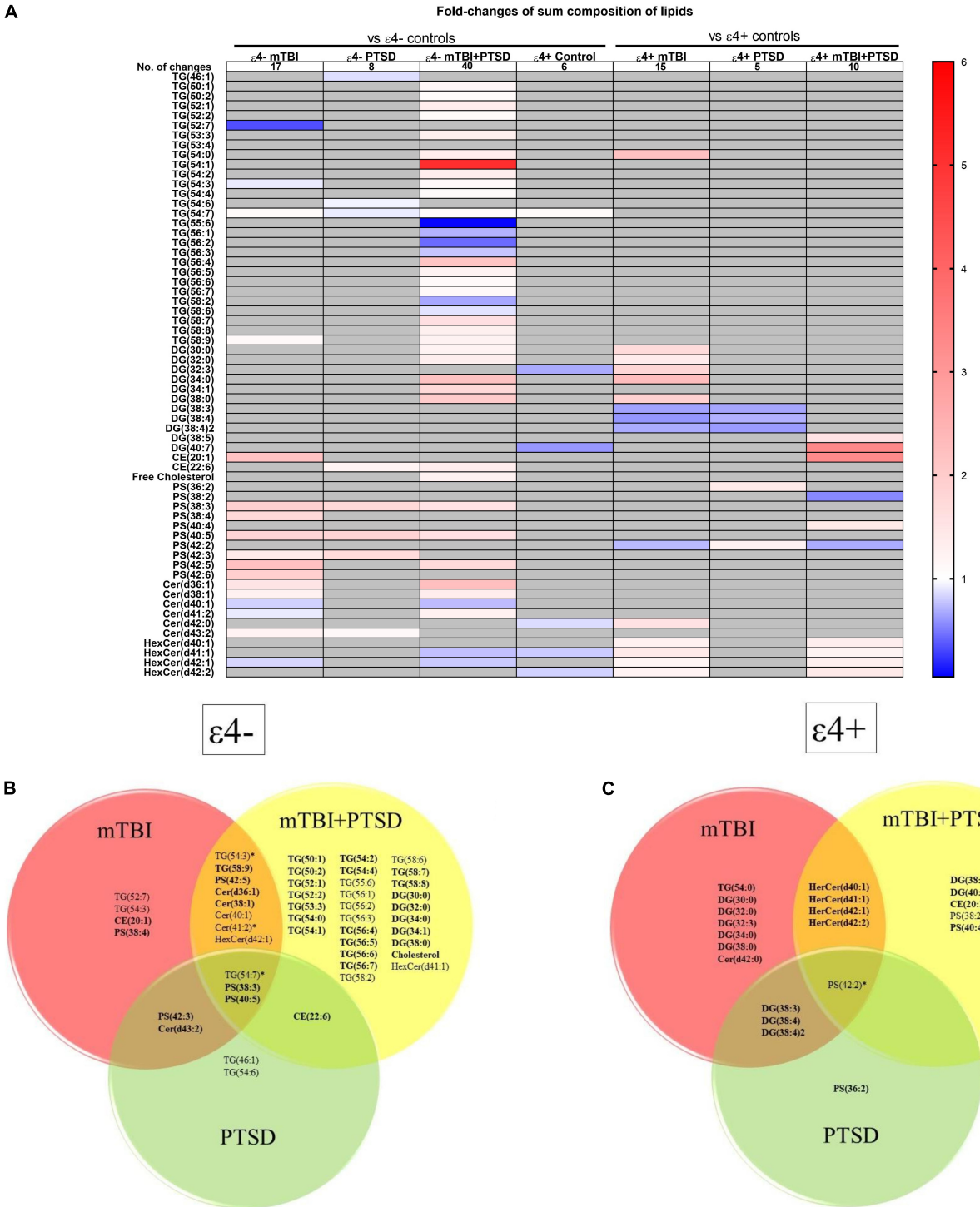

FIGURE 3 | Liquid chromatography-mass spectrometry examination of peripheral levels of sum composition lipids in mTBI, PTSD and mTBI + PTSD in individuals with and without the ε4 allele. **(A)** Heatmap of fold change of lipids that were significantly altered compared to their ε4− and ε4+ respective controls. **(B)** Venn diagram of sum composition lipids altered in ε4− diagnostic groups compared to their control. **(C)** Venn diagram of sum composition lipids altered in ε4+ diagnostic groups compared to their control. Statistical analyses; MLM with LSD *post hoc* and B–H correction, $p \leq 0.05$ cut-off. TG, Triglycerides; DG, Diglycerides; CE, Cholesterol esters; PS, Phosphatidylserines; Cer, Ceramides; HexCer, Hexosylceramides. DG(34:2)2 indicates a second peak integrated separately for that lipid. In Venn diagrams bolded lipids indicate an increase compared to controls while non-bolded indicates a decrease. Lipids marked by an asterisk indicate this lipid is increased in a group and decreased in another.

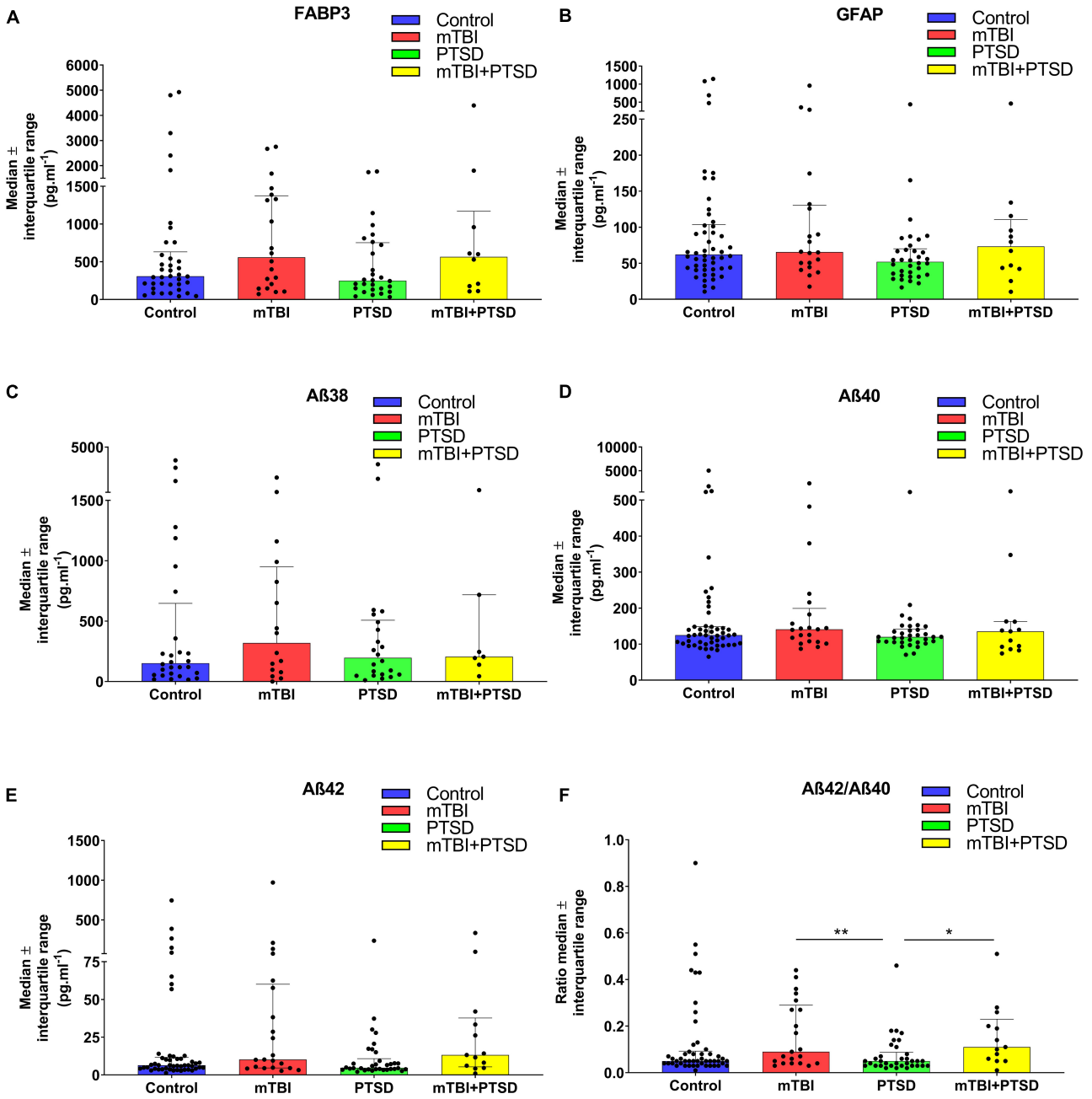

FIGURE 4 | Investigation of plasma protein biomarkers using immunoassays. **(A)** No significant changes were observed between diagnostic groups' FABP3 peripheral levels. **(B)** No significant differences were observed between diagnostic groups' GFAP peripheral levels. **(C)** A main effect of diagnosis was observed for Aβ38 ($p = 0.045$) but was non-significant in the ANOVA. **(D)** No significant differences were observed between diagnostic groups' Aβ40 peripheral levels. **(E)** No significant differences were observed between diagnostic groups' Aβ42 peripheral levels. **(F)** There was a main effect of diagnosis on Aβ42/Aβ40 ratio ($p = 0.028$). Statistical analyses; GLM for test of main and interaction effects, One Way ANOVA with LSD *post hoc* for multiple comparison, $p \leq 0.05$ cut-off. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$ vs. control. FABP3, Fatty acid binding protein 3; GFAP, Glial Fibrillary Acid Protein; Aβ, Amyloid-β.

association between lipid classes and mTBI and PTSD diagnoses. In particular, there was a significant interaction between both mTBI and mTBI + PTSD diagnostic groups and the ε4 carrier status on diverse lipid species, where ε4− participants with mTBI + PTSD showed the most pronounced changes in specific TG and DG species compared to controls. While the exact mechanisms behind peripheral lipid changes in response to CNS insults are yet to be fully elucidated, differences in these lipid signatures at non-acute timepoints, with inclusion of *APOE* genotyping, could help develop a lipid biomarker signature to assist clinicians with a differential diagnosis of these conditions.

Differential diagnosis of mTBI and PTSD is currently problematic due to symptom overlap and frequent comorbidity of these disorders, in both military cohorts and the general population (Laskowitz and Grant, 2016). Consistent with these findings, we observed shared symptomatology between TBI and PTSD using the NSI, where higher endorsement of symptoms generally thought to be associated with mTBI were reported among participants with PTSD in agreement with previous reports using the NSI (King et al., 2012; Porter et al., 2018). Although, NSI scores were somewhat lower in the mTBI group compared to the PTSD group, this might be because most individuals are asymptomatic within a few days following an mTBI (Kelly et al., 2012). Poorer sleep quality was also reported by participants with TBI and PTSD, which is a hallmark of both conditions (Gilbert et al., 2015). There were no significant differences between the controls and the diagnostic groups for stress and neurocognitive scores, which is not surprising given that the participants were young (mid-twenties) and more severe cases would not have been available due to injury or behavioral health issues identified at post-deployment medical screening. These observations further highlight the need for more objective biomarker measurements in TBI and PTSD.

Protein biomarkers currently used for TBI relate to either structural components of the brain (GFAP, neurofilaments, tau) or act as indicators of BBB injury (S100b, NSE) (Dadas et al., 2018). Therefore, these are likely to be more useful during the acute phases of the injury as a direct consequence of the physical damage but may not be as informative during sub-acute and chronic timepoints when biological changes likely reflect secondary consequences of injury, and the BBB is relatively intact, limiting their ability to enter the peripheral circulation. This is especially problematic since the initial features of injury do not necessarily correlate with long-term outcomes for patients (Dadas et al., 2018), particularly in mild injuries where immediate biological effects are subtle, often undetectable, and may only manifest as secondary consequences of the initial insult (Prince and Bruhns, 2017). Therefore, biomarkers associated with mTBI or PTSD could improve diagnosis to ensure appropriate care and management of these conditions. Although neuroimaging has been an invaluable tool for improving our understanding of TBI- and PTSD-related pathophysiology (Dadas et al., 2018; Dretsch et al., 2019) it is still limited in its utility over clinical measures (Dretsch et al., 2017a,b). Further, it is important to develop both cost effective and time efficient tools to track TBI-related changes while minimizing inconvenience to both patients and clinical staff. In our study, we did not observe any difference in protein markers, which appeared highly variable. This could be because some of these markers as previously discussed may be associated with more severe and acute injury, which were not present in our military sample, and therefore were not significantly elevated in our cohorts. Further, plasma levels of these protein biomarkers were within the range of control levels reported by others (Missler et al., 1998; Song et al., 2011; Catalucci et al., 2015).

Blood lipids are promising biomarkers as they are abundant, especially neutral lipids such as TG, DG, and CE which are present in the order of several μM to mM in blood (Burla et al., 2018), making them easily detectable. Lipid alterations have been reported for TBI and PTSD as well as other neuronal disorders, both centrally and peripherally (Adibhatla and Hatcher, 2007; Mellon et al., 2019), where hundreds of different molecular species are present and can inform on metabolic state (Burla et al., 2018). We examined total levels, the degree of unsaturation and chain lengths of lipid classes, as these can inform on lipid processes such as saturation and elongation, which may be disturbed following dysregulation of normal brain processes after TBI and PTSD. We also examined lipid species to determine whether specific lipid signatures could differentiate our three diagnostic groups. Functional magnetic resonance imaging and positron emission tomography have suggested metabolic changes in TBI (Dadas et al., 2018) and we have previously shown in these cohorts that several classes of plasma phospholipids including phosphatidylcholine (PC), lysoPC, phosphatidylethanolamine (PE), and lysoPE as well as sphingomyelin (SM) were all decreased in mTBI, PTSD, and mTBI + PTSD groups compared to controls (Emmerich et al., 2016). In the current study, we examined PS an additional PL as well as neutral lipids generally found in plasma lipid-rich lipoprotein particles responsible for transporting these lipids between various tissue including the brain and liver (Jonas and Phillips, 2008; Tracey et al., 2018). We also examined SM-related lipids, such as Cer and HexCer. While the exact mechanisms of changes in peripheral lipids after TBI and PTSD remain to be investigated, our present data show peripheral levels of TG, DG, CE, Cer, HexCer, and PS lipid classes to be differentially altered in mTBI, PTSD, and mTBI + PTSD groups which suggest a role of peripheral lipids, possibly requiring their transport into the brain for reparative and metabolic processes (Yu et al., 2011; Burla et al., 2018; Tracey et al., 2018). While in our previous study, PL differences were seen between controls and mTBI, PTSD, and mTBI + PTSD (Emmerich et al., 2016), in our current study, differences between controls and mTBI as well as mTBI + PTSD were notable for TG and DG and other individual species while only a few species differed between PTSD and controls and no class-wide differences were found in that group in agreement with what has been described by others (Jendrièko et al., 2009; Jergoviæ et al., 2015).

Different *APOE* isoforms differentially affect plasma lipids (Jonas and Phillips, 2008; Rasmussen, 2016). In particular, the presence of the ε4 allele is associated with impaired lipid transport in the periphery and the brain (Marais, 2019). Additionally, ε4 carriers have poorer outcomes after insults to the brain (Mota et al., 2017). In examining the influence of ε4 carrier status on the association between lipids and the diagnosis of TBI and PTSD, the greatest number of peripheral lipid changes were seen in the ε4− mTBI + PTSD group, while this was not the case in the ε4 + mTBI + PTSD group, indicating a differential response to injury in the presence of ε4 allele. Changes in TG and DG levels could reflect increased requirement for lipids to fuel the repair processes within the brain after injury (Tracey et al., 2018). Furthermore, TG and DG that contain certain long-chain PUFA require transport into the brain since approximately 35% of PUFA cannot be synthesized *de novo* and are acquired through diet (Liu et al., 2015). Differential profiles of PUFA with TG and DG following injury by ε4 carrier status may suggest differential

uptake of these lipids by the brain. Further, as Cer are central to the sphingolipid metabolism (Burla et al., 2018), and Hexcer are precursors to gangliosides which are abundant in the brain (Yu et al., 2011), blood changes in these lipids may be indicative of altered sphingolipid metabolism after injury. There was also an increase in several PS lipid species in all ε4− diagnostic groups. Phosphatidylserine is enriched in inner cell membranes and is involved in numerous important signaling pathways, apoptotic cell clearance, coagulation and response to histamine secretion (Leventis and Grinstein, 2010; Kim et al., 2014; Brelstaff et al., 2018). Hence, examination of the influence of ε4 on these lipids in relation to injury warrants further investigation.

While these lipid profile suggest their differential association with diagnosis and ε4 status, small sample size remains a limitation. Although precautions were taken to minimize sampling bias (i.e., evaluation of confounders and outliers), these studies require further validation in larger cohorts of other military populations. Further blood lipids can be affected by diet, lifestyle choices and chronic health conditions, such as cardiovascular risk factors (i.e., hypertension, high cholesterol, and diabetes). However, medication use data in our cohorts suggest that this was a healthy young cohort as the prevalence of cardiovascular medication was relatively low and our analyses showed that these were randomly distributed between diagnostic groups. Antidepressants was the only therapeutic category found to be unequally distributed across groups, but further analyses showed this did not confound the relationship between lipids and diagnosis in this study. Although limited data are available on dietary variations and lipid profiles, a recent study by Begum et al. (2016) examining inter- and intra-person variation in blood lipids showed that daily diet and time of the day only affected 8 out of 196 lipids which were examined. Specifically, they showed that PS, Cer, and HexCer lipids were not significant sources of variations over the course of the day (Begum et al., 2016). Unfortunately, data on diet and other lifestyle factors were unavailable in this cohort but given that they were from a military cohort, we may expect them to have similar lifestyles and diet at the time of deployment, as was suggested by our basic demographic data. Further, our semi-quantitative lipid data in control groups is consistent with what has been previously reported (Quehenberger et al., 2010). Therefore, our controls appear to be representative of the general population and should be able to control for some of the inter- and intra-individual variation expected. Additionally, our neurobehavioral and health questionnaire data suggests that these cohort are representative of military TBI and PTSD cohorts that have been previously described (King et al., 2012; Gilbert et al., 2015; Porter et al., 2018).

Expanding on our previous work these findings suggest that a panel of peripheral lipids may be useful to help differentiate mTBI from PTSD at subacute to chronic timepoints post-insult. Further, these data highlight the importance of knowing *APOE* genotype when examining and interpreting patients' blood lipid levels as we show that *APOE* genotype, specifically carrier status for the ε4 allele, has an important (sometimes opposing) influence when stratifying blood lipid profiles by diagnosis, which is consistent with our work in these cohorts examining different PL. Traumatic brain injury sequelae and PTSD are heterogeneous and currently assessed using criteria that are not necessarily well correlated with pathology, which is why using a lipidomics approach to examine a panel of lipid biomarkers could help pave the way toward building a better biological phenotype of these disorders. If changes seen in these peripheral lipids are replicated in other cohorts these could become useful clinical biomarkers in addition to available diagnostic tools. Longitudinal studies will be necessary to elucidate the potential of peripheral lipids to predict outcome and recovery after injury. Lastly, changes in composite variables and individual species could be further characterized in animal models, tissues and cells to identify altered networks which could, in turn, identify key regulators to target with potential therapeutics as we and others keep characterizing lipid profiles in neurological disease.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

The study was approved by the Institutional Review Board at Headquarters U.S. Army Medical Research and Materiel Command, Fort Detrick, MD (HQ United States MRMC IRB), and the protocol and procedures were carried out in accordance with the Declaration of Helsinki. Soldiers participated on a voluntary basis after receiving a study brief. An IRB approved informed consent was provided in accordance with international conference on harmonization guidelines and a signed consent was obtained from all participants in the presence of an ombudsperson. Approval was also obtained from brigade commanders.

AUTHOR CONTRIBUTIONS

CH wrote the manuscript, performed the immunoassay experiments, and analyzed the data. SO, HL, TD, and AN assisted with the experiments. AC and JE set-up the lipidomic assay. MD and FC conceived the original study design for biomarker investigation in these populations. MD, FC, and SF were involved in obtention of samples and processing. MM and FC provided institutional support. LA designed the experiments. LA, JE, MD, and FC reviewed and edited the manuscript.

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Polyunsaturated Fatty Acid Composition of Cerebrospinal Fluid Fractions Shows Their Contribution to Cognitive Resilience of a Pre-symptomatic Alzheimer's Disease Cohort

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Alzheimer's disease (AD) pathology is characterized by an early and prolonged decrease in the amyloid peptide (Aβ) levels concomitant with a later increase in phospho-tau concentrations in cerebrospinal fluid (CSF). We propose that changes in lipid metabolism can contribute to the abnormal processing of $A\beta_{42}$ in AD. Our aim was to determine if polyunsaturated fatty acid (PUFA) metabolism can differentiate pre-symptomatic AD from normal aging and symptomatic AD. Using neuropsychology measures and $A\beta_{42}$/T-tau in cerebrospinal fluid (CSF), we classify three groups of elderly study participants: cognitively healthy with normal $A\beta_{42}$/T-tau (CH-NAT), cognitively healthy with pathological $A\beta_{42}$/T-tau (CH-PAT), and AD individuals. We determined the size distribution and the concentration of CSF particles using light scattering and quantified PUFA composition in the nanoparticulate (NP) fraction, supernatant fluid (SF), and unesterified PUFA levels using gas chromatography combined with mass spectrometry. Four PUFAs (C20:2n-6, C20:3n-3, C22:4n-6, C22:5n-3) were enriched in NP of AD compared with CH-NAT. C20:3n-3 levels were higher in the NP fraction from AD compared with CH-PAT. When normalized to the number of NPs in CSF, PUFA levels were significantly higher in CH-NAT and CH-PAT compared with AD. In the SF fractions, only the levels of docosahexaenoic acid (DHA, C22:6n-3) differentiated all three clinical groups. Unesterified DHA was also higher in CH-NAT compared with the other clinical groups. Our studies also show that NP PUFAs in CH participants negatively correlate with CSF $A\beta_{42}$ while C20:4n-6, DHA, and n-3 PUFAs in the SF fraction positively correlate with T-tau. The profile of PUFAs in different CSF fractions that correlate with $A\beta_{42}$ or with T-tau are different for CH-NAT compared with CH-PAT. These studies show that PUFA metabolism is associated with amyloid and tau processing. Importantly, higher PUFA levels in the cognitively healthy study participants with abnormal $A\beta_{42}$/T-tau suggest that PUFA enhances the cognitive resilience of the pre-symptomatic AD population. We propose that interventions that

prevent PUFA depletion in the brain may prevent AD pathology by stabilizing $A\beta_{42}$ and tau metabolism. Further studies to determine changes in PUFA composition during the progression from pre-symptomatic to AD should reveal novel biomarkers and potential preventive approaches.

Keywords: Alzheimer's disease, cerebrospinal fluid, polyunsaturated fatty acids, mass spectrometry, cognition, resilience, brain-derived nanoparticles

INTRODUCTION

A hallmark of AD pathology is the formation of neurotoxic amyloid plaques and increased phosphorylation of total tau (T-tau) (Skoog et al., 2015). Lower levels of $A\beta_{42}$ and higher levels of T-tau are found in cerebrospinal fluid of AD subjects leading to a lower $A\beta_{42}$/T-tau ratio in AD compared with cognitively healthy (CH) study participants (Blennow et al., 2001; Harrington et al., 2013). These changes in $A\beta_{42}$/T-tau have been recognized as a sensitive biomarker of AD (Skoog et al., 2015). However, it is now clear that some CH individuals have $A\beta_{42}$/T-tau ratios similar to AD, suggesting that $A\beta_{42}$/T-tau ratio may not distinguish some asymptomatic CH subjects from AD. With a similar $A\beta_{42}$/T-tau for some CH and AD subjects, a major question that arises is whether there are other defining biochemical differences between these clinical populations. Secondly, what accounts for the cognitive resilience (Negash et al., 2013; Boros et al., 2017; Aiello Bowles et al., 2019) or reserve (Cummings et al., 1998; Persson et al., 2017; Menardi et al., 2018) in some elderly persons who have abnormal $A\beta_{42}$? Since amyloid precursor protein (APP) is a membrane-bound protein influenced by membrane biophysics and trafficking (Askarova et al., 2011; Tan and Gleeson, 2019), we propose that changes in the lipid environment in post-mitotic neurons may influence the processing and formation of amyloidogenic or non-amyloidogenic peptides.

Fatty acyls are a major component of membrane lipids and can influence AD pathology in several ways. Palmitoylation of APP influences $A\beta_{42}$ formation (Bhattacharyya et al., 2013; Andrew et al., 2017), and the ratios of different fatty acids in gangliosides have been shown to influence aggregation of $A\beta_{42}$ (Oikawa et al., 2009; Oikawa et al., 2015). The fatty acyl composition of cellular membranes contributes to their physical properties and the activities of transmembrane proteins. For example, the amount of cholesterol and the ratios of saturated to unsaturated fatty acids known to influence the biophysical properties of membranes have been shown to increase the interaction of APP with beta-secretase in early AD (Kametaka et al., 2003; Marlow et al., 2003; Avila-Munoz and Arias, 2015). In rodent studies, high saturated fat diets favor amyloid deposition, while PUFA (DHA) supplemented diets decrease amyloid accumulation and attenuate glial cell activation (Oksman et al., 2006). In addition to membrane fluidity, PUFAs are associated with cognitive function and memory (Cardoso et al., 2016). Supplementation with DHA/EPA maintains levels of pro-resolving or neuro-protecting mediators (Serhan et al., 2015; Thau-Zuchman et al., 2019), suggesting a protective role of PUFAs in post-mitotic neurons. Oxidation of PUFA by an enzyme or non-enzymatic pathways generates inflammatory and toxic products, and these are increased in AD (Montine and Morrow, 2005; Lukiw and Bazan, 2008; Grimm et al., 2016). Neuroinflammatory pathways are associated with cognitive decline, and PUFAs are the source of several lipid mediators of inflammation that are altered in AD (Pomponi et al., 2008). Moreover, several enzymes that hydrolyze PUFAs, such as phospholipases A_2, are altered in AD (Stephenson et al., 1996; Farooqui and Horrocks, 1998; Sanchez-Mejia and Mucke, 2010; Fonteh et al., 2013).

With these important associations with AD pathology, we examined PUFA composition in CH individuals with normal $A\beta_{42}$/T-tau (CH-NAT) compared with elderly CH study participants with pathological $A\beta_{42}$/T-tau (CH-PAT) and with AD (Harrington et al., 2013). We show that PUFA metabolism distinguishes pre-symptomatic AD from symptomatic AD, suggesting that PUFAs may contribute to the cognitive resilience of the pre-symptomatic population. PUFA levels in CH study participants negatively correlated with $A\beta_{42}$ and positively correlated with T-tau, suggesting that PUFAs contribute to the metabolism of these peptides. Our studies suggest that early changes in PUFA metabolism may contribute to AD pathology by disrupting brain membrane structures and initiating the abnormal processing and transport of denatured proteins. Therefore, detection of early PUFA changes in the brain may reveal mechanisms that account for pre-symptomatic AD progression and can be explored to prevent AD pathology.

MATERIALS AND METHODS

Recruitment and Classification of Study Participants

All study protocols and consent forms were approved by the Institutional Review Board of the Huntington Memorial Hospital, Pasadena, CA, United States (HMH-99-09). Written informed consent was obtained from all study participants. Demographic data, medical, and diagnostic procedures have been described (Harrington et al., 2013). Participants were included (**Table 1**) if they were classified as CH-NAT, CH-PAT, or with clinically probable AD (McKhann et al., 2011).

APOE Genotype

mRNA from peripheral blood lymphocytes was used for APOE genotyping and was performed using a polymerase chain reaction mixture of specific primers for E2, E3, and E4 (Calero et al., 2009). To determine the effects of ApoE on PUFA metabolism, we pegged each participant based on their risk from ApoE4: non-carriers of ApoE4 allele (E2/3 = 1, E3/E3 = 2) were grouped together while ApoE4 carriers (E2/E4 = 3, E3/E4 = 4) and

TABLE 1 | Demographic data, CSF chemistry, APOE genotype, and plasma lipid levels in the clinical subgroups.

Parameters	CH-NAT (*n* = 36)	CH-PAT (*n* = 34)	AD (*n* = 25)
Female (%)	61	62	52
		mean ± SD	
Age (years)	76.4 ± 7.1	78.0 ± 6.5	76.0 ± 9.1
CDR (mean)			
BMI[#1]	1.5 ± 0.8	1.8 ± 0.7	1.6 ± 0.7
Education[#2]	6.3 ± 1.8	6.0 ± 2.3	3.8 ± 2.5
$A\beta_{42}$ (pg/ml)	916 ± 211	518 ± 237	460 ± 196
T-tau (pg/ml)	197 ± 67	353 170	517 ± 205
$A\beta_{42}$/T-tau	5.1 ± 1.8	1.6 ± 0.6	1.0 ± 0.5
APOE risk[#3]	3.17 ± 1.1	2.82 ± 1.3	3.52 ± 1.1
CSF protein (μg/ml)	402 ± 16	396 ± 15	358 ± 12
Triglyceride	106 ± 52	106 ± 46	102 ± 39
Total cholesterol	187 ± 29	179 ± 33	183 ± 31
HDL	64 ± 15	61 ± 18	62 ± 15
LDL	111 ± 28	104 ± 25	110 ± 27

[#1]BMI – Underweight = 0, Normal weight = 1, Overweight = 2, Obese = 3. [#2]Education – Less than HS = 0; HS Diploma = 1; Technical or trade school = 2; Some college = 3; 2-year college = 4; College, more than 2-year degree but not a 4-year degree = 5, 4-year college degree = 6; Some post-graduate = 7; [#3]APOE Genotype – increasing risk from E4 (2/2 = 0; 2/3 = 1; 3/3 = 2; 2/4 = 3; 3/4 = 4; 4/4 = 5).

homozygous ApoE4 (E4/E4 = 5) formed different groups for high and highest risks, respectively.

CSF Collection, Total Protein, $A\beta_{42}$, and T-tau Measures

Cerebrospinal fluid was collected between 8:00 a.m., and 10:00 am after an overnight fast. Total protein concentrations, $A\beta_{42}$ and T-tau assays, were performed using CSF aliquots after a single thaw as previously described (Harrington et al., 2013).

Materials

HPLC grade water, chloroform, methanol, formic acid, and anhydrous acetonitrile required for lipid extraction were purchased from VWR (West Chester, PA, United States). Hydrochloric acid and butylated hydroxytoluene (BHT) were purchased from Sigma (St. Louis, MO, United States). Linoleic Acid-d_4, α-Linolenic Acid-d_{14}, Arachidonic Acid-d_8, Eicosanoic Acid-d_3, Eicosapentaenoic Acid-d_5, Docosanoic Acid-d_{43}, and Docosahexaenoic Acid-d_5 (Avanti Polar Lipids, Alabaster, AL, United States) were used as internal standards.to monitor PUFA extraction recovery and for quantification. Non-deuterated FA standards containing a mixture of 50 free fatty acids were purchased from NuChek Prep (Elysian, MN, United States). Pentafluorobenzyl bromide (PFBBr) from Thermo Fisher Scientific (Bellafonte, PA, United States) and NN-diisopropylethanolamine (DIPE) from Sigma-Aldrich were used for synthesizing PFBBr-derivatives of PUFAs.

CSF Fractionation and Fatty Acid Extraction

CSF supernatant fluid (SF) or nanoparticle (NP) fractions were obtained by centrifugations as previously described (Harrington et al., 2009). After the addition of a deuterated fatty acid standard cocktail (100 ng each), fatty acids were extracted from each fraction using a modified Bligh and Dyer procedure (Bligh and Dyer, 1959).

Acid Hydrolysis of Extracted Lipids

We obtained the PUFA composition of each CSF fraction by first hydrolyzing aliquots (250 ul) of the lipid extracts, as previously described (Aveldano and Horrocks, 1983). The fatty acid-enriched $CHCl_3$ extract was washed using 2 mL NaCl (1 M) before the addition of 1 mL CH_3OH containing 0.1 mg/mL BHT.

Derivatization of Lipid Extracts

Hydrolyzed fatty acids from the NP or SF fractions were dried under a stream of N_2 and then converted to pentafluorobenzyl esters using a mixture of PFBBr in acetonitrile solution (1:19 v/v, 50 μL) and DIPE in acetonitrile solution (1:9 v/v, 50 μL) for 20 min at 45°C with vortexing every 10 min (Quehenberger et al., 2008). After the removal of reagents using N_2 drying, derivatized FAs were extracted with 1 mL hexane (x2) (Chilton et al., 1993). The combined hexane extract was dried under N_2, and the derivatized fatty acids were dissolved in 50 μL dodecane before transfer into GC-MS vials.

GC-MS Analyses of Derivatized Fatty Acids

We obtained fatty acid levels in CSF fractions using GC negative ion chemical ionization MS (Chilton et al., 1993). Single ion monitoring was used to measure carboxylate ions for deuterated standards and samples. We used the same list for carboxylate ions (m/z) of non-deuterated and deuterated fatty acid standards (Fonteh et al., 2014).

CSF Nanoparticle (NP) Sizing

Particle size number and distribution in CSF was determined using a NanoSight NS300 instrument (Malvern Panalytical Inc., Westborough, MA, United States). Briefly, freshly collected CSF was centrifuged (3000 RCF, x 3 min), and 1 ml aliquots were frozen. CSF was diluted 10-fold (dd-H_2O) and continuously infused into the NS300 that had been calibrated with polystyrene beads (30 nm, 100 nm, and 400 nm). The sample temperature was maintained at 25°C, and recordings were obtained at 432 nm for 60 s (x5) with 10 s delay between recordings. The data were acquired and processed using the Nanoparticle Tracking Analysis software (Malvern Panalytical Inc.).

Data Analyses

MassHunter Workstation Software (Agilent) was used to analyze GC-MS fatty acid data. Calibration and standard curves were obtained using deuterated fatty acid standards All CSF samples and standards were analyzed in replicates, followed by automatic peak integration for most fatty acids.

Statistical Analyses

ANOVA with Tukey's Multiple Comparison tests or the Mann–Whitney *U* tests were performed to determine significant differences in fatty acid levels between CH-NAT, CH-PAT, and AD study participants. Spearman's rank analyses were used to determine the correlation of PUFAs with $A\beta_{42}$ or T-tau. Receiver operating characteristic (ROC curve) was performed to determine if larger or smaller values of fatty acids can classify AD or CH-NAT over the CH-PAT subjects. All statistical analyses were performed using GraphPad Prism software (La Jolla, CA, United States) or MetaboAnalyst software and data were considered significant if adjusted P for false discovery rate was <0.05 (Chong et al., 2018). Briefly, data in an Excel sheet was coded for CH-NAT (=1), CH-PAT (=2), and AD (=3), and the file was converted to tab-delimited text (.txt) prior to import into the MetaboAnalyst Statistical Analysis platform. The data were normalized using globalized logarithm transformation (glog) and then scaled by mean-centering before ANOVA with Tukey *post hoc* analyses (Chong et al., 2019). This data processing resulted in a Gaussian distribution and scaling enabled us to compare PUFA levels that are several orders of magnitude in CSF fractions.

RESULTS

Clinical Demographics and AD Risk Factors

The demographic data, AD risk factors, and CSF $A\beta_{42}$, and T-tau levels are shown in **Table 1**. The AD group was less educated than the other groups, as we and others previously reported (Roe et al., 2008; Harrington et al., 2013). The rank order of CSF $A\beta_{42}$ was CH-NAT > CH-PAT > AD while this was reversed for T-tau. This resulted in a significantly lower CSF $A\beta_{42}$/T-tau in CH-PAT and AD compared with CH-NAT (**Table 1**). Thus, the $A\beta_{42}$/T-tau pathology distinguished two CH groups, one set with higher $A\beta_{42}$/T-tau (CH-NAT) and another group (CH-PAT) with $A\beta_{42}$/T-tau values similar to AD. We describe this group as a pre-symptomatic AD cohort because 4 years longitudinal follow-up study shows that 25% of these participants progress to MCI or AD while no CH-NAT participants deteriorate in the same period (Harrington et al., 2019).

Dietary Fatty Acids and AD

We obtained fasting levels of triglycerides, cholesterol, HDL, and LDL to isolate dietary influence on fatty acid metabolism. No differences were detected in each lipid class in our clinical groups (**Table 1**). Using a validated self-reported Dietary Health Questionnaire (DHQ), energy and consumption, and dietary fatty acid compositions were accessed. No significant differences were measured in the dietary levels of fatty acids by CH-NAT and CH-PAT (**Table 2**).

Fatty Acid Composition of CSF Fractions

We next measured PUFA levels in CSF fractions: nanoparticles derived from brain membranes (npPUFA), supernatant fluid representative of interstitial metabolism (sfPUFA), and unesterified PUFA levels (uPUFA) resulting from lipolytic enzyme activities that increase in AD. The proportion of several n-6 and n-3 species varied between the various fractions (**Figures 1A–C**). Our data show the differential distribution of PUFAs in CSF fractions. The proportion of n-6 PUFAs was highest in the SF fraction (**Figure 1D**), while n-3 was highest in the unesterified fractions (**Figure 1E**). Total PUFA was lowest in the NP fraction (**Figure 1F**). Differences in the distribution of PUFAs in CSF fractions are supported by principal component analyses, showing the interaction of npPUFA with uPUFA but not with sfPUFA (**Figure 1G**).

TABLE 2 | Energy and dietary lipid consumption – Cognitively healthy study participants completed an online DHQ, and their lipid consumption was estimated using DietCal (NCI-NIH).

DHQ item	CH_NAT Mean (SEM)	CH_PAT Mean (SEM)
Food energy (kcal)	1824 ± 124	1656 ± 110
Total fat (g)	63 ± 24	55 ± 21
Carbohydrate (g)	244 ± 18	234 ± 16
Protein (g)	65 ± 4	57 ± 4
Cholesterol (mg)	190 ± 14	161 ± 14
Saturated fat (g)	21 ± 2	18 ± 1
Monounsaturated fat – g	23 ± 2	20 ± 2
Polyunsaturated fat – g	14 ± 1	13 ± 1
Fatty acid 20:4 – g	0.09 ± 0.007	0.07 ± 0.008
Fatty acid 20:5 – g	0.03 ± 0.006	0.04 ± 0.008
Fatty acid 22:6 – g	0.06 ± 0.008	0.06 ± 0.01
Fatty acid 10:0 – g	0.4 ± 0.05	0.3 ± 0.04
Fatty acid 14:0 – g	1.9 ± 0.2	1.6 ± 0.2
Fatty acid 16:1 – g (CSFII)	1 ± 0.1	0.9 ± 0.07
Fatty acid 18:2 – g (CSFII)	12.7 ± 0.9	11.3 ± 0.9
Fatty acid 18:3 – g	1.4 ± 0.1	1.2 ± 0.1
Fatty acid 22:5 – g	0.01 ± 0.002	0.01 ± 0.002
18:2 *TRANS* (*trans*-octadecadienoic acid [linolelaidic acid]) – g (NDS-R)	0.5 ± 0.04	0.4 ± 0.04
Dietary fiber – g (CSFII)	21 ± 2	21 ± 2
Total dietary fiber – g (NDS-R)	20 ± 2	20 ± 2
Insoluble dietary fiber – g (NDS-R)	13 ± 1	13 ± 1
Soluble dietary fiber – g (NDS-R)	7 ± 1	7 ± 1

Changes in Fatty Acid Composition in the CSF Fractions

PUFA Concentration and Composition in NP Fractions (Figure 2 and Supplementary Table 1)

The concentrations of n-3 npPUFA (**Figure 2A**), n-6 npPUFA (**Figure 2B**), and npPUFA (**Figure 2C**) were similar in all clinical groups. However, when expressed as a proportion of all fatty acids in the NF fraction, n-6 npPUFA was significantly lower in AD than in CH-PAT (**Figure 2E**) while n-3 npPUFA and npPUFA trended lower in AD than in CH-NAT (**Figure 2D**) and CH-PAT (**Figure 2F**). Further ANOVA analyses of individual PUFAs

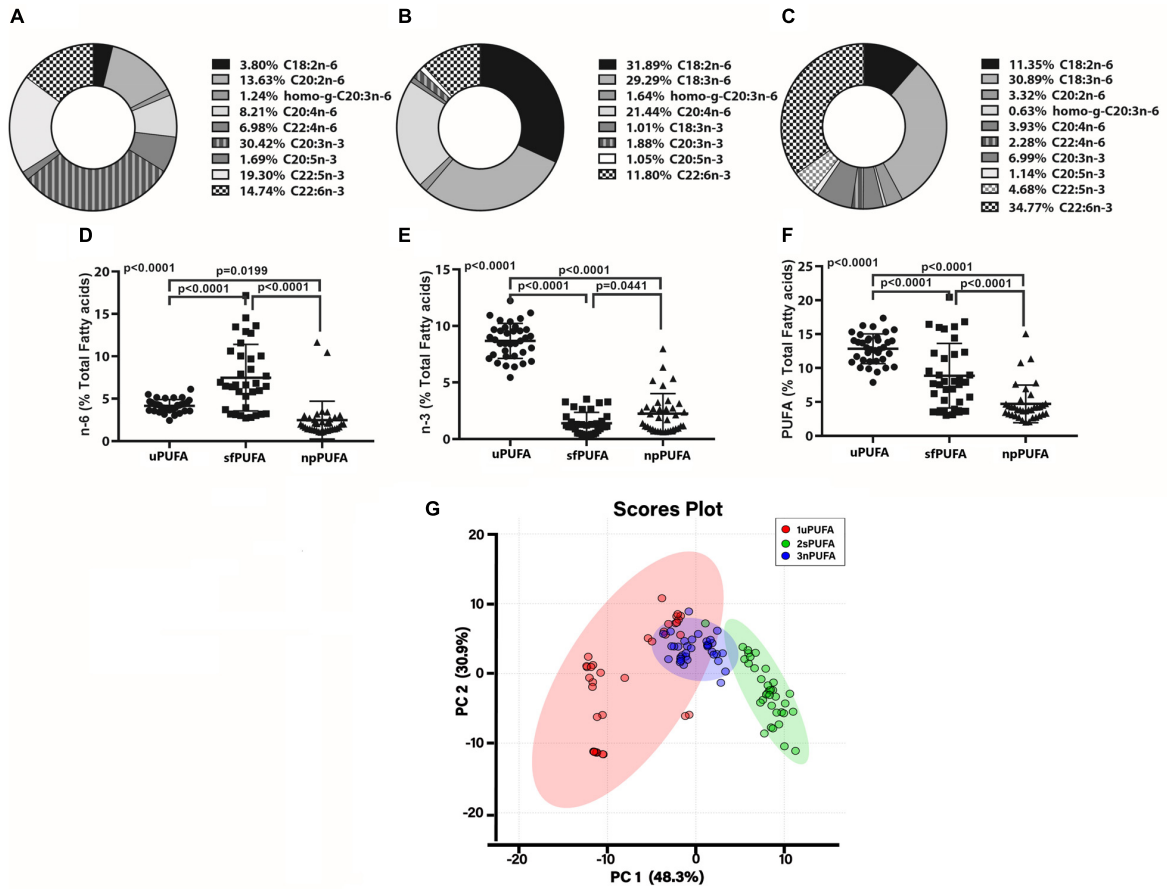

FIGURE 1 | PUFA distribution in CSF fraction – The doughnut graphs show the proportion of n-3 and n-6 PUFAs quantified in the NP fraction of CSF **(A)**, the supernatant fluid fraction **(B)**, or as unesterified fatty acids **(C)**. Comparison of the n-6 PUFA amounts in the three CSF fractions **(D)**, n-3 PUFA in the fractions **(E)**, and total PUFAs **(F)** using 1-way ANOVA with Dunn's multiple tests showing adjusted *p*-values. PCA of the distribution of PUFA species in the CSF fractions **(G)**.

showed that four PUFAs (C20:2n-6, C20:3n-3, C22:4n-6, C22:5n-3) were enriched in nanoparticles of AD compared with CH-NAT (**Supplementary Table 1**).

NP Concentration Adjusted for Nanoparticle Counts

Nanoparticle Tracking Analysis showed that the CSF is enriched with billions of nanoparticles. The average number of particles in CH-NAT and CH-PAT was lower than in AD. Levels of n-3 npPUFAs per billion particles trended lower in AD than in CH-NAT and CH-PAT (**Figure 2G**). N-6 npPUFA and n-3 npPUFA were significantly lower in AD than CH-NAT and CH-PAT (**Figures 2H,I**, respectively).

PUFA Concentration and Composition in the SF Fraction (Figure 3, Supplementary Table 2)

The concentration of n-3 sfPUFA was significantly lower in AD than in CH-NAT and CH-PAT (**Figure 3A**) while the concentration of n-6 sfPUFA (**Figure 3B**), and sfPUFA (**Figure 3C**) were similar in all clinical groups. The proportion of n-3 sfPUFA, n-6 sfPUFA, and sfPUFA was similar in all clinical groups (**Figures 3D–F**, respectively). Further ANOVA analyses of individual fatty acids showed levels of three PUFAs (C18:3n-6, C20:4n-6, and C22:6n-3) were ranked CH-PAT > CH-NAT > AD (**Supplementary Table 2**). However, only DHA reached statistical significance with the highest level in CH-PAT compared with AD (**Supplementary Table 2**).

Unesterified PUFA Concentration and Composition (Figure 4, Supplementary Table 3)

The concentration of n-3 uPUFA was significantly lower in AD than in CH-NAT and CH-PAT (**Figure 4A**), while the concentration of n-6 uPUFA was similar in all clinical groups. Similar to n-3 uPUFA, uPUFA levels were lower in AD compared with CH-NAT and CH-PAT (**Figure 4C**). The proportions of n-3 uPUFA, n-6 uPUFA, and uPUFA were higher in CH-PAT than in AD (**Figures 4D–F**, respectively). Of several uPUFAs species quantified in CSF, the general trend was CH-NAT > CH-PAT > AD for C18:2n-6 and C20:4n-6 (**Supplementary Table 3**).

Correlation of PUFAs With $A\beta_{42}$ and Tau (Table 3)

To further test our hypothesis that changes in PUFA composition can influence amyloid-beta metabolism, we performed Spearman

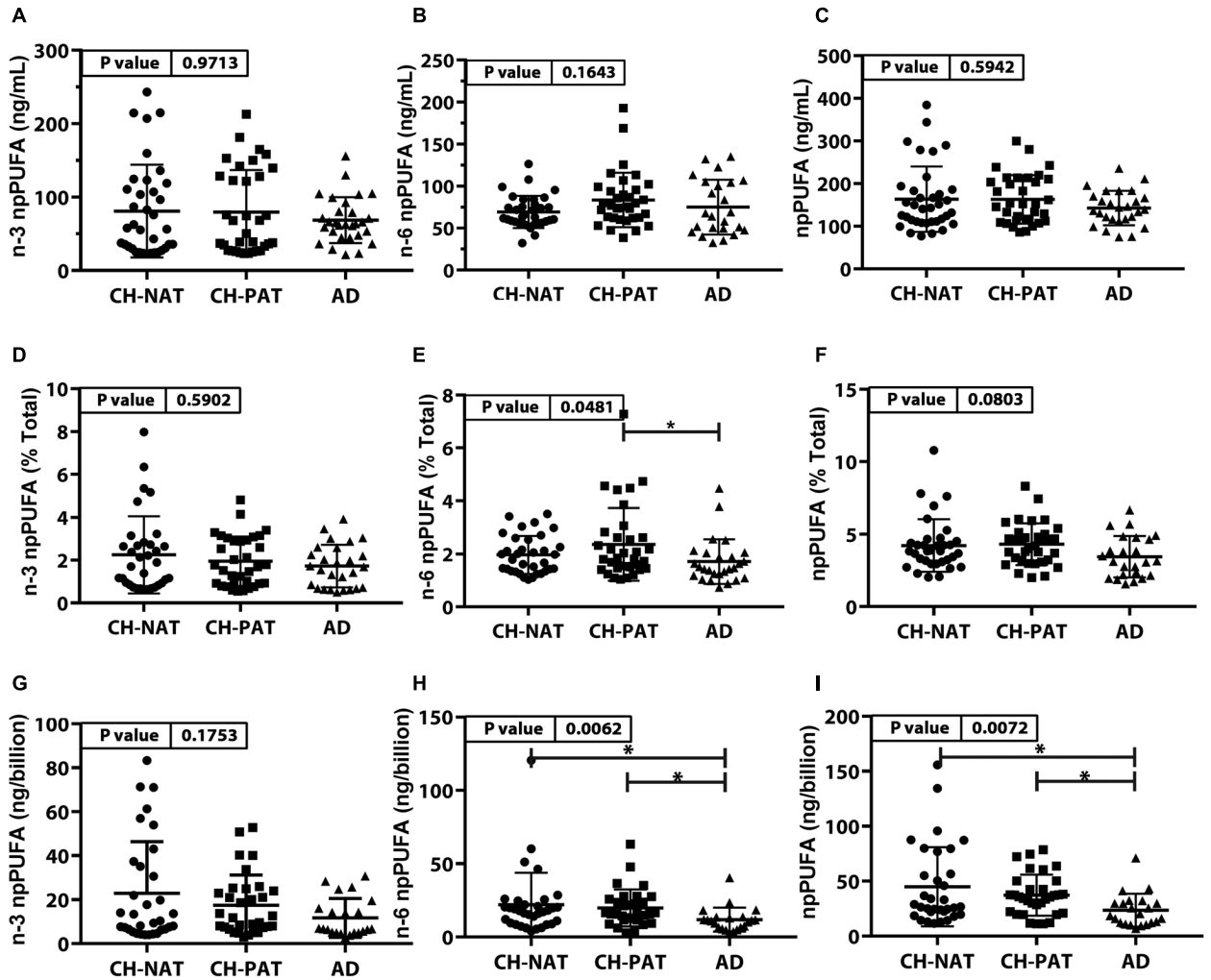

FIGURE 2 | PUFAs in nanoparticles from CH-NAT, CH-PAT, and AD – Scatter plots (mean ± SD) of the concentrations of n-3 npPUFAs **(A)**, n-6 npPUFA **(B)**, and npPUFA **(C)** showing *p*-values determined using 1-way ANOVA and adjusted *p*-values < 0.05 indicated by an asterisk (*). Scatter plots of the proportions of n-3 PUFAs **(D)**, n-6 npPUFA **(E)**, and npPUFA **(F)** showing *p*-values obtained using 1-way ANOVA and adjusted *p*-values < 0.05 indicated by an asterisk (*). Concentrations of npPUFAs per billion nanoparticles for n-3 npPUFAs **(G)**, n-6 npPUFA **(H)**, and npPUFA **(I)** showing *p*-values obtained using 1-way ANOVA and adjusted *p*-values < 0.05 indicated by an asterisk (*).

ranked correlation analyses comparing fatty acids in each CSF fraction with CSF amyloid and T-tau levels in all study participants and in each subgroup. We did not find any correlation between CSF PUFA and $A\beta_{42}$/T-tau in all study participants, probably due to the complex pathology of the three clinical groups. Therefore, we examined the CH, the CH-NAT, and CH-PAT groups separately.

CH– For all CH participants, C18:2n-6 and n-6 npPUFA in the NP fractions negatively correlated with $A\beta_{42}$ levels (**Table 3**). For the SF PUFAs, C20:4n-6, C22:6n-3, and n-3 positively correlated with $A\beta_{42}$.

CH-NAT – No NP PUFAs correlated with $A\beta_{42}$ levels. For uPUFAs, our studies show that three PUFAs (C22:5n-3, C22:6n-3), total PUFA, and n-3 PUFA inversely correlated with $A\beta_{42}$. The ratio C20:4n-6/(C20:5n-3+C22:6n-3) positively correlated with $A\beta_{42}$ levels. C20:4n-6 in NP and C22:6 in SF positively correlated with total T-tau. C18:2n-6 in the SF fraction negatively correlated with T-tau levels.

CH-PAT. In the NP fraction, C18:3n-6, n-6/n-3 PUFA ratio,C20:4n-6/C22:6n-3 and C20:4n-6/(C20:5n-3+C22:6n-3) ratios were inversely correlated with CSF $A\beta_{42}$ levels. C20:4n-6, C22:6n-3 and n-3 PUFAs positively correlated with T-tau in the SF fraction.

ROC Analyses to Determine Fatty Acid Classifiers of Clinical Groups (Figure 5)

ROC analyses were performed between clinical groups to determine if PUFA levels in CSF fractions could differentiate the three clinical groups. Among all PUFAs analyzed, only two were able to classify between CH-NAT and CH-PAT subjects. Both were NP derived n-6 PUFA fatty acids and higher levels classified

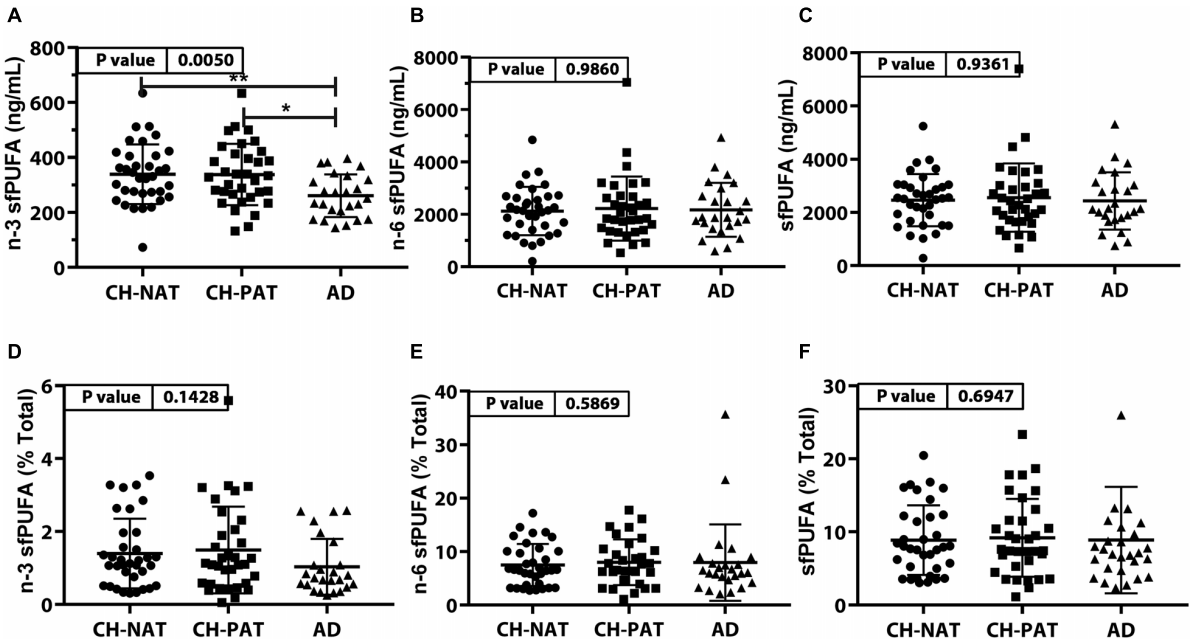

FIGURE 3 | PUFAs in supernatant fluid (sf) – Scatter plots (mean ± SD) of the concentrations of n-3 sfPUFA **(A)**, n-6 sfPUFA **(B)**, and sfPUFA **(C)** showing *p*-values determined using 1-way ANOVA and adjusted *p*-values < 0.05 indicated by an asterisk (*). Plots of the proportions of n-3 sfPUFA **(D)**, n-6 sfPUFA **(E)**, and sfPUFA **(F)** showing *p*-values obtained using 1-way ANOVA and adjusted *p*-values < 0.05 indicated by an asterisk (*).

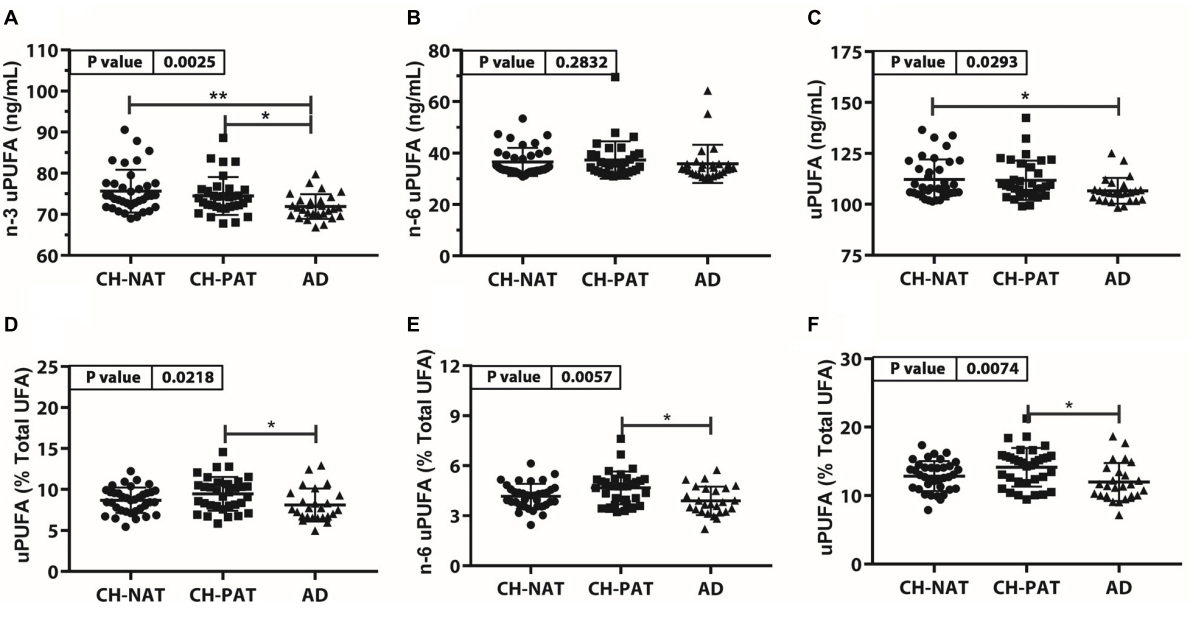

FIGURE 4 | Unesterfied PUFAs (uPUFA) in CH-NAT, CH-PAT, and AD – Scatter plots (mean ± SD) of the concentrations of n-3 uPUFAs **(A)**, n-6 uPUFA **(B)**, and uPUFA **(C)** showing *p*-values determined using 1-way ANOVA and adjusted *p*-values < 0.05 indicated by an asterisk (*). Plots of the proportions of n-3 uPUFAs **(D)**, n-6 uPUFA **(E)**, and uPUFA **(F)** showing *p*-values obtained using 1-way ANOVA and adjusted *p*-values < 0.05 indicated by an asterisk (*).

CH-PAT with AUC of 0.65 ($P = 0.027$) and 0.64 ($P = 0.042$) for C18.2n-6 and C20:4n-6, respectively.

The most striking changes were noticed when ROC analyses were performed for CH-NAT compared with AD. Four (C20:2n-6, C20:3n-3, C20:3n-6, and C22:5n-3) of the nine free PUFAs and levels of n-3 PUFAs were able to perform as binary classifiers of AD. In the SF fraction, two PUFAs (C20:3n-3, C22:6n-3), and levels of N-3 PUFAs performed as binary classifiers of AD subjects. Of the ten NP PUFAs analyzed, five were found to perform as binary classifiers of AD. ROC curve analysis showed

TABLE 3 | Fatty acids that correlate with CSF Aβ42 or T-tau levels.

CSF fraction	CH	CH-NAT	CH-PAT
Fatty acid levels that correlate with CSF $A\beta_{42}$			
NP PUFA	C18:2n-6, (−0.3)*		C18:3n-6, (−0.34)*
	n-6, (−0.25)*		N-6/N-3 npPUFA, (−0.38)*
			AA/DHA, (−0.37)*
			AA/(EPA+DHA), (−0.35)*
Unesterified PUFA		C22:5n-3, (−0.38)*	
		C22:6n-3, (−37)*	
		n-3 uPUFA, (−0.38)*	
		uPUFA, (−0.37)*	
		AA/(EPA+DHA), (0.46)**	
Fatty acid levels that correlate with CSF T-tau levels			
NP PUFA		C20:4n-6, (0.38)*	
SF PUFA	C20:4n-6, (0.26)*	C18:2n-6, (−0.38)*	C20:4n-6, (0.35)*
	C22:6n-3, (0.43)***	C22:6n-3, (0.40)*	C22:6n-3, (0.54)**
	n-3, (0.29)*		n-3, (0.48)**

Spearman correlation coefficient (ρ) with $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.005$ (***).

that lower levels of C20:5n-3 classified AD while higher levels of C20:3 (n-3), C20:4, C22:4, and C22:5n-3 were shown to be classifiers of AD.

We next examined whether PUFAs in CSF fractions distinguished CH-PAT from AD. None of the PUFAs in the NP fractions were classifiers of CH-PAT/AD (data not shown). However, the proportion of PUFA, n-6 PUFA, C18:3n-6, and C18:2n-6 were significant classifiers of CH-PAT and AD (**Figure 5A**). More significantly is the expression of NP PUFA per billion NPs that showed that PUFAs ($p < 0.005$), n-6 PUFA ($p < 0.005$), C22:4n-6 ($p = 0.05$), C30:4n-6 (AUC = 0.70, $p < 0.01$), C18:3n-6 ($p < 0.005$), and C18:2n-6 ($p < 0.005$) were binary classifiers of CH-PAT and AD (**Figure 5B**). For PUFAs in the SF fraction, n-3 PUFA ($p < 0.005$), C22:6n-3 ($p < 0.05$), and C20:3n-3 ($p < 0.01$) (**Figure 5C**), were accurate classifiers of CH-PAT and AD, while the proportion of PUFAs in the SF fraction did not significantly classify these clinical groups (**Figure 5D**). In the unesterified fractions, levels of PUFA, n-3, C22:6n-3, and C20:2n-6 were binary classifiers of CH-PAT and AD (**Figure 5E**). When expressed as a proportion of all unesterified fatty acids, PUFA, n-3, n-6, C22:6n-3, C20:4n-6, and C20:2n-6 were binary classifiers of CH-PAT and AD (**Figure 5F**).

DISCUSSION

As a neurodegenerative disease, AD is irreversible and progresses over many years before symptoms are evident (Filley, 1995). While CSF $A\beta_{42}$ is recognized as an early indicator of AD, it is known that this pathology can present for several decades before the onset of clinical symptoms, while p-Tau levels are closely linked with symptoms (Wallin et al., 2006). We identified a group of cognitively normal subjects with CSF $A\beta_{42}$ similar to symptomatic AD (Harrington et al., 2013). Given that amyloid precursor protein (APP) is a membrane-bound protein, we hypothesize that changes in membrane lipid components will influence APP processing, and these changes are potential early indicators of AD. To test this hypothesis, we examined fatty acid levels in three clinical groups; our CH population, into a group with normal $A\beta_{42}$ and T-tau ratio (CH-NAT) and a second group with pathological $A\beta_{42}$/T-tau ratio (CH-PAT) similar to AD subjects. The aim of our study was to determine if PUFA differences in CSF fractions can classify these subgroups and isolate CH-PAT from AD that has similar CSF $A\beta_{42}$/Tau ratios. The major findings of our study include: (1) No differences in plasma LDL and HDL, and no significant differences in self-reported consumption of fatty acids between the three clinical groups. (2) Significant differences in some PUFA levels in CSF fractions between our three clinical groups. (3) The progressive decrease in the amount of PUFA per billion nanoparticles in CH-NAT to CH-PAT to AD. (4) Correlation of some PUFA species with CSF $A\beta_{42}$ or with CSF T-tau. (5). ROC analyses showing that some CSF PUFAs can perform as effective binary classifiers of our clinical subgroups. The implications of our study are that early changes in brain PUFA compositions are reliable indicators of AD pathology. Importantly, PUFAs contribute to the cognitive resilience in our pre-symptomatic AD population.

Figure 6 illustrates the factors that may influence PUFA metabolism and the clinical consequences of such changes. Several risk factors acting independently or interacting with each other can influence plasma PUFA metabolism, resulting in changes in plasma and CSF PUFA levels. Our studies show changes in PUFA species in CSF fractions that may be representative of changes in brain cells. The three different fractions have different PUFA composition and differentially affected by AD pathology. There is a similarity in the PUFA composition of nanoparticles (npPUFA) and with unesterified species (uPUFA). These data suggest that membrane-bound phospholipases that have been shown to localize with amyloid plaques may be involved in the release of uPUFA from npPUFA. Considering differences in our clinical groups, CH-NAT versus AD represented the greatest changes in PUFA. We also noticed that DHA was the most consistent difference between CH-PAT and AD, suggesting that the cognitive resilience shown by the CH-PAT participants may be attributed to the higher DHA concentrations in their fractions. Overall, CH-NAT study participants display homeostasis in PUFA metabolism and are, therefore, cognitively healthy. In CH-PAT, there are early signs of dysfunction in PUFA metabolism, while in the AD brain, there is dysfunction in PUFA metabolism. Dysfunction in PUFA metabolism contributes to neuronal apoptosis that subsequently results in the neuropsychological deficits that characterize AD.

Since LDL/HDL levels and self-reported fatty acid consumption are similar in our clinical groups, it implies that changes we observe in CSF fractions are due to transport to the brain or *in vivo* metabolism of fatty acids. Of all fatty acids detected, we found significant changes in several PUFAs, including AA (C20:4n-6) and DHA (C22:6n-3). These changes in

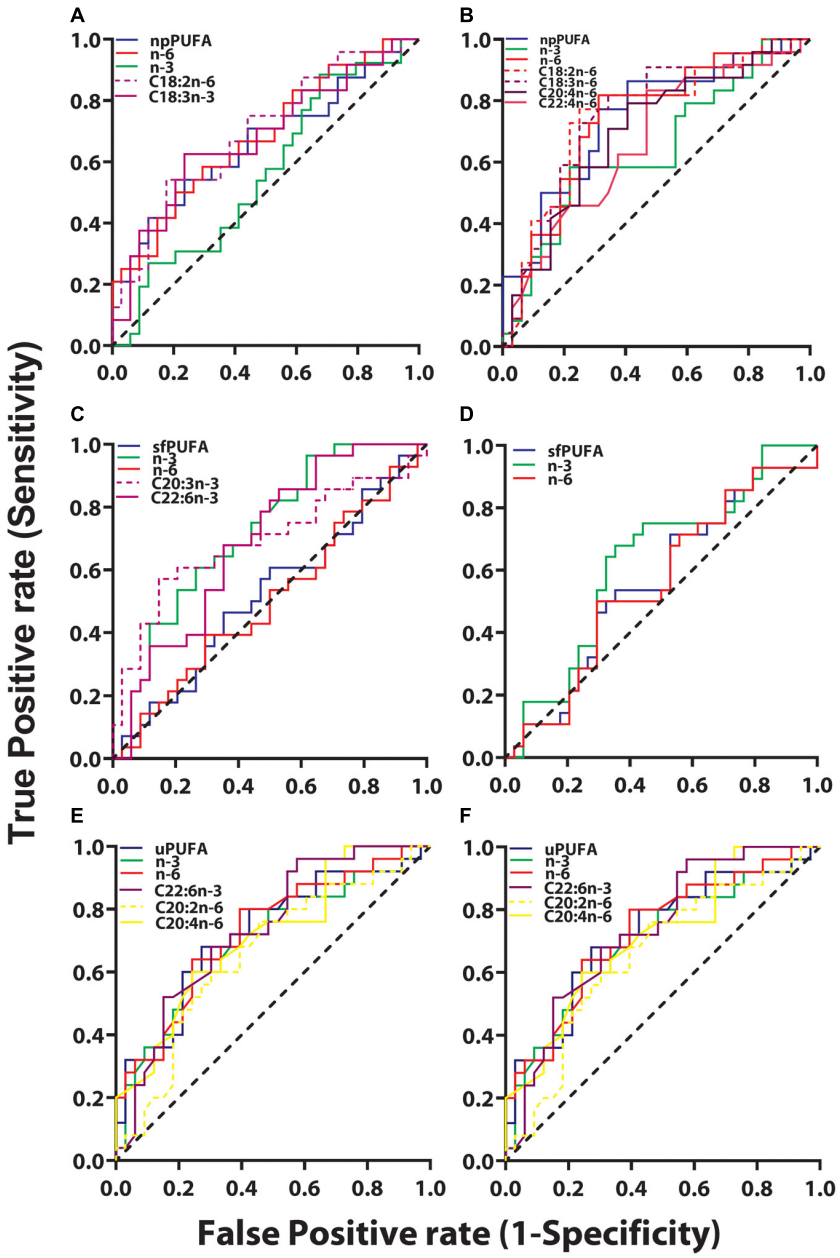

FIGURE 5 | ROC for CH-PAT versus AD – *ROC of PUFA concentrations in the NP fraction (npPUFA)*- The proportion of PUFA (AUC = 0.66, $p = 0.0344$), n-6 PUFA (AUC = 0.68, $p = 0.0195$, C18:3n-6 (AUC = 0.68, $p = 0.0221$), and C18:2n-6 (AUC = 0.69, $p = 0.0126$) were significant classifiers of CH-PAT and AD **(A)**. More significantly is the expression of NP PUFA per billion NPs that showed that PUFAs (AUC = 0.75, $p = 0.0023$), n-6 PUFA (AUC = 0.74, $p = 0.0031$), C22:4n-6 (AUC = 0.67, $p = 0.0348$), C30:4n-6 (AUC = 0.70, $p = 0.0116$), C18:3n-6 (AUC = 0.76, $p = 0.0015$), and C18:2n-6 (AUC = 0.75, $p = 0.0019$) were binary classifiers of CH-PAT and AD **(B)**. *ROC of PUFA concentrations in the SF fraction (sfPUFA)*- For PUFAs in the SF fraction, n-3 PUFA (AUC = 0.72, $p = 0.0032$), C22:6n-3 (AUC = 0.69, $p = 0.0113$), and C20:3n-3 (AUC = 0.69, $p = 0.0096$) **(C)**, were accurate classifiers of CH-PAT and AD, while the proportion of PUFAs in the SF fraction did not significantly classify these clinical groups **(D)**. *ROC for PUFA concentrations in the unesterified fraction (uPUFA).* In the unesterified fractions, levels of PUFA (AUC = 0.70, $p = 0.0083$), n-3 (AUC = 0.68, $p = 0.0197$), C22:6n-3 (AUC = 0.66, $p = 0.0334$), C20:2n-6 (AUC = 0.72, $p = 0.0046$) were binary classifiers of CH-PAT and AD **(E)**. When expressed as a proportion of all fatty acids, PUFA (AUC = 0.70, $p = 0.0094$), n-3 (AUC = 0.72, $p = 0.0041$), n-6 (AUC = 0.73, $p = 0.0032$), C22:6n-3 (AUC = 0.73, $p = 0.0024$), C20:4n-6 (AUC = 0.71, $p = 0.0059$), and C20:2n-6 (AUC = 0.66, $p = 0.0428$) were binary classifiers of CH-PAT and AD **(F)**.

PUFA metabolism may play an important role in the progression of AD pathology by not only influencing $A\beta_{42}$ formation but also affecting mitochondrial energy homeostasis and the generation of inflammatory or pro-resolving and immune modulators.

PUFA and Cognitive Function

PUFAs influence cognitive outcomes with several studies showing that n-3 supplementation improved cognitive measurements (Chiu et al., 2008). Changes in n-3 PUFAs

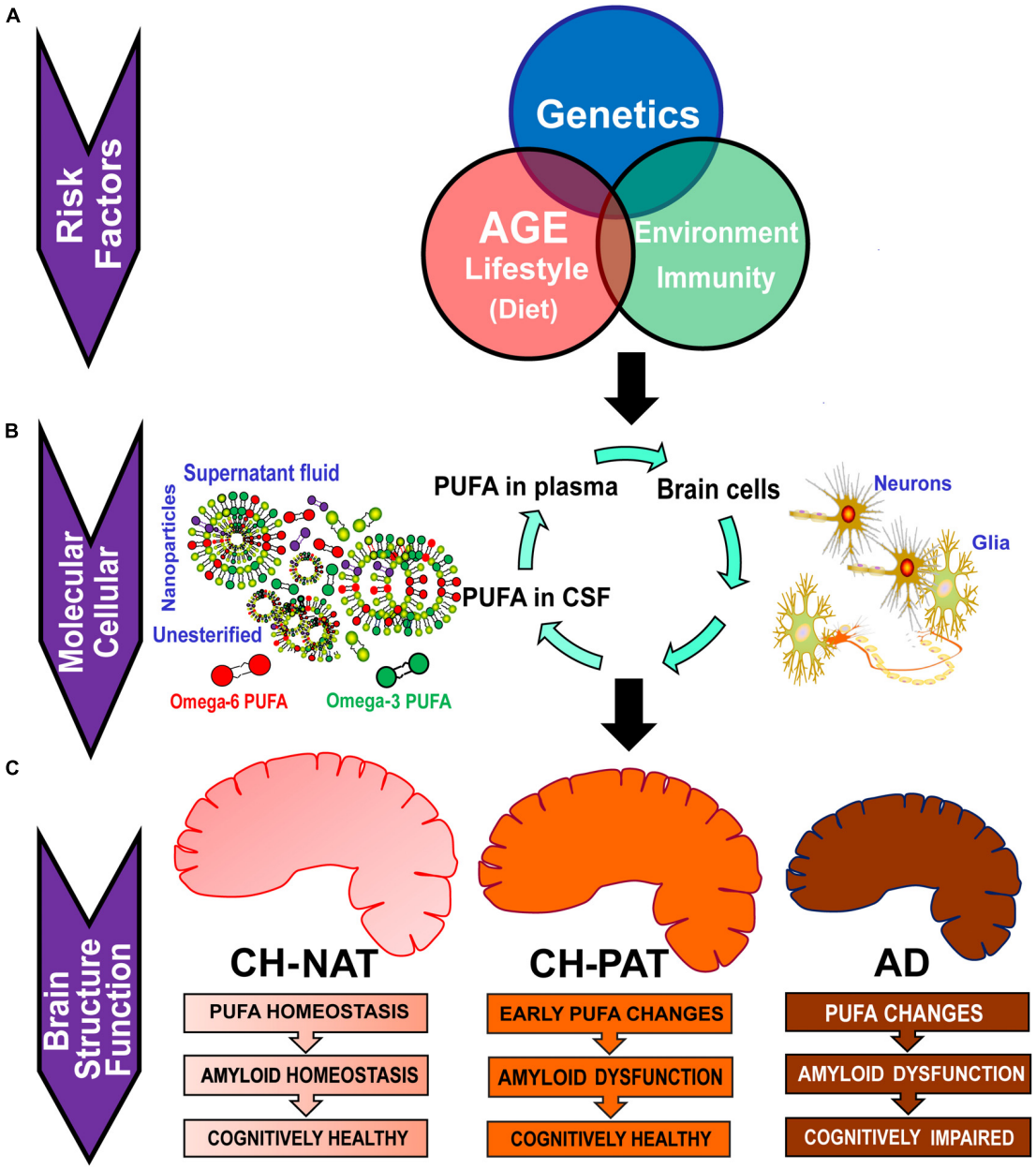

FIGURE 6 | Potential factors that contribute to the dysfunction in PUFA homeostasis in AD. (A) Risk factors such as genetic variants, age, lifestyle, and environmental exposure can influence plasma PUFA metabolism. **(B)** Changes in plasma and CSF PUFA levels are manifested at the molecular and cellular levels. Plasma PUFAs are transported into the brain where they are used for signaling, immune response, or for the energy needs of the brain. CSF PUFA is considered a fair representation of brain metabolism. PUFAs in CSF are found in nanoparticles (npPUFA), in the supernatant fluid (sfPUFA), or as unesterified species (uPUFA). **(C)** In CH-NAT study participants, there is homeostasis in PUFA metabolism resulting in cognitively healthy brain function. In the CH-PAT brain, there are early signs of dysfunction in PUFA metabolism but not significant enough to cause cognitive decline, while in the AD brain, there is dysfunction in PUFA metabolism. Whereas $A\beta_{42}$ levels are similar in CH-PAT and AD, cognitive resilience in CH-PAT is allied with the higher PUFA levels in the brain. We conclude that dysfunction in PUFA homeostasis contributes to neuronal apoptosis that subsequently results in the neuropsychological deficits that characterize AD.

levels in red blood cells are linked to visual memory, executive function, and abstract thinking (Tan et al., 2012). Another study found that lower n-6 to n-3 ratios predicted executive function with adolescents performing better on tests of cognitive function and holding shorter processing times on memory tasks (Sheppard and Cheatham, 2013), In contrast, increased inflammatory n-6 cascade results in heightened IL-1β and a subsequent decline in working memory in a rodent model (Matsumoto et al., 2004). N-3 PUFAs are known to influence brain structure by increasing synaptic protein expression leading to increases in numbers of c-Fos-positive neurons and hippocampal neurogenesis (Wang et al., 2010). Our study shows

that n-6 and n-3 fatty acids inversely and directly correlate with $A\beta_{42}$ or with T-tau, respectively. In addition, whereas $A\beta_{42}$ levels are similar in CH-PAT and AD, several PUFAs in CSF fractions are significantly lower in AD than in CH-PAT. While many n-3 fatty acids decreased, higher levels of two NP n-6 FAs (C20:4n-6 and C22:4n-6) classified AD participants (**Figure 5**). These data suggest that PUFA changes are closely linked to cognitive resilience in our CH-PAT population than $A\beta_{42}$ levels. However, the inverse correlation of PUFA with $A\beta_{42}$ suggests there is an association between PUFA and the clearance of neurotoxic $A\beta_{42}$ from the brain. Thus, changes in different PUFA types and ratios may impact brain structure and result in cognitive changes associated with AD pathology.

PUFA and $A\beta_{42}$

We recently showed differences in fatty acid composition in CSF fractions from CH, MCI, and AD individuals (Fonteh et al., 2014). Fatty acyls incorporated into glycerophospholipids or sphingolipids are major components of the cell membrane of brain cells. These membrane lipids create the appropriate environment for ion channels, receptors, structural proteins, and transmembrane proteins, including APP, needed for the proper functioning of brain cells. Disruption of membrane components, including fatty acid composition, can alter interactions with these proteins. For example, physical measurements show that the ratio of saturated to PUFA influences how $A\beta_{42}$ binds to BACE (Cole and Frautschy, 2006; Grimm et al., 2011; Eto et al., 2019; Marwarha et al., 2019). Similar changes in fatty acids that we measured in CSF fractions, and their correlation with $A\beta_{42}$ and T-tau in our clinical groups confirm the role of fatty acids in AD severity and progression.

Fatty acids are also involved in posttranslational modification of membrane proteins. The palmitoylation of APP is known to influence its processing (Bhattacharyya et al., 2016). We also found that DHA is higher CH than in AD, free DHA negatively correlates with $A\beta_{42}$ in CH-NAT, and positively correlates with T-tau in SF from CH-NAT and CH-PAT. Given studies showing the non-amyloidogenic and the anti-amyloidogenic role of DHA and its product, neuroprotectin D1 (Sahlin et al., 2007; Eckert et al., 2011; Grimm et al., 2011; Zhao et al., 2011; Grimm et al., 2016), these changes in DHA and other PUFAs in CSF fraction could determine APP processing and changes in cognitive performance of our study population.

PUFA, Inflammation, and Oxidative Stress

Modifications of PUFAs by enzyme oxidation or auto-oxidation can alter the physical properties of neuronal membranes, generate inflammatory mediators, or pro-resolving anti-inflammatory mediators (Qu et al., 2015; Bazan et al., 2017; Poreba et al., 2017). Auto-oxidation forms isoprostanes that are indicators of oxidative stress and are neurotoxic. On the other hand, resolvins and neuroprotectins resolve inflammation and are involved in the repair of post-mitotic brain cells (Heras-Sandoval et al., 2016; Ho et al., 2018). Therefore, a balance in the levels of n-6 to n-3 levels can impact neuronal function and survival. The importance of DHA is revealed by studies showing that supplementation showed a correlative relationship with immunoregulation in AD (Freund-Levi et al., 2014). Similarly, hematocytes treated with EPA have reduced IL-1β/IL-10 ratio and IL-6/IL-10 ratio (Serini et al., 2012). This anti-inflammatory role for n-3 PUFA is counteracted by the inflammatory effects of n-6 PUFA AA. AA metabolites are associated with cellular redox increase of Cox-2 expression, while n-3 PUFA decreases Cox-2 expression (Gravaghi et al., 2011; Mitjavila and Moreno, 2012). Several studies have documented increased oxidative stress in AD brain (Butterfield et al., 1999; Ansari and Scheff, 2010; Bonda et al., 2010; Mosconi et al., 2008; Raukas et al., 2012; Lee et al., 2013; Eckman et al., 2018) typified by increases in reactive oxygen species (ROS). ROS contribute to neurodegeneration and atrophy in neurites (Munnamalai and Suter, 2009). N-3 PUFAs have been found to lower oxidative stress and impact aging (Kiecolt-Glaser et al., 2013). In agreement with these studies, we report a decrease in DHA and an increase in AA in NP of CH-PAT compared with AD study participants. Moreover, several PUFAs are negatively or positively correlated with Aβ42 and T-tau levels in CSF, respectively (**Table 3**). These data suggest that dysregulation of PUFA metabolism occurs in preclinical AD and may be linked with a balance in inflammatory and anti-inflammatory signaling pathways. Inflammatory pathways in CH-PAT are counteracted by higher n-3 fatty acids in CSF fractions compared with AD subjects. N-3 to n-6 PUFA homeostasis may determine the inflammatory capacity of the brain with higher or lower n-3 to n-6 ratio signaling protection or progression from pre–symptomatic to symptomatic AD, respectively.

PUFA and Brain Energy

It is now recognized that neurological disorders are associated with metabolic syndromes (Farooqui et al., 2012; Lucke-Wold et al., 2014). The brain's principal normal source of energy is from sugars, but liver-derived ketone bodies become a relevant source of brain energy during fasting (Zhang et al., 2013; Wu et al., 2018). In AD, ketogenic diets do not only provide an energy source to mitigate oxidative damage associated with metabolic stress but may be crucial in mitochondrial biogenesis. Recently, ketone bodies have been shown to have an inflammasome activity (Neudorf et al., 2019). Thus, changes in ketone body precursor fatty acids that we measure in CSF may reflect metabolic stress in the brain associated with AD. Dysfunctional brain energy may impact repair of post-mitotic neuronal membranes and clearance of neurotoxic or damaged macromolecules such as $A\beta_{42}$. Although there is an increase in inflammatory n-6 fatty acids in CH-PAT, this is counteracted by increases in n-3 PUFAs that maintain pro-resolving mediators in the brain. This process protects the CH-PAT brain and maintains cognitive function, although $A\beta_{42}$ levels would indicate otherwise.

CONCLUSION

These studies support our hypothesis that brain PUFA metabolism is altered in the early phases of AD. The balance in fatty acid metabolism in CSF fractions when comparing cognitively normal subjects with normal and abnormal amyloid

to the T-tau ratio shows that PUFA metabolism is important in the clinical progression of dementia. We propose that dietary approaches that maintain normal PUFA levels, especially supplementation studies that favor pro-resolving fatty acids over inflammatory fatty acids may enhance cognitive function in an elderly population. The use of antioxidants or regulators of PUFA metabolism may maintain n-3 to n-6 balance and prevent cognitive decline in aging seniors. Further longitudinal studies to determine the rate and causes of PUFA changes are required to reveal novel biomarkers and validate potential preventive approaches.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Huntington Memorial Hospital, Pasadena, California IRB #HMH-99-09. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AF and MH contributed to the conceptualization and study design, writing of original draft and manuscript preparation, project administration, resources, and funding acquisition. AF, MH, MC, AC, and SE contributed to the methodology. AF contributed to the validation and supervision. AC, SE, XA, and AF contributed to the formal analysis. AC, MC, and AF contributed to the data curation. XA, MH, and AF contributed to the manuscript review and editing.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2020.00083/full#supplementary-material

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REVIEW
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Lipid and Lipoprotein Metabolism in Microglia

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Microglia, once viewed as static bystanders with limited homeostatic functions, are now considered key players in the development of neuroinflammatory and neurodegenerative diseases. Microglial activation is a salient feature of neuroinflammation involving a dynamic process that generates multitudinous microglial phenotypes that can respond to a variety of situational cues in the central nervous system. Recently, a flurry of single cell RNA-sequencing studies have defined microglial phenotypes in unprecedented detail, and have highlighted robust changes in the expression of genes involved in lipid and lipoprotein metabolism. Increased expression of genes such as Apolipoprotein E (ApoE), Triggering Receptor Expressed on Myeloid Cells 2 (TREM2) and Lipoprotein Lipase (LPL) in microglia during development, damage, and disease, suggest that increased lipid metabolism is needed to fuel protective cellular functions such as phagocytosis. This review describes our current understanding of lipid and lipoprotein metabolism in microglia, and highlights microglial lipid metabolism as a modifiable target for the treatment of neurodegenerative diseases such as Alzheimer's disease and multiple sclerosis.

Keywords: lipid and lipoprotein metabolism, microglia, neurodegenerating diseases, Alzheimer's disease, lipid, lipoprotein, multiple scleorsis (MS), APOE

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INTRODUCTION

Microglia are functionally distinct brain-resident macrophages that are seeded developmentally and maintained by self-proliferation (Ajami et al., 2007; Alliot et al., 1991; Askew et al., 2017). Once considered static bystanders with limited homeostatic functions, it is now becoming increasingly clear that microglia interact with all CNS components and have a marked impact on brain health and disease (Li and Barres, 2018). Microglia are highly plastic and respond to a variety of environmental cues by switching to appropriate activation states. While some activation states are adaptive and contribute to homeostatic functions, others are maladaptive and associated with neuroinflammation. Microglial activation and dysfunction are salient features of neuroinflammatory and neurodegenerative diseases (NDs), such as Alzheimer's disease (AD), Parkinson's disease (PD), and multiple sclerosis (MS) (Colonna and Butovsky, 2017). Thus, it has been increasingly recognized that understanding the heterogeneity of microglial activation in the context of disease may facilitate the design of therapeutics that dampen the detrimental effects of microglial activation, while augmenting the beneficial effects of "alternatively" activated microglia (Li and Barres, 2018).

Recently, several comprehensive single-cell RNA-sequencing (scRNAseq) analyses of microglia *ex vivo* have defined the transcriptomic identities of microglia with temporal (Grabert et al., 2016; Hammond et al., 2019; Li et al., 2019), regional (Grabert et al., 2016; Hammond et al., 2019; Li et al., 2019), and disease state specificity (Hammond et al., 2019; Keren-Shaul et al., 2017; Mathys et al., 2017). Various clusters of microglia with similar gene expression profiles have been mapped to specific regions and developmental or disease stages. A striking feature of these studies is the identification of microglial clusters with common metabolic characteristics. For example, Keren-Shaul et al. (2017) used the 5XFAD murine model of AD to define Disease Associated Microglia (DAMs) that express a distinct set of genes associated with lipid and lipoprotein metabolism (e.g., Apolipoprotein E [ApoE], Lipoprotein Lipase [LPL], and Triggering Receptor Expressed On Myeloid Cells 2 [TREM2]) (Keren-Shaul et al., 2017). This transcriptional signature represents a preference for lipids as fuel substrates that fulfill the greater bioenergetics needs of activated microglia (Keren-Shaul et al., 2017). Interestingly, these DAMs bear a striking resemblance to microglial clusters observed during early postnatal life (P4/P5) (Hammond et al., 2019), and in proliferative-region-associated-microglia (PAMs), which reside in regions of the early postnatal brain with active gliogenesis and neurogenesis (Li et al., 2019). A similar signature is also observed in microglia of the demyelinating brain (Hammond et al., 2019), and in the later stages of a CK-p25 murine model of DNA damage and neurodegeneration (Mathys et al., 2017).

Even though these studies have varied in their experimental approach, they have repeatedly implicated consistent changes in microglial metabolism during microglial activation. These studies have reignited our interest in "immunometabolism," particularly in the context of neurodegenerative disease (ND). Here, we review our current understanding of lipid and lipoprotein metabolism in microglia to identify potentially targetable metabolic processes, which may ameliorate detrimental microglial responses and help develop novel therapeutic interventions for ND and beyond.

Abbreviations: 11β-HSD1, 11β-hydroxysteroid dehydrogenase type 1; 15d-PGJ_2, 15-deoxy-Δ^{12-14}-PGJ_2; 18-HEPE, 18-hydroxyeicosapentaenoic acid; 24-OHC, 24(S)-hydroxycholesterol; 5XFAD, 5 familial AD mutations; 7KC, 7-ketocholesterol; Aβ, amyloid beta; AA, arachidonic acid; ABC, ATP-binding cassette transporter; ACAT, Sterol O-acyltransferase; acyl-CoA, acyl-coenzyme A; AD, Alzheimer's disease; AIBP, ApoA-1 binding protein; Akt, Protein kinase B; ALA, alpha-linolenic acid; ApoA-1, apolipoprotein A-1; ApoB-100, apolipoprotein B-100; ApoE, Apolipoprotein E; ApoJ, apolipoprotein J; APP, Amyloid precursor protein; ARG1, Arginase 1; ATGL, Adipose triglyceride lipase; ATP, adenosine triphosphate; BAM, Border-Associated Microglia; BBB, blood brain barrier; CD11b/CD18, C3 receptor; CE, cholesterol ester; CLEM, Correlative light and electron microscopy; CLU, Clusterin; CM, chylomicron; CNS, central nervous system; COX2, cyclooxygenase 2; cPLA2, cytosolic group IV of PLA2; CPT1A, Carnitine Palmitoyltransferase 1A; CSF, cerebrospinal fluid; CSF-1R, Colony stimulating factor 1 receptor; CX3CL1, chemokine fractalkine; CX3CR1, C-X3-C Motif Chemokine Receptor 1; CXCL1, chemokine (C-X-C motif) ligand 1; CYP46A1, cholesterol 24S-hydroxylase; DAG, diacylglycerol; DAM, disease associated microglia; DAP12, DNA polymerase III-Activation Protein 12; db/db, leptin deficient mouse model of type 2 diabetes; DHA, docosahexaenoic acid; EGFP, enhanced GFP; EPA, eicosatetraenoic acid; ER, endoplasmic reticulum; fAβ, fibrillar Aβ; FA, fatty acid; FABP, FA binding protein; FAD, familial AD; FAO, FA oxidation; FATP, FA transport protein; FFA, free FA; GFP, green fluorescent protein; GL, glycolipid; GPAT, Glycerol-3-phosphate acyltransferase; GPR109A, G-protein-coupled receptors 109A.; GWAS, Genome Wide Association Studies; HDL, high-density lipoprotein; HFD, high-fat diet; HMGB1, High Mobility Group Box 1; HMGCR, 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase; HSL, hormone sensitive lipase; Iba1, Ionized calcium binding adaptor molecule 1; IDL, intermediate-density-lipoproteins; IFN, interferon; IGF1, Insulin growth factor 1; IHC, immunohistochemistry; iMG, induced microglia-like cell culture; IP, intraperitoneal; LA, linoleic acid; LC, long chain; LCAT, Lecithin–cholesterol acyltransferase; LDL, low-density lipoprotein; LDLR, LDL receptor; LOX, lipoxygenase; LPA, lysophosphatidic acid; LPC, lysophosphatidylcholine; LPL, Lipoprotein Lipase; LPS, lipopolysaccharide; LRP1, LDL Receptor Related Protein 1; LXR, liver X receptor; mAβ, monomeric Aβ; MFG-E8, Milk fat globule epidermal growth factor-8; MFP2, multi-functional protein-2; Mfsd2a, Major Facilitator Superfamily Domain Containing 2A; MS, multiple sclerosis; MS4A7, Membrane-spanning 4-domains subfamily A member 7; mTOR, mammalian target of rapamycin; MUFA, monounsaturated FA; n-3, omega-3; n-6, omega-6; ND, neurodegenerative disease; NF-kB, Nuclear Factor kappa-light-chain-enhancer of activated B cells; NPC, neural progenitor cell; NSC, neural stem cell; oAβ, oligomeric Aβ; OL, oligodendrocyte; OPC, oligodendrocyte precursor cell; oxLDL, oxidized LDL; OXPHOS, oxidative phosphorylation; PAM, proliferative-region-associated-microglia; PARP, poly (ADP-ribose)-polymerase; PC, phosphatidylcholine; PD, Parkinson's disease; PE, phosphatidylethanolamine; PET, Positron emission tomography; PI3K, Phosphoinositide 3-kinase; PL, phospholipid; PLA2, phospholipase A2; PPAR, peroxisome proliferator activated receptor; PPP, pentose phosphate pathway; PS, phosphatidylserine; PS1, Presenilin-1; PSD95, postsynaptic density protein 95; PUFA, polyunsaturated FA; ROS, reactive oxygen species; RXR, retinoid X receptor; SAD, sporadic late onset AD; SCARA, class A scavenger receptor gene; SCARB, class B scavenger receptor gene, SCARC, class C scavenger receptor gene, FAT, FA translocase; scRNAseq, single-cell RNA-sequencing; SFA, saturated fatty acid; siglec-11, sialic acid-binding Ig-like lectin; Slc2a5, Solute carrier family 2 member 5; SNAP25, Synaptosome Associated Protein 25; SPM, specialized pro-resolving mediator; Spp1, Secreted phosphoprotein 1; SR-B1, class B scavenger receptor protein 1; SREBP, sterol regulatory element binding proteins; STED, stimulated emission depletion; SZ, schizophrenia; TBI, traumatic brain injury; TG, triglyceride; TGFβ, Transforming growth factor β; TLR, toll-like receptor; Tmem119, Transmembrane Protein 119; TREM2, Triggering Receptor Expressed on Myeloid Cells 2; VLDL, very-low-density-lipoprotein; VLDLR, VLDL receptor.

IMPORTANCE OF MICROGLIA IN BRAIN HEALTH AND DISEASE

Development

Microglia represent 5 to 15% of the adult CNS cell population and constitute the largest population of immune cells in the brain. They play a major role in the development of the CNS, maintain homeostasis within the healthy brain, and can initiate, propagate, and/or resolve inflammatory responses. To understand how these cells are capable of such feats, it is important to understand the origin and cell lineage of microglia. Microglia originate from yolk sac erythromyeloid precursor cells in mice at embryonic day 7.5 (E7.5) (Alliot et al., 1991). These cells infiltrate both the neuro-epithelium and cephalic mesenchyme at days E8.5/E9.0 and undergo dramatic expansion until the second postnatal week (Alliot et al., 1991, 1999; Tambuyzer et al., 2009; Ginhoux et al., 2010; Mizutani et al., 2012; Crotti and Ransohoff, 2016). Infiltration into the CNS is not uniform, and location-dependent heterogeneity in density, morphology, and gene expression is observed (Lawson et al., 1990). Using scRNA-seq in conjunction with RNA immunohistochemistry (IHC) *in situ* across developmental time points, microglial heterogeneity within parenchyma, (PAM), and meninges, border-associated microglia (BAM), is now mapped into specific subpopulations by gene cluster (Goldmann et al., 2016; Prinz et al., 2017; Li et al., 2019; Ochocka et al., 2019;

Van Hove et al., 2019). Although microglial ontogeny is mostly preserved across subpopulations, the region of the brain in which the microglia reside significantly affects the phenotype and response to their microenvironment (Grabert et al., 2016). At E9.5, yolk sac progenitors develop into embryonic microglia (Rosenbauer and Tenen, 2007). These embryonic microglia are a distinct tissue specific macrophage population, which is different from circulating myeloid cells. Specifically, erythromyeloid precursor cells develop into $CD45^{+}$, c-kit^{lo}, C-X3-C Motif Chemokine Receptor 1 (CX3CR1)immature cells, when in turn mature into $CD45^{+}$, c-kit^{-}, $CX3CR1^{+}$, which invade the brain using specific metalloproteases (Kierdorf et al., 2013). Following infiltration, factors such as Transforming growth factor β (TGFβ), IL-34 and Colony stimulating factor 1 receptor (CSF-1R) are necessary for the terminal differentiation of resident microglia (Butovsky et al., 2014). Although it remains somewhat controversial, recent studies suggest that in addition to canonical, non-*Hoxb8* expressing microglia that infiltrate the brain at E9.5, there is a "second wave" of yolk-sac progenitors that are greatly expanded in the fetal liver prior to infiltrating the brain at E12.5 (De et al., 2018). Importantly, this second wave of cells may give rise to microglia-like cells, with distinct characteristics such as expression of Membrane-spanning 4-domains subfamily A member 7 (Ms4a7) and ApoE (Bennett and Bennett, 2020).

At E11/E16, neural stem cells (NSCs) at the dorsoventral boundary begin a massive expansion and differentiate into neural progenitor cells (NPCs), oligodendrocyte precursor cells (OPCs), and mature oligodendrocytes (OLs) (Naruse et al., 2017). Over half of these mature OLs eventually undergo apoptosis (Barres et al., 1992). Many of these mature apoptotic OLs are phagocytosed by an early phagocytic subset of PAM (Li et al., 2019), highlighting the role of microglia in primary myelination. In addition, a subtype of microglia associated with white matter and axon tracts express high levels of LPL and Secreted Phosphoprotein 1 (Spp1) (Hammond et al., 2019), which could potentially facilitate clearance of myelin-derived lipid debris. Chemokines such as chemokine (C-X-C motif) ligand 1 (CXCL1), expressed in high quantities by neurons, stimulate microglial migration (Harrison et al., 1998; Jakovcevski et al., 2009), potentially to sites with active myelinogenesis. It is thought that microglia regulate primary myelinogenesis through a critical source of neuroprotective Insulin growth factor 1 (IGF1), known to regulate OL function (Wlodarczyk et al., 2017). Mature OLs are lipid-rich, requiring cholesterol and both essential and non-essential fatty acids (FAs) to effectively produce myelin (Mathews and Appel, 2016; Dimas et al., 2019). Although astrocytes provide OLs with cholesterol via effluxed lipoprotein particles, OLs can also produce cholesterol *de novo* via Phosphoinositide 3-kinase/Protein kinase B/mammalian target of rapamycin (PI3K)/(Akt)/(mTOR) signaling (Chen et al., 2013; Mathews and Appel, 2016). The precise role of microglia in OL lipid metabolism is unclear, however, studies outlined above suggest a key role in the phagocytosis of lipid-rich debris from myelin and apoptosed OPCs and OLs.

While recent studies have shown that microglia are extremely diverse during early development, it is clear that they become less heterogeneous during adulthood (Hammond et al., 2019). Nonetheless, the dynamic transcriptional signatures of microglia exposed to injury, aging or disease, suggest that the adult microglia are readily poised to adapt to challenges in the local environment.

Synaptic Pruning

It has been previously observed in healthy brain that so-called "resting microglia" are in fact very active. They are highly mobile and take, on average, a 5 min sample of one neuronal synapse per hour (Davalos et al., 2005; Nimmerjahn et al., 2005; Wake et al., 2009). Additionally, synapses in ischemic areas are turned over following microglial detection (Wake et al., 2009). This sampling of synapses hints at the possibility that microglia are involved in the crucial process of synaptic pruning. The expression of the chemokine fractalkine (CX3CL1), a strong microglial chemoattractant and whose receptor in the CNS is expressed solely in microglia, is up-regulated in neurons during development (Harrison et al., 1998; Jung et al., 2000; Cardona et al., 2006; Liang et al., 2009). Through GFP-labeling of microglia and immunohistochemistry against postsynaptic density protein 95 (PSD95), a marker of postsynaptic density, stimulated emission depletion (STED) microscopy revealed colocalization of PSD95 and GFP. Moreover, electron microscopy of these microglia show the presence of both clathrin-coated and non-clathrin-coated vesicles containing PSD95 as well as Synaptosome Associated Protein 25 (SNAP25), a marker of presynaptic density (Paolicelli et al., 2011). These data strongly suggest that microglia are actively involved in synaptic pruning. A closer look at hippocampal synapses via light sheet fluorescence microscopy and 3D ultrastructural characterization using Correlative light and electron microscopy (CLEM) found that microglia do not directly phagocytose entire synapses (Weinhard et al., 2018). Instead, they trogocytose—selective partial phagocytosis—the membranes of presynaptic boutons and axons without any evidence for elimination of dendritic spines (Weinhard et al., 2018). The exact mechanism of trogocytosis has yet to be elucidated.

Phagocytosis

Microglia play an important role as the immune effector cells of the central nervous system. In line with this role, microglia express many cell surface factors that are also expressed by peripheral myeloid cells and macrophages such as integrin's, toll-like receptors, scavenger receptors, and TREM2 (Aldana, 2019). Although these similarities have made the isolated study of microglia somewhat challenging, there has been a concerted effort to define the characteristics that set microglia apart from other myeloid cells. For example, while microglia and bone marrow derived macrophages both express CD11B and CD45 (although high for macrophages and low for microglia), microglia express higher levels of Transmembrane Protein 119 (Tmem119), CX3CR1 and Solute Carrier Family 2 Member 5 (Slc2a5) (Haage et al., 2019). It is likely that this differential expression represents the homeostatic functions that are specific to microglia and not peripheral cells such as synaptic pruning, neuronal survival, and synaptogenesis (Bilimoria and Stevens, 2015). Such neuroprotective functions are largely due to the

phagocytic nature of microglia (Janda et al., 2018). Microglia are highly efficient phagocytes that remove apoptotic or necrotic cells (Green et al., 2016), and unfolded proteins such as amyloid beta (Aβ) or neuromelanin. Engulfment of myelin debris is also a key function of microglial sub-populations associated with remyelination and repair (Olah et al., 2012). Furthermore, microglia are an important part of the innate immune system and are activated in response to infections in order to directly phagocytose potentially pathogenic microorganisms (Nau et al., 2014). Microglial phagocytosis initiates the adaptive arm of the immune system via antigen presentation (Litman et al., 2005). Overall, the immune functions of microglia showcase their plasticity and ability to respond to a wide variety of stimuli. However, the response can sometimes become chronic and maladaptive. This prolonged microglial activation is a salient feature of many NDs such as AD, Parkinson's disease and MS.

Disease

Metabolic Disorders

Metabolic disorders such as obesity have implicated in the pathological activation of microglia largely due to an increase in systemic inflammation. For example, the leptin deficient mouse model of type 2 diabetes (db/db), shows increased inflammatory chemokine and cytokine expression in the CNS (Kumar et al., 2014). In addition, several studies have shown that exposure to a high-fat diet (HFD) can increase microglial activation, even without peripheral inflammation (Ziko et al., 2014; Kang et al., 2016). In fact, recent work as shown that only 3 days of HFD exposure is sufficient to promote gliosis in the hypothalamus (Thaler et al., 2012). Since microglia are seeded developmentally and are particularly long-lived, they are a potential conduit for the transmission of "developmentally programmed" dietary exposures. In support of this hypothesis, microglia in the paraventricular nucleus of the hypothalamus can be programmed to an active state following HFD exposure during very early life; a phenotype that persists into adulthood (Morari et al., 2014). It is likely that circulating inflammatory mediators and lipids and lipoproteins have greater penetrance to the hypothalamus than other regions of the brain, which may explain the rapid metabolic polarization of microglia in this region.

Schizophrenia

Dysregulation of synaptic pruning has been increasingly implicated in the pathophysiology of both NDs and psychiatric disorders. In schizophrenia (SZ), patients have decreased gray matter thickness and reduced overall brain volume (Ziermans et al., 2012; Cannon et al., 2015). This is correlative with a decrease in synaptic density (Glantz and Lewis, 2000; Glausier and Lewis, 2013). To study this phenomenon, peripheral monocytes were induced into a validated microglia-like (iMG) cell culture model (Sellgren et al., 2019). Interestingly, iMGs from patients with SZ that are co-cultured with neurons display elevated internalization of PSD95 and SNAP25 as well as a utilization of the complement system, suggesting that microglial synaptic pruning is increased in patients with ongoing SZ (Sellgren et al., 2019). Microglia-mediated degradation of synapses is also seen in AD (Lacor et al., 2004; Chu et al., 2010; Schafer et al., 2012; Bialas and Stevens, 2013; Sekar et al., 2016; Panayiotou et al., 2017; Shi et al., 2017).

Alzheimer's Disease

Alzheimer's Disease is the most common cause of dementia in the elderly, characterized by gradual memory loss and cognitive decline. The first phase of the disease involves the subclinical, gradual buildup of extra-cellular monomeric Aβ (mAβ) that coalesces into oligomers and eventually larger amyloid fibrils/plaques. This leads to the clinically significant second phase of the disease, involving the formation of hyperphosphorylated tau neurofibrillary tangles that are associated and the destruction of neurons; the hallmark of AD. Although cognitive decline occurs most drastically during the second phase, the gradual phase has also been implicated in the deterioration of synaptic density as a result of increased microglia-mediated synapse loss (Carroll, 2004; Gasque, 2004; Reichwald et al., 2009; Ransohoff, 2016). This is primarily facilitated by fibrillar Aβ (fAβ) and oligomeric Aβ (oAβ) aggregation onto neuronal post-synaptic terminals leading to complement deposition, microglial activation following synapse elimination and neural network dysfunction (Carroll, 2004; Gasque, 2004; Reichwald et al., 2009; Pan et al., 2011; Ransohoff, 2016). C3 receptor (CD11b/CD18) wielding microglia are the key mediators of Aβ clearance and show a differential phagocytic response depending on the morphology of the encountered Aβ (Carroll, 2004; Gasque, 2004; Reichwald et al., 2009; Ransohoff, 2016). Microglial exposure to fAβ induces a classical phagocytic response, which may help to facilitate the removal of fAβ and prevent plaque formation (Pan et al., 2011). In support, recent scRNA-seq studies in the 5XFAD model of AD have shown a profound increase in DAM, which have elevated expression of factors involved in lipid uptake and phagocytosis, likely in an attempt to clear fAβ at later stages of the disease (Keren-Shaul et al., 2017). Although the role of microglia in AD pathogenesis is a research-intensive area, whether specific microglial subpopulations contribute or prevent AD progression remains to be empirically determined.

The clearance of Aβ and its tendency to aggregate can be significantly altered by lipid and lipoprotein metabolism (see **Figure 1A**). Interestingly, Alois Alzheimer originally described an increased quantity of "lipoid granules" in the AD brain as a pathological hallmark, suggesting that irregular lipid and lipoprotein metabolism may be a driving factor (Foley, 2010). Increased free-cholesterol containing lipid rafts (Riddell et al., 2001; Ehehalt et al., 2003; Hattori et al., 2006; Marquer et al., 2011), which have been shown to increase Aβ aggregation (Wisniewski et al., 1994; Bhattacharyya and Kovacs, 2010; Bryleva et al., 2010) may be a potential underlying mechanism. Additionally, ApoE, the most abundant apolipoprotein in the CNS, is also known to modulate Aβ aggregation in an isoform dependent manner (LaDu et al., 1994). Furthermore, gangliosides (which contain ceramide) within Aβ aggregates may contribute to the persistence of amyloid plaques by evading microglial detection via their interaction with sialic acid-binding Ig-like lectin (siglec-11),

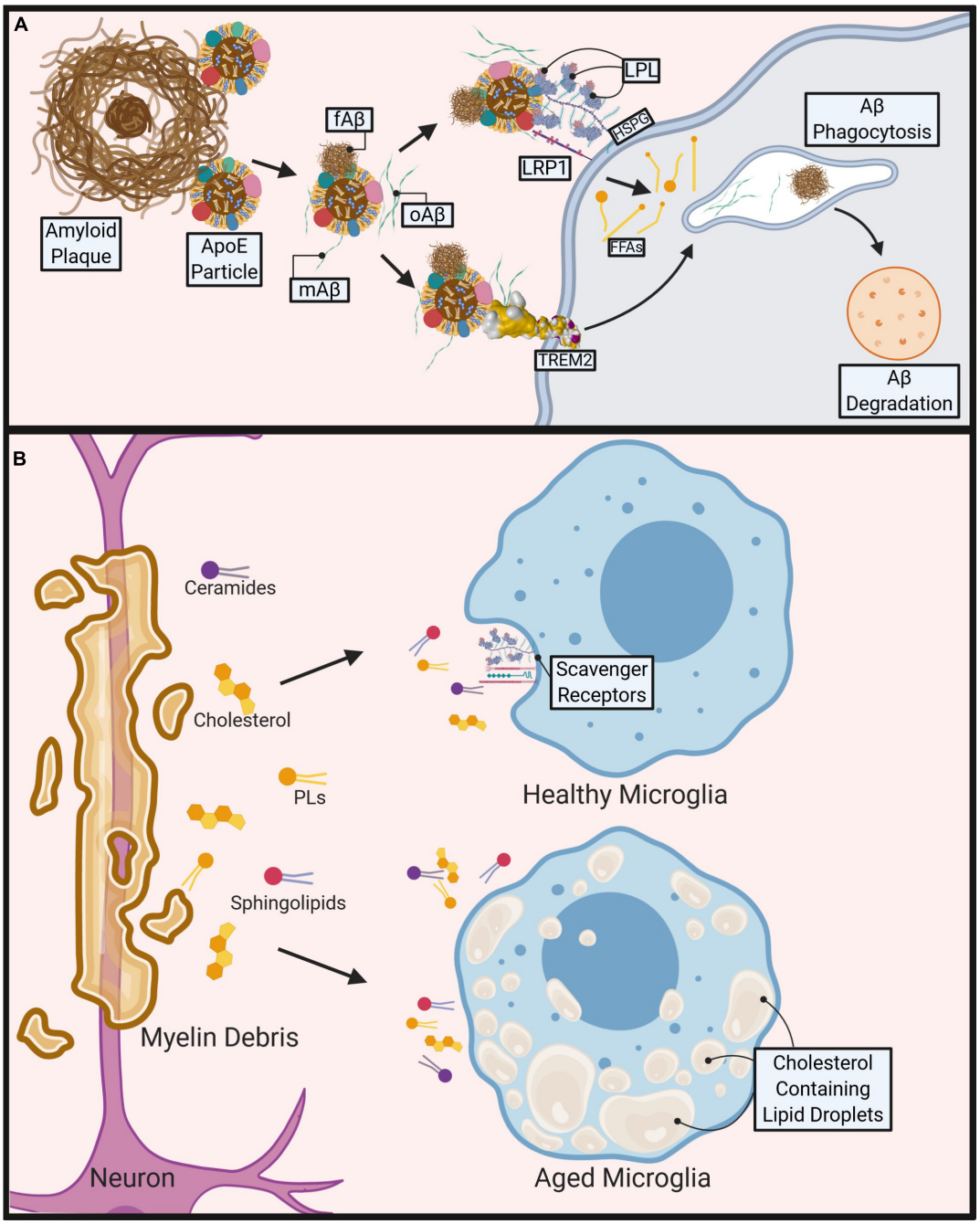

FIGURE 1 | Role of microglial lipid metabolism in the pathogenesis of Alzheimer's disease (AD) and Multiple Sclerosis (MS). **(A)** During AD, Apolipoprotein E4 (ApoE4) containing particles are thought to cause polymerization of Amyloid-Beta (Aβ) contributing to fibrillar Aβ (fAβ), plaque formation and AD pathogenesis. Aβ phagocytosis/clearance may prevent plaque formation. TREM2 binds to oligomeric Aβ (oAβ) and may bind to ApoE containing lipoprotein particle-bound monomeric Aβ (mAβ) and fAβ to facilitate phagocytosis and degradation of Aβ. Lipoprotein Receptor-like protein 1 (LRP1) and Lipoprotein Lipase (LPL) tethered to the microglial cell surface via Heparan sulfate proteoglycans (HSPGs) may also bind to Aβ directly or via an interaction with Aβ-bound ApoE containing lipoproteins, to facilitate Aβ uptake and degradation. **(B)** During MS, demyelination causes release of myelin-derived lipids such as ceramides, cholesterol, phospholipids (PL), and sphingolipids. Healthy microglia clear lipids via cell surface scavenger receptors (e.g., LRP1, LPL). However, aged, or pro-inflammatory microglia accumulate cholesterol and other neutral lipids, which impairs their ability to phagocytose myelin debris, leading to reduced and abnormal remyelination. Created with Biorender.com.

a negative immune receptor (Salminen and Kaarniranta, 2009). There is an increasing focus toward microglia-mediated lipid and lipoprotein metabolism in the brain and their effects on systemic and neuroinflammatory diseases (Di Paolo and Kim, 2011; Bruce et al., 2017; Reale and Sanchez-Ramon, 2017; Xicoy et al., 2019).

Multiple Sclerosis

In progressive forms of MS, the progression of chronically active demyelination lesions leads to worsening disability (Absinta et al., 2019). Within these lesions, myelin, which is composed mostly of lipid, is destroyed by microglia and lipid-laden infiltrating macrophages amongst other immune cells (Raine et al., 1981). It is here that lipid metabolism is most crucial in the modulation of disease. The presence of myelin debris itself is known to cause a sustained inflammatory response (Clarner et al., 2012; Cantuti-Castelvetri et al., 2018; Kopper and Gensel, 2018). Additionally, cholesterol-breakdown products are significantly higher within the brain and CSF of these patients. 7-ketocholesterol (7KC) is among these products and is associated with mitochondrial dysfunction and attenuated lipid processing through FA oxidation (FAO) in OLs (Diestel et al., 2003; Leoni et al., 2017). In microglia, 7KC rapidly enters the nucleus and activates poly (ADP-ribose)-polymerase (PARP)-1, which induces a pro-inflammatory phenotype (Diestel et al., 2003; Kauppinen and Swanson, 2005). In some ways this is helpful, as activated microglia are more mobile and significantly increase their phagocytic activity in order to clear debris effectively (Fu et al., 2014). However, a recent study has shown that as phagocytic microglia age they lose the capacity to effectively efflux cholesterol, form intracellular cholesterol crystals, and propagate a maladaptive pro-inflammatory response (Cantuti-Castelvetri et al., 2018) (see **Figure 1B**). In contrast, omega-3 (n-3) polyunsaturated FAs (PUFAs) have been shown to reduce inflammation and induce an anti-inflammatory phenotype within microglia (Hopperton et al., 2016; Chen et al., 2018; Layé et al., 2018). Similarly, an increased intake of total dietary PUFAs is associated with a reduced risk of developing MS (Bjornevik et al., 2017). In addition, we have previously shown that LPL, the rate-limiting enzyme in the hydrolysis of triglyceride (TG) rich lipoproteins, is associated with an anti-inflammatory microglial phenotype due to its complex role in lipid uptake and metabolic reprogramming (Bruce et al., 2018).

METABOLIC REPROGRAMMING IN MACROPHAGES AND MICROGLIA

It is well established that macrophages change their metabolic profile to meet the increased bioenergetic demands of activation. Although a similar "metabolic reprogramming" also occurs in microglia, our understanding of this process is less well understood. For this reason, it is useful to review the literature describing the interaction between metabolism and inflammation in macrophages to serve as a relative model for microglia. When macrophages are homeostatic, or quiescent, they catabolize various substrates (glucose, amino acids, fatty acids) to be utilized in the TCA cycle to produce electrons (carried in the form of NADH/FADH2). These electrons are then used during mitochondrial oxidative phosphorylation (OXPHOS) to drive adenosine triphosphate (ATP) production (Mehta et al., 2017). In contrast, pro-inflammatory macrophages show a metabolic shift toward glycolysis and away from OXPHOS (Van den Bossche and Saraber, 2018). The metabolic preference for energy derived from glycolysis over OXPHOS in normoxic conditions is reminiscent of the metabolic profile of many types of tumors. In these tumor cells, glycolysis predominates despite sufficient oxygen for oxidative metabolism to proceed; a phenomenon first described by Otto Warburg in 1927, thus termed the "Warburg effect" (Warburg et al., 1927). In macrophages, metabolic polarization to a Warburg-like metabolism can follow exposure to a range of stimuli, including LPS, the toll-like receptor (TLR) 3 ligand poly(I:C), and type 1 interferon (IFN) (Kelly and O'Neill, 2015). It has been suggested that activated macrophages shift toward glycolytic metabolism to preserve the macromolecules that may be needed to synthesize new proteins required for a given activation state (Rambold and Pearce, 2018). For example, glucose metabolism feeds the pentose phosphate pathway (PPP), which increases the production of purines and pyrimidines, important for biosynthesis in the activated cell. Increased flux through the PPP also provides NADPH for NADPH oxidase, which generates reactive oxygen species (ROS) that can be used as an anti-bacterial mechanism (West et al., 2011). It is also thought that the increase in glycolysis generates ATP quickly, and although inefficient in comparison to oxidative metabolism, provides the necessary energy to support cell activation in the shortest time frame. Importantly, recent studies using microglia isolated from 5XFAD mice have shown similar impairments in metabolism including shifts toward glycolysis, which can be reversed following INF-γ treatment (Baik et al., 2019). There are a number of mechanisms thought to contribute to the metabolic reprogramming of macrophages and microglia, although here we will focus on those involving alterations in lipid metabolism, there are several reviews that discuss the mechanisms in more detail (Kelly and O'Neill, 2015; Langston et al., 2017).

Macrophage lipid metabolism is profoundly altered during polarization. For example, following macrophage activation, the TCA cycle is interrupted and acetyl-CoA is shunted to the synthesis of lipid precursors for inflammatory mediators (Jha et al., 2015). Specifically, following intraperitoneal (IP) injection of LPS or zymosan (a ligand found on surface of yeast) there is a dramatic increase in the synthesis of cholesterol ester (CE) and FAs in peritoneal macrophages (Posokhova et al., 2008). Furthermore, activated macrophages take up significantly more native and acetylated low-density-lipoproteins (LDL), which contributes to CE synthesis, whereas non-acetylated LDL uptake is inhibitory to CE synthesis in control cells (Wang et al., 2007). In addition, LPS treatment leads to TG accumulation in macrophages, which is coupled with decreased FAO and TG lipolysis, without altered expression of intracellular lipases (Adipose triglyceride lipase [ATGL] and hormone sensitive lipase [HSL]) (Feingold et al., 2012). In contrast, 'alternative activation' of macrophages is associated with increased FAO, raising the possibility that controlling fatty acid metabolism may attenuate inappropriate activation (Namgaladze and Brune, 2016). Several targets have been identified that may control FA metabolism in the context of inflammation, with a particular emphasis on mitochondrial FAO. For example, promoting mitochondrial β-oxidation has been shown to attenuate endoplasmic reticulum (ER) stress that can be induced by the saturated fatty acid (SFA) palmitate

(C16:0) (Namgaladze et al., 2014). Conversely, knockdown, or pharmacological inhibition of mitochondrial FAO leads to exacerbated ER stress and inflammation in response to palmitate (Namgaladze et al., 2014). In support, over expression of a mutant from of Carnitine Palmitoyltransferase 1A (CPT1A) that is insensitive to malonyl-CoA inhibition (and thus shows enhanced mitochondrial FAO) prevents palmitate-induced inflammation, ER stress, and oxidative damage (Malandrino et al., 2015). While there may be deleterious effects of increased FAO, particularly in tissue-specific or hypoxic conditions, overall it has been suggested that a major consequence of increased macrophage FAO is a diversion of fatty acid flux away from the formation of FA metabolites that cause ER stress and are precursors for inflammatory mediators.

Until recently, our understanding of how microglial metabolism relates to activation and function has been somewhat extrapolated from the peripheral macrophage literature. However, recent scRNA-seq studies mentioned above have highlighted that microglial metabolism may be similarly altered in response to changing bioenergetics during the development of NDs. For example, several studies have highlighted the need for increased expression of genes associated with lipid metabolism (e.g., TREM2, LPL and ApoE) during development, damage and disease (Keren-Shaul et al., 2017; Hammond et al., 2019). While this suggests a need for increased FAO in these activation states, further studies are needed to empirically define the role of these factors in microglial lipid metabolism and inflammation, particularly in the context of ND. It is important to point out that while the metabolism of microglia may be similar to macrophages in many aspects, it is likely that access to limited metabolic substrates in the CNS (e.g., no very-low-density-lipoproteins [VLDL] or LDL) has led to the development of somewhat distinct metabolic profiles, that have not yet been clearly defined.

FATTY ACID METABOLISM IN MICROGLIA

Overview

The brain's dry weight is made up of about 50% lipid, most of which is comprised of myelin enriched white matter (Hamilton et al., 2007). Myelin is a specialized OL membrane that contains ∼43% phospholipid (PL), ∼28% glycosphingolipid, and ∼28% cholesterol of its total lipid content (Morell, 1999). PLs are derived from FAs in a biosynthetic pathway that is initiated by fatty acyl-CoA synthase, which combines a FA with acyl-coenzyme A (acyl-CoA) using ATP to create fatty acyl-CoA (Yamashita et al., 2014). Glycerol-3-phosphate acyltransferase (GPAT) adds a glycerol backbone, creating a single-tailed lysophosphatidic acid (LPA) (Yamashita et al., 2014). CoA replenishes it's acyl group from acyl-carnitine via the carnitine transport system within the mitochondria (Yamashita et al., 2014). Within the ER, LPA is further modulated to give rise to TGs, diacylglycerols (DAGs), and PLs (Yamashita et al., 2014). These PLs are transported through the Golgi, to other organelles, and to the polymorphic cell surface along with proteins, cholesterol, and other FA containing glycolipids (GLs). PLs are important for providing membrane integrity, lipid raft formation, signal transduction, providing curvature to the cell membrane, vesicle formation, apoptosis, and in the production of pro- or anti-inflammatory mediators. Moreover, they are becoming increasingly implicated in the pathophysiology of NDs.

Phospholipids

Phospholipids constitute 45% of the total dry weight of the brain, and are key to several pathways, such as the synthesis and turnover of neuronal and glial membranes and signaling. Since these processes are critical to CNS health, it is not surprising that there are substantial energy demands associated with PL metabolism, with 5% of the brains ATP being used for the turnover of 1,2-diacyl type PLs (Purdon et al., 2002). A number of reports have suggested that FAs and PLs are increased during pathological conditions such as inflammation, hypoxia and ischemia, and it is likely that this represents release of FAs from brain PLs, or indeed PLs from vital brain structures. For example, in individuals with PD, total serum PL levels are increased without any signs of cognitive impairment and positively correlate with the progression of their cognitive impairment (Li et al., 2015). This finding suggests that increased PL levels could be an early marker for PD onset and disease progression. Furthermore, the enzymes involved in synthesizing phosphatidylserine (PS), phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are increased in the substantia nigra of PD patients (Ross et al., 2001). The increase in total serum PL may indicate cell membrane damage in the substantia nigra, which triggers a compensatory increase in PS, PE, and PC synthesis. Interestingly, while total serum PL is increased, PS 40:4 is decreased (Zhang et al., 2017). In non-apoptotic cells, PS is asymmetrically present within the inner layer of the cell membrane. However, in PD, apoptotic neurons extrovert PS to the surface of the cell membrane, via inactivated flippase and activated scramblase enzymes, to signal phagocytes to remove the cell (Wei et al., 2013). Microglia are known to target these apoptotic cells via expression of PS-specific receptors (Fadok et al., 2000). In recent years several microglia-derived factors that participate in PS-stimulated phagocytosis have been identified. For example, Milk fat globule epidermal growth factor-8 (MFG-E8) is secreted by microglia and links exposed neuronal PS to vitronectin receptors, which modify the actin cytoskeleton to stimulate microglial phagocytosis of dying neurons (Fricker et al., 2012). Therefore, it is plausible that activated microglia may have increased uptake of PS during inflammation, which could potentially drive decreased serum levels observed in PD. It is also plausible that elevated serum PL, could indicate defective microglial phagocytosis.

PUFAs

Polyunsaturated fatty acids have more than one carbon-carbon double bond within the FA hydrocarbon chain. n-3 PUFAs are characterized by a double bond three atoms away from the ω-methyl group. While mammals cannot synthesize n-3 PUFAs, they are available from the diet in the form of essential FAs, such as alpha-linolenic acid (ALA). n-3 PUFAs

are associated with a range of benefits: from preventing age related cognitive decline to preventing metabolic syndrome and improving cardiovascular risk factors (Schwinkendorf et al., 2011; Layé et al., 2015; Trépanier et al., 2016). In contrast, omega-6 (n-6) PUFAs, which have their last double bond 6 atoms away from the terminal methyl group, are generally associated with the worsening of many chronic diseases such as obesity, neuroinflammatory diseases, and asthma (Simopoulos, 2016; Brigham et al., 2019). Within the cell membrane, the ratio of n-3 to n-6 PUFAs can influence cell functions (Hennig and Watkins, 1989; Schwinkendorf et al., 2011; Simopoulos, 2016). Both n-3 and n-6 PUFAs are synthesized using the same elongases and desaturases, so they must compete for their creation and integration into the cell membrane (Simopoulos, 2011). PUFAs are abundant within the CNS, making up 30% of the brain's FAs; n-3 PUFAs account for a third of these (Hamilton et al., 2007). The distribution of PUFAs isn't homogenous across lipid class (Simopoulos, 2008). For example, TGs and CEs contain eicosatetraenoic acid (EPA), 20:5, and ALA, whereas ALA is scarcely present in PLs, which contain a high concentration of docosahexaenoic acid (DHA), 22:6, and EPA (Simopoulos, 2008). PUFAs are also heterogeneously located across different brain regions. About 30% of all fatty acids in the outer segment membrane of retinal photoreceptors are n-3 PUFAs (Bazan et al., 1990). Consequently, a diet rich in n-3 PUFAs significantly slows the decline in visual acuity in patients with retinitis pigmentosa (Berson et al., 2012). In addition, the most prevalent n-3 PUFA in the body, DHA, is found at concentrations that are several hundred-fold higher than EPA in the brain and retina (Arterburn et al., 2006). This may be caused by the brain's preferential usage of EPA as an energy source that is rapidly catabolized via FAO (Chen and Bazinet, 2015). Importantly, DHA cannot be synthesized *de novo* in the brain, and until fairly recently, the mechanism of long chain (LC)-FA transportation across the blood brain barrier (BBB) has remained unknown. However, recent studies have shown that a member of the Major Facilitator Superfamily Domain Containing 2A (Mfsd2a) can transport DHA, in the form of lysophosphatidylcholine (LPC) (Nguyen et al., 2014). In support, Mfsd2a-knockout mice show markedly reduced levels of DHA in the brain, accompanied by neuronal loss and cognitive deficits (Nguyen et al., 2014). Although initially studies report the expression of Mfsd2a largely in the endothelial cell, subsequent studies have also shown that Mfsd2a is present, albeit at a lower level in OLs, OPCs and astrocytes (Chan et al., 2018). Moreover, increased numbers of activated microglia are observed in the subretinal space of Mfsd2a knockout mice compared to wild-type controls (Wong et al., 2016).

Omega-3 and omega-6 PUFA containing PLs bring about dichotomous secondary mediators when turned over at the level of the cell membrane. The phospholipase A_2 (PLA_2) superfamily of enzymes, hydrolyze FAs from the sn-2 position of membrane PLs, and contain 15 groups that differ in their ability to recognize and respond to various PL substrates. Among these groups, cytosolic group IV of PLA_2 ($cPLA_2$) releases arachidonic acid (AA), whereas calcium dependent group VI PLA_2 releases DHA (Jenkins et al., 2004). Typically, free DHA is enzymatically metabolized by cyclooxygenase 2 (COX2) and lipoxygenases (LOXs) into resolvins, protectins, and maresins, which are known to be potent anti-inflammatory specialized pro-resolving mediators (SPMs) (Serhan, 2014; Calder, 2015). Free AA, however, is metabolized into prostaglandins, prostacyclins, thromboxanes, and leukotrienes. With a few exceptions, these AA derived mediators are known to produce potent pro-inflammatory effects (Lukiw and Bazan, 2000). Positron emission tomography (PET) has been used to study AA incorporation into the brain (Robinson et al., 1992; Rapoport, 1999), and made important contributions to our understanding of AA turnover and metabolism in various contexts. For example, PET imaging has revealed that AA uptake is elevated in widespread cortical regions in patients with AD compared to healthy controls, which is consistent with the notion that elevated AA is a marker for neuroinflammation (Esposito et al., 2008).

Within microglia, the downstream effects of n-3 PUFA metabolism has been observed to be beneficial in the context of neuroinflammatory diseases. n-3 PUFA supplementation attenuates activation by deacetylation of the High Mobility Group Box 1/Nuclear Factor kappa-light-chain-enhancer of activated B cells (HMGB1)/(NF-kB) pathway in a traumatic brain injury (TBI) mouse model (Chen et al., 2018). This in turn leads to decreased inflammatory markers and neuroprotective effects following injury (Chen et al., 2018). Although n-6 PUFAs are generally considered pro-inflammatory, this is not the case for all n-6 PUFAs. For example, supplementation with palmitic acid, a 16-C SFA, can activate microglia (Tu et al., 2019). However, supplementation with n-6 linoleic acid (LA) is sufficient to reverse this activation (Tu et al., 2019). In addition to microglial polarization, a growing body of evidence suggests that different FA classes uniquely alter energy metabolism irrespective of activation status (Button et al., 2014; Chausse et al., 2019). In a study with real-time metabolic measurements coupled with lipidomic analysis, both oleate, a monounsaturated FA (MUFA), and palmitate, a SFA, promoted oxidative metabolism whereas LPS increased glycolysis (Chausse et al., 2019). Although both FAs promoted OXPHOS, oleate treatment increased CD36 abundance (an important scavenger receptor and LC-FA transporter), as well as PUFA-containing TGs, while palmitate incorporated more PUFAs into PLs (Chausse et al., 2019). It is likely that SFA-induced incorporation of PUFAs into PLs contributes to inflammation since PUFAs are more readily peroxidized (Chausse et al., 2019). It is also of note that LPS activation can decrease MUFAs while increasing SFA concentrations, both of which can induce a pro-inflammatory effect (Button et al., 2014). Further studies using metabolomic and lipidomic analysis need to be conducted to fully elucidate the metabolic alterations that take place in microglia in response to different FAs.

Omega-3 PUFA supplementation has also been shown to enhance microglial phagocytosis of myelin (Chen et al., 2014). Several studies have demonstrated that increasing the n-3 to n-6 ratio in fat-1 mice, which express an n-3 FA desaturase that converts n-6 PUFAs to n-3 PUFAs, has beneficial results on the inflammatory status of microglia (Ma et al., 2006; Kang, 2007; Zhang et al., 2017). A recent

study used the cuprizone-model of demyelination and remyelination to compare fat-1 mice to WT (Siegert et al., 2017). The CNS lipid profile revealed increased remyelination in fat-1 mice as well as increased EPA levels and EPA metabolites, such as 18-hydroxyeicosapentaenoic acid (18-HEPE) (Siegert et al., 2017). As previously stated, EPA has a relatively low quantity in the CNS lipid profile, and is rapidly utilized as a carbon source in FAO (Chen and Bazinet, 2015). Taken together, perhaps the energy provided from FAO of EPA is critical in fueling phagocytosis of myelin and production of secondary mediators that further aid in the process of remyelination. It is also possible that EPA maintains oxidative metabolism and prevents the shift toward glycolysis that occurs during pro-inflammatory and metabolic reprogramming in microglia.

In addition to fueling oxidative metabolism, PUFAs are also agonists for nuclear receptors with important roles in microglial activation and function. For example, the peroxisome proliferator activated receptors (PPARs) are a family of nuclear receptors with key roles in lipid and glucose metabolism, inflammation, and proliferation. All three PPAR isoforms (α, β/δ and γ) are activated by naturally occurring FAs such as AA and EPA. Moreover, the downstream metabolite of AA, 15-deoxy-Δ^{12-14}-PGJ_2 (e.g., 15d-PGJ_2), was the first reported endogenous ligand for PPAR-γ (Forman et al., 1995; Kliewer et al., 1995). Differences in the concentration required to activate PPAR-γ *in vitro*, and the concentrations naturally present, have called the physiological relevance of 15d-PGJ_2 into question. Nonetheless, PPAR-γ is expressed in primary microglial cells, and its expression is enhanced following 15d-PGJ_2 supplementation (Bernardo et al., 2000). Interestingly LPS-stimulated primary microglia synthesize large amounts of 15d-PGJ_2, which is able to downregulate microglial activation through both PPAR-γ-dependent and independent mechanisms (Bernardo et al., 2003). Since these initial findings, several studies have now shown that natural and synthetic PPAR-γ agonists can down-regulate surface antigens and inflammatory mediators (Fumagalli et al., 2018), while increasing expression of anti-inflammatory factors such as Arginase 1 (ARG1) and IL-4 (Song et al., 2016). It is likely that the pivotal role of PPAR-γ in microglial activation lies with its role in metabolic reprogramming. PPAR-γ signaling directs microglia toward protective functions, such as increased FAO and OXPHOS (Fumagalli et al., 2018). Therefore, PPAR-γ agonists have been extensively studied as potential therapeutics to ameliorate aberrant microglial activation during ND, with mixed results.

FAs in Oxidative Metabolism

Although the brain is only 2% of the total body mass, it utilizes ∼20% of the body's total ATP. While glucose is the primary source of energy for the brain, it has since been estimated that 20% of the brain's energy production stems from FAO (Ebert et al., 2003; Bélanger et al., 2011). FAs have also been shown to enter the mammalian brain (Dhopeshwarkar and Mead, 1970; Dhopeshwarkar et al., 1971; Taha et al., 2016). Recent evidence suggests that FA uptake by the brain is both a multifaceted and protein-mediated process (Murphy, 2017). It is likely that technical advances may lead to a deeper understanding of brain metabolism and may challenge the dogma of (largely) exclusive glucose utilization by the brain. In support, several studies have shown that FAs are oxidized in the brain during development and adulthood (Allweis et al., 1966; Warshaw and Terry, 1976). It is believed that FAO occurs predominantly in astrocytes (Ebert et al., 2003). However, since isolated neuronal and glial cells, and mitochondria from both, are able to use FAs to fuel oxidative metabolism (Chausse et al., 2019), it is not implausible that FAs provide an alternative source of energy to neurons and glia, either directly, or via astrocyte-derived lipoproteins (see **Figure 2**). Thus, a growing body of literature suggests that in addition to the role of lipids as components of myelin debris, or precursors to secondary messengers, FAs may also be an important driver of microglial oxidative metabolism. For example, many genes involved in fatty acid oxidation are expressed in microglia, but this expression is repressed following LPS or INF-γ mediated activation (Mauerer et al., 2009). Importantly, this repression is damped by DHA supplementation (Mauerer et al., 2009).

IFN-β is known to increase FAO and OXPHOS in macrophages (Wu et al., 2016). Although an IFN-β-mediated increase in microglial FAO acid oxidation has not been empirically determined, IFN-β is a well-known therapy for MS, and patients undergoing therapy show in an increase in FAO in peripheral myeloid cells (Croze et al., 2013). Moreover, recent studies have shown that supplementation with the IFN-β in a rat model of AD is sufficient to ameliorate microglial activation, reduce ROS and lipid peroxidation, suggesting that IFN-β helps polarize microglia to a phenotype that favors oxidative metabolism (Mudo et al., 2019). These studies highlight the therapeutic potential of IFN-β and FAO to dampen aberrant microglial activation in ND.

Previously, we have shown microglia lacking LPL have significantly reduced FAO, suggesting that lipid uptake fuels lipid oxidation (Bruce et al., 2018). Conversely, L-carnitine supplementation has been shown to increase FAO and attenuate the LPS induced inflammatory response in microglia leading to improved cognitive and neuronal functions (Gill et al., 2018; Singh et al., 2018). Recent studies have also shown that both oleate and palmitate supplementation can sustain mitochondrial respiration in BV-2 microglia, while LPS treatment diminishes oxidative metabolism in favor of increased glycolysis (Chausse et al., 2019). Since LC-PUFAs are important for microglial function, it is likely that peroxisomal β-oxidation is vital to microglial metabolism. Indeed, studies in mice lacking the multi-functional protein-2 (MFP2), a pivotal enzyme in peroxisomal β-oxidation, become profoundly pro-inflammatory (Beckers et al., 2019). Although further studies are needed to identify the principle components of microglial oxidative metabolism, it is likely that FAO is necessary for physiologic energy production and maintenance of an anti-inflammatory phenotype.

To utilize FAs for energy, they must first be taken up by the cell. FA transport proteins (FATPs), FA binding proteins (FABPs), and scavenger receptors that are categorized as class

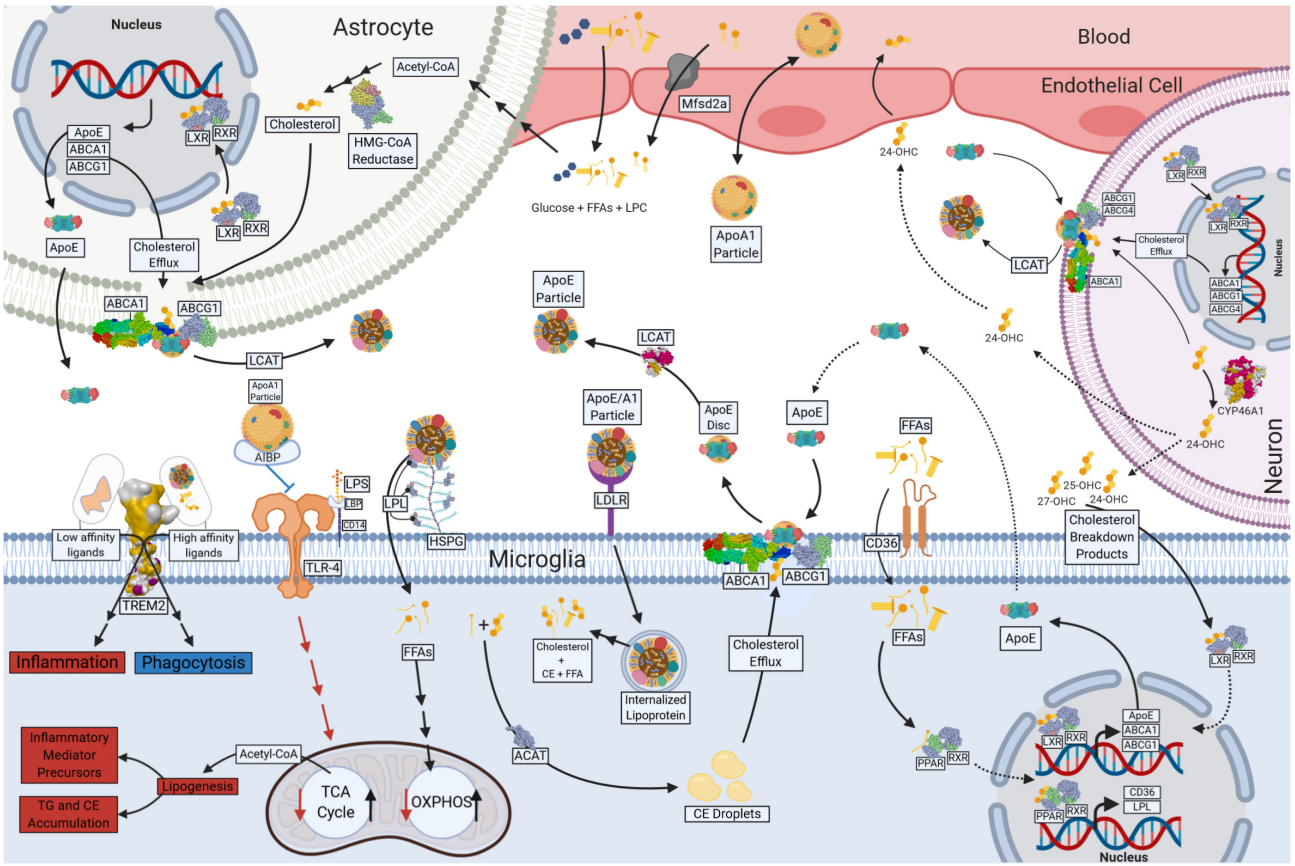

FIGURE 2 | Lipid and Lipoprotein metabolism in microglia. Free fatty acids (FFAs) and glucose cross the blood brain barrier (BBB) and provide energy substrates for neurons and glia. Apolipoprotein A1 (ApoA1) containing lipoproteins traverse the BBB and may play a role in lipid delivery to neurons and glia. Astrocytes are major source of CNS-derived lipoproteins and produce Apolipoprotein E (ApoE) containing lipoprotein particles, which is lipidated following ATP Binding Cassette Subfamily A Member 1 (ABCA1) and ATP Binding Cassette Subfamily G Member 1 (ABCG1)-mediated cholesterol efflux. Astrocyte-derived ApoE particles interact with Triggering Receptor Expressed on Myeloid Cells 2 (TREM2), Toll Like Receptor 4 (TLr-4), Low-Density-Lipoprotein Receptor (LDLR) and Lipoprotein Lipase (LPL) tethered to Heparan sulfate proteoglycans (HSPG) on the cell surface of microglia. Internalized lipoprotein particles or components provide a source of FFAs, phospholipids and cholesterol to microglia, driving oxidative metabolism and anti-inflammatory functions. FFAs act a ligand for Peroxisome Proliferator Activated Receptors (PPARs), which in turn drives transcription of genes involved in cholesterol efflux (ABCG1 and ABCA1) and lipid uptake (LPL and CD36). Phospholipids are used in the generation of cell membranes, and the FAs can be cleaved by Phospholipase A2 (PLA2) resulting in production of downstream metabolites such as Prostaglandins (PGs). In the absence of lipid uptake low affinity ligands bind TREM2, and glycolysis and pro-inflammatory functions such as increased inflammatory mediator production and neutral lipid accumulation (TG and CE). Cholesterol and myelin breakdown products (24-OHC, 25-OHC, and 27-OHC) from neurons also provide ligands for nuclear receptors (Liver X receptor [LXR] and Retinoic acid receptor [RXR]) that regulate ABCA1, ABCG1, and ApoE transcription and the production of microglial derived Lipoproteins. Created with Biorender.com.

A (SCARA), B (SCARB), or C (SCARC) among others are utilized in both transportation of esterified and non-esterified FAs across the BBB and the monocytic cell membrane. Importantly, these factors are all expressed in microglia, and are regulated during development and disease. CD36, also known as FA translocase (FAT), is a SCARB that plays a major role in microglial LC-FA uptake, oxidized LDL (oxLDL) and fAβ adhesion as well as activation of the innate immune response and ROS production (Coraci et al., 2002; Doens and Fernández, 2014). Interestingly, CD36 is predominantly expressed in microglia of the cerebellum, which may represent increased requirement for FA substrates in a region of the brain where microglia are relatively sparse and are required to survey larger areas (Grabert et al., 2016). Importantly, FAs are often not transported freely. They are instead transported in the form of esterified FAs and CE within lipoproteins.

CHOLESTEROL METABOLISM IN MICROGLIA

Lipoprotein derived CE is broken down by CE hydrolase to produce cholesterol and free FAs (FFAs). Although relatively understudied, recent proteomic analysis of specific cell types in the brain has shown that neutral CE hydrolase 1 is abundant in neurons and glia (Viader et al., 2016). Cholesterol is an essential component of the cell membranes that are especially important within the brain. The CNS contains 23% of the

body's total cholesterol content; most of which is a major cell membrane component of neurons and myelin (Hamilton et al., 2007). Unlike other organs, most of the cholesterol comes not from the liver, but from synthesis within the brain itself. *De novo* cholesterol synthesis is not very efficient within neurons, and is primarily achieved by astrocytes (Nieweg et al., 2009). Synthesis of cholesterol, within astrocytes is regulated by sterol regulatory element binding proteins (SREBPs) as well as 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMGCR) (Ye and DeBose-Boyd, 2011). Cholesterol synthesis is vital in the developing CNS, however, in the healthy adult brain, there is a very low level of cholesterol synthesis (Lavrnja et al., 2017). Cholesterol synthesis is high during development due to increased levels of myelinogenesis (Saher et al., 2005), and as previously stated, cholesterol makes up ∼28% of the total lipid volume of myelin (Morell, 1999). In neuroinflammatory diseases, destruction of myelin leads to a buildup of myelin debris that can stimulate microglial phagocytosis, directly inhibit remyelination, and propagates a pro-inflammatory response (Clarner et al., 2012; Kopper and Gensel, 2018). Cholesterol clearance is an important function of microglia (Lavrnja et al., 2017; Cantuti-Castelvetri et al., 2018). In fact, high concentrations of cholesterol are required for the survival of microglia (Bohlen et al., 2017). Additionally, cholesterol is necessary for phagocytosis as well as cytokine release (Churchward and Todd, 2014). Cholesterol homeostasis within the healthy brain also requires its constant elimination from the CNS compartment (Dietschy and Turley, 2004).

The major mechanism for elimination of cholesterol from the brain is its conversion to 24(S)-hydroxycholesterol (24-OHC) which freely exits neurons and the brain (Lund et al., 1999). This is achieved by cholesterol 24S-hydroxylase (CYP46A1) which is solely expressed in neurons in healthy brain (Lund et al., 1999). In neuroinflammatory disease states such as AD and MS, however, CYP46A1 is also highly expressed in both microglia and infiltrating macrophages (Lavrnja et al., 2017). CYP46A1 has been shown to be neuroprotective in the striatum of the R6/2 murine model of Huntington's disease (Boussicault et al., 2016). These findings highlight the importance of cholesterol elimination and its therapeutic potential. What happens when cholesterol elimination is inadequate, and cholesterol accumulates in the microenvironment? Importantly, CE accumulation is observed in both familial AD (FAD) and sporadic late onset AD (SAD) (van der Kant et al., 2019). Specifically, CE-containing lipids droplets have been shown to accumulate in the SAD brain (Chan et al., 2012). These findings have also been corroborated with observations of increased CE in mouse models of AD (Yang et al., 2014).

In chronic neuroinflammatory states, myelin debris containing phagocytes become "foamy" due to a buildup of FAs, cholesterol, and their breakdown products (Cantuti-Castelvetri et al., 2018). As previously stated, there is a buildup of 7KC in the CSF of patients with MS (Diestel et al., 2003; Leoni et al., 2017). 7KC metabolism is not well understood, however studies suggest that 7KC degradation is facilitated by either 27-hydroxylase or 11 β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) (Lyons and Brown, 2000; Mitiæ et al., 2013). Therefore, a buildup of 7KC is likely associated with ineffective microglial cholesterol metabolism. For example, MS patients have increased CSF cholesterol, likely as a result impaired cholesterol elimination (van de Kraats et al., 2013). These breakdown products are natural ligands for the liver X receptor (LXR) (see **Figure 2**). When ligand-bound LXR creates a heterodimer with retinoid X receptor (RXR), which transcriptionally regulates expression of cellular cholesterol efflux proteins such as ApoE and ATP-binding cassette transporters (ABCs) (Zhao and Dahlman-Wright, 2010). ABCA1 and ABCG1 are vital for microglial lipoprotein metabolism as they are responsible for the efflux of cholesterol which gets incorporated into ApoE containing high-density-lipoprotein (HDL)-like particles (Hirsch-Reinshagen et al., 2004). Foamy phagocytes contain increased levels of 24-OHC, 25-OHC, and 27-OHC as well as up-regulated expression of LXR, ABCA1 and ABCG1 (Zhao and Dahlman-Wright, 2010), which may be an attempt to rid foamy microglia or perivascular macrophages of the cholesterol containing myelin debris (Nathanael et al., 2012).

As previously stated, aged microglia have defective phagocytic cholesterol clearance which limits remyelination in MS (Cantuti-Castelvetri et al., 2018). Myelin debris accumulates in the cells' cytosol as lipid droplets and needle-shaped cholesterol crystals (Cantuti-Castelvetri et al., 2018). Moreover, debris accumulates within microglial lysosomes, leading to lysosomal rupture, and inflammasome stimulation (Cantuti-Castelvetri et al., 2018). Altogether, the balance between cholesterol intake and cholesterol efflux may play a major role in the pathogenesis and progression of neuroinflammatory diseases. Perhaps increased cholesterol efflux may reverse metabolic derangements. For example, a recent study has demonstrated that apolipoprotein A-1 (ApoA-1) binding protein (AIBP) binds to TLR4, a component of the innate immune response, within the cell membranes of activated microglia to stimulate removal of excess cholesterol via efflux to HDL or ApoA-1 (see **Figure 2**) (Woller et al., 2018). Furthermore, intrathecal AIBP reduces TLR4 dimerization and neuroinflammation in the spinal cord in facilitated pain states (Woller et al., 2018). Although microglial cholesterol efflux is important, a major contributor of cholesterol and oxysterol efflux is achieved by astrocytes and neurons (Chen et al., 2013). Astrocytes also use ABCA1 and ABCG1 for cholesterol efflux into lipoproteins, whereas neurons use ABCA1, ABCG1, and ABCG4 (Chen et al., 2013). Cholesterol efflux seems to have therapeutic potential and can be modulated via lipoprotein metabolism.

LIPOPROTEIN METABOLISM IN MICROGLIA

Tightly regulated lipid metabolism in the CNS is critical for maintaining normal brain functions. However, because lipids such as cholesterol and triglycerides are insoluble in water, they are transported with apoproteins, which creates an emulsion that can be transported to other cells via the extracellular milieu and/or peripheral circulation. The resulting lipoproteins are complex particles composed of a central hydrophobic core of neutral lipids such as CEs and TGs, surrounded

by an amphipathic monolayer of PLs, free cholesterol, and apolipoproteins.

Although lipoprotein metabolism in the circulation and the CNS vary considerably, it is useful to outline what we have learned from circulating lipoproteins for a clearer understanding of lipoprotein metabolism as a whole. In the peripheral circulation, exogenous lipoprotein metabolism begins with TG-rich chylomicrons (CMs) that are hydrolyzed by LPL to produce CM remnants, which are then taken up into the liver. The liver initiates endogenous lipoprotein production via the formation of VLDL carrying a range of apoproteins including apolipoprotein B-100 (ApoB-100) and ApoE. VLDL is hydrolyzed further by LPL to form intermediate-density-lipoproteins (IDL) and low-density-lipoproteins (LDL), also carrying ApoB-100 and ApoE, which are then bound to LDL receptors and internalized by cells of key metabolic tissues such as muscle, heart, adipose tissue and liver (Grunfeld and Feingold, 2018). There are no reports of VLDL and LDL in the CNS. However, it is tempting to speculate that lower-density-lipoproteins could penetrate the BBB in pathological conditions such as increased inflammation. In addition, it is possible that larger lipoproteins could have enhanced penetrance in the circumventricular organs of the brain; regions devoid of a typical BBB with intimate contact between the blood and CSF such as the median eminence (Gross and Weindl, 1987).

In contrast, HDL or HDL-like lipoproteins may traverse the BBB to enter the CSF under physiologic conditions (Stukas et al., 2014). In the periphery, the first step in HDL formation involves the synthesis of ApoA-I by the liver and intestine. After secretion, ApoA-I acquires cholesterol and PLs that are effluxed by hepatocytes and enterocytes. Cholesterol efflux is regulated by ABCA1, ABCG1 and class B scavenger receptor protein (SR-B1). It is thought that increased intracellular cholesterol leads to oxysterol formation, which triggers the activation of the LXR-RXR heterodimer and ABCA1 and ABCG1 expression in order to efflux/remove cholesterol from the cell. During lipidation, Lecithin–cholesterol acyltransferase (LCAT) transfers a FA from PLs to free cholesterol forming CE, which can then be transferred to the HDL-core (Grunfeld and Feingold, 2018). Sterol O-acyltransferase (ACAT) shares a similar function to LCAT, however it catalyzes the formation of CEs from cholesterol and LC-fatty acyl-CoA and plays a role in hepatic lipoprotein assembly (Li et al., 1999). Although ApoA-I is not synthesized in the brain, fluorescently labeled ApoA-I rapidly accumulates in the CSF after intravenous injection (Stukas et al., 2014), highlighting a plausible role in cholesterol delivery to and from the brain (Mahley, 2016).

ApoA-I is a major apoprotein of HDL-like particles in the brain, second only to ApoE. Unlike ApoA-I, ApoE is synthetized within the CNS predominantly by astrocytes (including specialized astrocytic cells, Bergmann glia, tanycytes, pituicytes, and retinal Muller cells) (Boyles et al., 1985). It has been suggested that astrocytes secrete cholesterol-rich ApoE/HDL through an interaction with ABCA1, to provide cholesterol to other cells of the CNS (Ito et al., 2014). Astrocyte derived ApoE/HDL cholesterol is involved in the provision of lipids for axonal growth and regeneration (Vance et al., 2000), and synaptogenesis (Mauch et al., 2001). Importantly, other cell types can increase ApoE production when the need for lipid substrates is greater, such as during development, injury or stress. For example, in mice expressing enhanced GFP (EGFP) ApoE, injured neurons produce high levels of EGFP and ApoE mRNA in the hippocampus (Xu et al., 2006). ApoE is also expressed in type 1 NSCs (Hong et al., 2016), and can directly regulate proliferation and spine density (Hong et al., 2016). Similarly, scRNA-seq studies have shown that microglia express higher levels of ApoE in the embryonic mouse brain (E14.5) (Hammond et al., 2019), during LPC mediated demyelination injury (Hammond et al., 2019), and in the late stages of disease pathogenesis in murine models of AD (Keren-Shaul et al., 2017). Although the precise role of increased ApoE production in microglia is unknown and may be activation-state and/or microglial subpopulation specific, it is likely that increased ApoE may be a response to increased intracellular cholesterol accumulation. For example, during late embryogenesis a significant number of OLs undergo apoptosis (discussed above). In addition to microglial phagocytosis of dying cells and myelin debris, resident microglia may also increase ApoE production concurrently in order to efflux accumulating cholesterols and oxysterols in an attempt to maintain normal microglial function. Although this has not been empirically determined, aging microglia show reduced ApoE production and massive cholesterol accumulation, with major detriment to their phagocytic capacity (Cantuti-Castelvetri et al., 2018). Interestingly, LXR agonists have been shown to reduce neurodegeneration and pathology in neurogenerative animal models, suggesting that ABCA1/ABCG1-mediated cholesterol efflux promotes homeostatic glial functions (Cantuti-Castelvetri et al., 2018).

In addition to cholesterol efflux, apoproteins and lipoproteins have been repeatedly implicated in microglial phagocytosis. For example, ApoE is known to bind to LDL receptors (e.g. LDL receptor [LDLR], LDL Receptor Related Protein 1 [LRP1] and VLDL receptor [VLDLR]) on the surface of microglia and become internalized via receptor-mediated endocytosis (Pocivavsek et al., 2009). Recently, several other cell surface factors that may participate in lipoprotein uptake and/or endocytosis have been identified. TREM2 is a recently discovered AD risk gene that encodes a single-transmembrane protein that is selectively expressed in the microglia in the CNS (Klesney-Tait et al., 2006). Although the physiological role of TREM2 is still under investigation, it is thought to be protective in the context of AD since TREM2 deficiency in the 5XFAD mouse model causes increased amyloid deposition (Wang et al., 2015), whereas overexpression of TREM2 in the Amyloid precursor protein/Presenilin-1 (APP)/(PS1) mouse ameliorates neuropathology (Jiang et al., 2014). Since Aβ is known to bind to ApoE, the protection offered by TREM2 maybe conferred through its ability to bind to phospholipids and apoproteins (ApoE and apolipoprotein J [ApoJ]) and to increase phagocytosis (Atagi et al., 2015). TREM2 has been

shown to interact with Aβ-bound lipoproteins to facilitate Aβ uptake by microglia and human macrophages (Yeh et al., 2016). In fact, TREM2 serves as an extracellular lipid and lipoprotein receptor that modulates the inflammatory profile of microglia depending on the affinity of ligand binding (Lessard et al., 2018). Specifically, high avidity ligands, such as lipids and ApoE, bind TREM2 causing phosphorylation of two tyrosine residues within the adjacent transmembrane DNA polymerase III-Activation Protein 12 (DAP12) (Lessard et al., 2018). This initiates a cellular cascade culminating in survival, proliferation, phagocytosis, and motility depending on the ligand (Lessard et al., 2018). Conversely, when TREM2 is associated with low avidity ligands, an intracellular cascade is initiated that may promote inflammatory functions within the cell (Lessard et al., 2018). Recent work suggests that PS and PE from apoptotic neurons (which are not exposed on the surface of healthy cells) act as signals for TREM2-mediated microglial activation (Shirotani et al., 2019). This topic has been more comprehensively reviewed in a number of recent peer-reviewed articles (Jay et al., 2017; Gratuze et al., 2018).

Human ApoE exists as three major isoforms ApoE2, ApoE3 and ApoE4, which are encoded by three allelic variants at a single gene locus on the long arm of chromosome 19. Each isoform differs by 2 amino acid substitutions (ApoE2 [Cys112, Cys158], ApoE3 [Cys112, Arg158], and ApoE4 [Arg112, Arg158]), which significantly alters their lipid and receptor binding affinities (Mahley and Rall, 2000; Wu and Zhao, 2016). ApoE2 has a lower affinity for LDLRs compared to ApoE3 and ApoE4. In addition, ApoE2 and ApoE3 preferentially bind to small, PL-rich HDL whereas ApoE4 preferentially binds to larger, TG-rich lipoproteins (Wu and Zhao, 2016). ApoE3 is present in approximately 75% of the population and is believed to play a neutral role in AD. In contrast, ApoE2 is relatively rare (5% incidence) and is considered to be protective against AD (Wu and Zhao, 2016). While the mechanism of this protection is unclear, it is plausible that its differential association with CNS derived HDL-like lipoprotein particles plays a major role. Importantly, the ApoE4 isoform (present in 20% of the population), is present in nearly 50% of AD patients (Wu and Zhao, 2016). Although there has been a major research effort attempting to understand the increased risk of AD conferred by ApoE4, the mechanisms remain elusive. Nonetheless, in murine models of AD, it has been established that ApoE isoforms differentially effect Aβ accumulation in a dose- and isoform- dependent manner, with hippocampal Aβ burden: ApoE2 < ApoE3 < ApoE4 (Castellano et al., 2011). Findings from *in vitro* studies have shown that ApoE4 can inhibit TREM2 expression in primary murine microglia (Li et al., 2015), therefore potentially blocking the appropriate microglial response to Aβ accumulation.

ApoE4 is not the only apoprotein associated with increased AD risk. In 2009, two Genome Wide Association Studies (GWAS) identified ApoJ (otherwise known as Clusterin [CLU]) as a novel SAD-risk gene (Harold et al., 2009; Lambert et al., 2009). ApoJ is now considered the third greatest risk factor for SAD. Although a number of possible mechanisms have been proposed, including its role in TREM2-mediated microglial lipoprotein and Aβ clearance (Yeh et al., 2016), its precise function is has yet to be determined (Foster et al., 2019).

THE ROLE OF LPL IN MICROGLIA FUNCTION AND METABOLISM

In the periphery, LPL is primarily involved in the hydrolysis of CM-TG and VLDL-TG. In the murine brain LPL is predominantly expressed in microglia/macrophages and the OPC (Zhang et al., 2014), whereas in the human brain LPL is predominantly expressed in microglia (Zhang et al., 2016). Although the role of LPL in microglia is not well understood, LPL has been repeatedly implicated in AD pathogenesis in humans. For example, patients with AD have reduced LPL immunoreactivity in the dentate gyrus and reduced LPL activity in their CSF (Gong et al., 2013). In addition, loss of function LPL polymorphisms have been linked to reduced enzymatic activity, increased VLDL-TG, and increased AD risk (Ren and Ren, 2016). In contrast, patients with a gain-of-function LPL polymorphism (447Ter) have increased LPL activity concomitant with lower VLDL-TG, higher HDL, and significantly reduced hippocampal amyloid plaque formation (Baum et al., 2000). Moreover, there is a growing body of literature highlighting the importance of LPL in microglial function. For example, in the 5XFAD mouse model of AD, LPL expression is dramatically increased in DAM (Keren-Shaul et al., 2017). Importantly, markedly enhanced LPL expression repeatedly observed in microglial subpopulations, particularly in the context of development (Hammond et al., 2019), demyelination (Hammond et al., 2019), and disease (Mathys et al., 2017). In both mouse and human brains, LPL co-localizes with microglia that have internalized Aβ (Keren-Shaul et al., 2017), suggesting that microglial-LPL may also play a role in Aβ uptake. Indeed, LPL has been shown to directly bind Aβ and play a role in uptake and degradation in astrocytes (Nishitsuji et al., 2011). However, exactly how LPL may interact with Aβ remains to be determined. Since LPL is known to directly interact with LRP1 (Beisiegel et al., 1991; Chappell et al., 1992), which is major receptor for both ApoE and Aβ (Herz and Strickland, 2001), it is plausible that LPL could facilitate Aβ uptake through interaction with other factors expressed on the microglia cell surface. LPL also directly interacts with ApoE (Baum et al., 2000), and it is plausible that the strength of the interaction may vary depending on the isoform's specific lipid binding avidity. The role of LPL in microglia could conceivably link both lipid metabolism and inflammation. We have previously shown that the BV-2 microglia is polarized to a robust inflammatory phenotype following LPL loss, a phenotype that is recapitulated in primary microglia (Bruce et al., 2018). Moreover, loss of LPL shifts the metabolic profile of microglial cell lines toward increased glycolysis and reduced oxidative metabolism, reminiscent of the Warburg-like metabolism observed in inflammatory macrophages (Bruce et al., 2018). Our findings suggest that the importance of LPL in

microglia may extend beyond phagocytosis of Aβ to regulation of metabolic and inflammatory phenotype.

FUTURE STRATEGIES

The findings highlighted in this review suggest that lipid and lipoprotein metabolism is a tightly regulated component of microglial immunometabolism. The changes seen in microglial lipid metabolism during damage and disease are some of the most drastic and profound responses that have been observed to date. This suggests that microglial lipid, cholesterol, and lipoprotein metabolism may be novel therapeutic targets for the treatment of CNS disorders such as ND. Since pro-inflammatory microglia and perivascular macrophages that may contribute to ND progression exhibit increased glycolysis but decreased OXPHOS and FAO, it is plausible that modulating substrate utilization in microglia could ameliorate neuroinflammation. Although the optimal method to switch substrate usage in microglia has not been defined, several studies have shown that dietary approaches are sufficient to modulate microglial immunometabolism. Ketogenic diets typically contain very low carbohydrate, but very high fat levels and therefore have the capacity to promote FAO and limit glycolytic flux. In a recent study using a murine model of glaucoma, a ketogenic diet (10.4% protein, 0.1% carbohydrate, and 89.5% fat) was able to reduce markers of microglial activation such as Ionized calcium binding adaptor molecule 1 (Iba1) (Harun-Or-Rashid and Inman, 2018). It has also been shown that ketones can directly modulate inflammation in microglia. For example, β-hydroxybutyrate activates G-protein-coupled receptors 109A (GPR109A) to attenuate NF-κB signaling and pro-inflammatory cytokine production (Fu et al., 2015). These studies suggest that ketogenic diets may be a promising strategy; however, it is important that we do not halt the waves of microglial activation that may be potentially protective in the contexts of certain disease states. In addition, it is critical to carefully evaluate the lipid composition of these diets, since only those including LC-FAs would promote mitochondrial oxidative metabolism. It is also important to note that ketogenic diets may not be optimal for overall metabolic health, and other strategies, such as caloric restriction, and fasting may increase ketone delivery to the brain to down-regulate microglial activation without markedly elevating lipid consumption (Loncarevic-Vasiljkovic et al., 2012; Tu et al., 2012; Ghosh et al., 2018).

CONCLUDING REMARKS

In summary, microglia are dynamic cells that are not only critical for homeostatic brain functions but in mitigating the response to a variety of stimuli such as primary myelination, and demyelination. During their response, microglia tightly regulate lipid and lipoprotein metabolism in order to fuel greater bioenergetics needs, to phagocytose and process lipid-rich debris, and to produce precursors for secondary messengers. Although recent studies have galvanized the importance of lipid and lipoprotein metabolism in microglia, this emerging field is likely to reveal further important mechanisms going forward. In addition, further study into the role of lipid metabolism in the polarization of microglial inflammatory status may highlight novel approaches that modulate metabolism to ameliorate neuroinflammatory and NDs.

AUTHOR CONTRIBUTIONS

KB devised the framework of the manuscript. BL designed and created the figures. BL and KB shared equal parts in drafting and editing the manuscript.

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REVIEW
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Involvement of Lipids in Alzheimer's Disease Pathology and Potential Therapies

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Lipids constitute the bulk of the dry mass of the brain and have been associated with healthy function as well as the most common pathological conditions of the brain. Demographic factors, genetics, and lifestyles are the major factors that influence lipid metabolism and are also the key components of lipid disruption in Alzheimer's disease (AD). Additionally, the most common genetic risk factor of AD, APOE $\epsilon 4$ genotype, is involved in lipid transport and metabolism. We propose that lipids are at the center of Alzheimer's disease pathology based on their involvement in the blood-brain barrier function, amyloid precursor protein (APP) processing, myelination, membrane remodeling, receptor signaling, inflammation, oxidation, and energy balance. Under healthy conditions, lipid homeostasis bestows a balanced cellular environment that enables the proper functioning of brain cells. However, under pathological conditions, dyshomeostasis of brain lipid composition can result in disturbed BBB, abnormal processing of APP, dysfunction in endocytosis/exocytosis/autophagocytosis, altered myelination, disturbed signaling, unbalanced energy metabolism, and enhanced inflammation. These lipid disturbances may contribute to abnormalities in brain function that are the hallmark of AD. The wide variance of lipid disturbances associated with brain function suggest that AD pathology may present as a complex interaction between several metabolic pathways that are augmented by risk factors such as age, genetics, and lifestyles. Herewith, we examine factors that influence brain lipid composition, review the association of lipids with all known facets of AD pathology, and offer pointers for potential therapies that target lipid pathways.

Keywords: amyloid precursor protein, apolipoproteins, blood-brain barrier, energy metabolism, inflammation, late-onset Alzheimer's disease, mitochondria, myelination

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BACKGROUND

The Importance of Cellular Lipid Membranes

Cell membranes are composed of several lipid classes and membrane-bound proteins/receptors that interface cellular organelles, and cells with their environment. It is now recognized that these membrane lipids are important in maintaining cellular functions. Several studies show that perturbation of membrane lipids can have devastating consequences on the brain. These changes

underlie Alzheimer's disease (AD) pathology depicted in **Figure 1**. We will examine factors that affect lipid metabolism, describe the functions of brain lipids, and examine the consequences and contributions of lipid dyshomeostasis on AD pathology.

Brain Lipids in Healthy Aging and AD Pathology

Most of the brain is composed of lipids, which can be grouped as sphingolipids, glycerophospholipids, and cholesterol (Svennerholm et al., 1994; O'Brien and Sampson, 1965; Kishimoto et al., 1969). The brain consists of straight-chain monocarboxylic acids ranging from C_{12} to C_{26}, and omega-3 (n-3) and omega-6 (n-6) fatty acids are most abundant (Kishimoto et al., 1969; Siegel, 1999). Docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) are prominent polyunsaturated fatty acids (PUFA) in the brain that are derived from alpha-linolenic acid (ALA), an omega-3 fatty acid (Chappus-McCendie et al., 2019). Arachidonic acid (AA) and docosatetraenoic acid (DPA) constitute a large proportion of PUFA's that are derived from linolenic acid (LNA), an omega 6 fatty acid (Leonard et al., 2000; Sinclair et al., 2007).

FACTORS THAT AFFECT BRAIN LIPIDS

Demographic Factors That Influence Brain Lipids

Brain Lipids Changes in Aging

These PUFA's are incorporated into membrane phospholipids and therefore play a significant role in structural integrity and function of cell membranes. Lipid metabolism is changed during aging (Montanini et al., 1983; Yehuda et al., 2002; Whelan, 2008; Denis et al., 2015; Cutuli, 2017; Chappus-McCendie et al., 2019), as shown by a decline in omega-3 fatty acids and an increase in lipid peroxidation (Chen et al., 2017). Omega-3 fatty acids have antioxidant properties, and a lack of these fatty acids in one's diet may accelerate neuronal degeneration (Yehuda et al., 2002; Janssen and Kiliaan, 2014). Susceptibility of lipids to peroxidation increases with age (Bourre, 1991; Spiteller, 2010; Denis et al., 2015; Chen et al., 2017), which supports using the level of oxidative stress as a critical determinant of neuronal health and longevity (Hulbert et al., 2006). Previous studies have suggested that DHA and EPA may protect against peroxidation and the effects of age-related brain pathology (Hasadsri et al., 2013; Chen et al., 2017). Lipids are involved in cellular signaling, energy balance, blood-brain barrier (BBB), and inflammation (Song et al., 2008; Willis et al., 2009), and such age-dependent lipidome changes that disrupt these functions may contribute to neurodegenerative diseases (Arnoldussen et al., 2016; Bos et al., 2016; Hooper et al., 2018; Luo et al., 2018; McNamara et al., 2018; Lepping et al., 2019), such as Alzheimer's disease (AD) (Schmitt et al., 2014; Hussain et al., 2019).

Lipids and Race/Ethnicity

Race and ethnicity play a significant role in the risk of AD and related disorders. In 2014, nearly 5 million people over the age of 65 had been diagnosed with Alzheimer's disease or related dementias (ADRD) (Matthews et al., 2019). African Americans and Hispanics had the highest prevalence of ADRD (13.8% and 12.2%, respectively), while ADRD was least common in Asian and Pacific Islanders (8.4%), followed by American Indian/Alaska Natives (9.1%), non-Hispanic whites (10.3%), and people with two or more races (11.5%) (Matthews et al., 2019). Ethnic and racial backgrounds impact many aspects of health, including diet, stress, access to medical treatment, and biological factors of disease. From past research, we can clearly see the ways in which ethnicity, race, and lipids overlap. Most clearly seen in the high incidence of both dyslipidemia, or abnormal amounts of lipids in the blood, and cardiovascular disease observed in minority populations (Frank et al., 2014), race/ethnic disparities affect the regulation of lipid metabolism. Increased concentrations of triglycerides (TG) and decreased levels of lipid carriers, such as HDL-C (high-density lipoprotein-cholesterol) in the blood of Mexican, Filipino, Indian, and Vietnamese people compared to whites may provide a possible explanation for higher risk of both ADRD and cardiovascular disease within these populations (Frank et al., 2014; Gazzola et al., 2017). HDL-C is often referred to as "good cholesterol," has beneficial antioxidant and anti-inflammatory effects in the body, and has been observed to modulate ß-amyloid (Aß) production in the brain, a key biomarker of AD pathogenesis (Reitz, 2012; Hottman et al., 2014). Lowered levels of HDL-C have been associated with increased cognitive decline and poor cardiovascular health outcomes (Hottman et al., 2014). TG, which is increased in almost every minority population, except African Americans, has been shown to relate to central leptin- and insulin resistance in the brain and decreases in cognition (Sumner, 2009; Frank et al., 2014; Banks et al., 2018). In light of the less marked changes in lipid make-up and metabolism seen in African American populations at increased risk of ADRD, it has been suggested that African Americans are underdiagnosed with metabolic syndromes and vascular-cognitive disorders (Sumner, 2009). Furthermore, it has been observed that there is a differential expression of various molecular biomarkers of AD (phosphorylated tau and total tau) in African Americans compared to whites (Morris et al., 2019), suggesting even small, imperceptible changes in lipid distribution in this population may be sufficient to affect cognition negatively. It is important to note that despite the disproportionate impact ADRD has on minority populations, these individuals continue to be considerably underrepresented in ADRD research, contributing to large gaps in our understanding of brain lipid metabolism as it pertains to race and ethnicity (Gilmore-Bykovskyi et al., 2019).

Lipids and Sex

Sex continues to be one of the largest risk factors for developing AD. Females not only makeup two-thirds of all cases of AD diagnoses but also possess a greater lifetime risk of dementia compared to men due to longer life expectancy (Viña and Lloret, 2010; Mielke, 2018). Increased prevalence and risk of AD and other age-related disorders among females have been attributed to not only extended life expectancy but

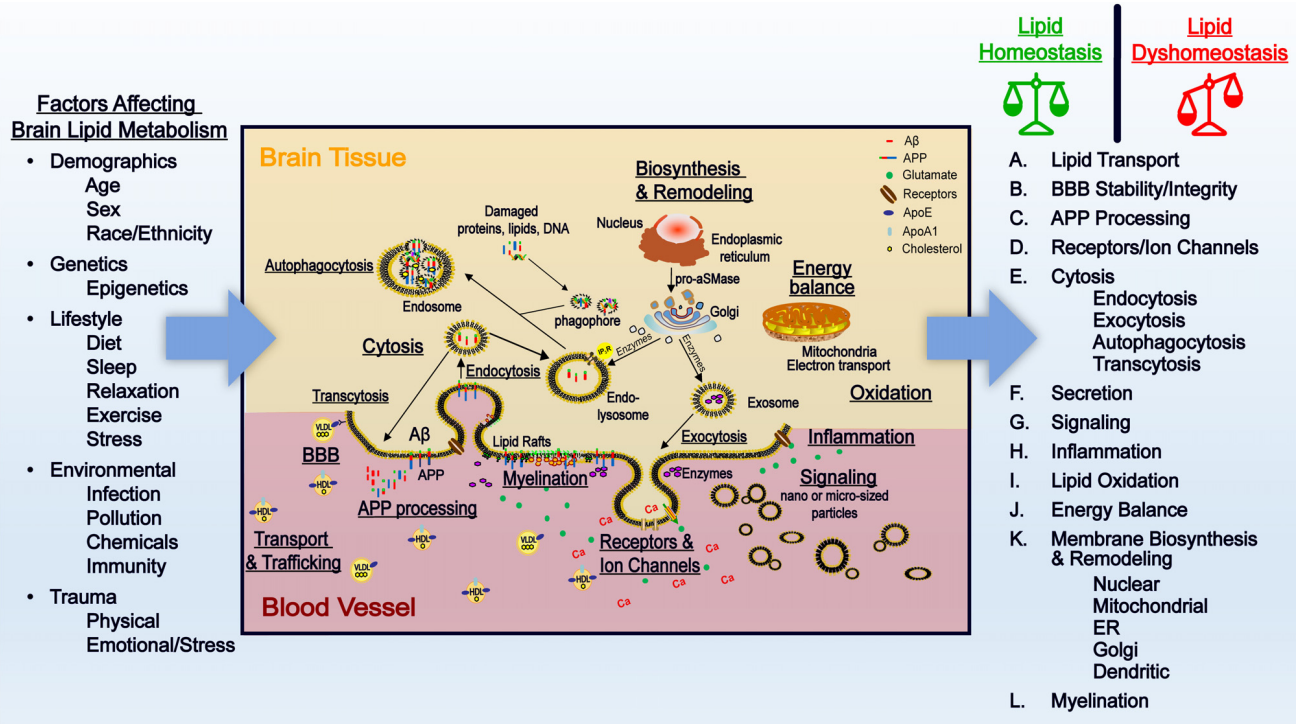

FIGURE 1 | Factors that affect brain lipid metabolism and the importance of lipids in healthy aging and AD. *Factors that affect brain lipid metabolism* – Demographic factors, genetics, lifestyle, the environment, and trauma can influence lipid metabolism in the brain. Interestingly, these factors that influence lipid metabolism are also recognized risk factors of AD. Abnormalities in lipid metabolism can contribute to dysfunctional brain networks that associate with AD pathology. *Importance of lipid metabolism in brain function and AD pathology* – In healthy aging, normal transport of lipids through apolipoproteins contribute to the function of the brain. Homeostatic control of the brain lipid environment is responsible for sustaining a normal BBB, providing the right environment for normal APP processing, the right composition for ion channels and receptors, cytosis, vesicle formation, and secretion, signaling, inflammation, oxidation, energy balance, and membrane biosynthesis and remodeling. Dyshomeostasis in lipid delivery into the brain and its metabolism attributes to disturbed BBB, abnormal APP processing, disturbance in cytosis, signaling, energy balance, and enhanced/sustained inflammation and oxidation. Over time, these processes lead to neuronal death that is the hallmark of AD pathology.

also to sudden decreases in estrogen post-menopause, among many other factors including education level and mental health status (Viña and Lloret, 2010; Mielke, 2018). Despite the many factors that may contribute to increased risk of AD in women, the contribution of sex-hormone levels and differential lipid distribution play evident roles in cognitive decline are not fully understood. Not only is fat in the form of TG distributed differently in the adipose tissue of male and females, which can be attributed in part to sex-hormone signaling, but concentrations of long-chain PUFAs (LC-PUFAs) have also been observed to be increased in women pre-menopause compared to men (Decsi and Kennedy, 2011; Lohner et al., 2013). Correspondingly, a positive association has been established between omega-3 LC-PUFA biosynthesis, i.e., the production of EPA and DHA, and circulating concentrations of estrogen and progesterone (Childs et al., 2008). Estrogen, an ovarian steroid hormone, is hypothesized to affect lipid metabolism at several points during biosynthesis, including playing a key role in lipid transport and exchange, increasing expression of metabolic enzymes, and reducing the oxidation of α-linoleic acid (ALA), the deriving fatty acid in n-3 LC-PUFA production (Childs et al., 2008; Decsi and Kennedy, 2011; Lohner et al., 2013; Palmisano et al., 2018). Estrogen has also been directly associated with inhibiting memory function impairment in premenopausal women following the surgical removal of their ovaries and loss of the ability to produce estrogen endogenously (Duka et al., 2000; Sherwin, 2012). In a study of *trans*-sexual subjects, those transitioning from male to female and receiving estrogen observed an increase in DHA plasma levels while those transitioning from female to male and receiving testosterone treatment experienced a marked decrease in plasma DHA (Giltay et al., 2004). The decrease in estrogen levels, as seen in post-menopausal women, has also been associated with increased TG content and lower HDL-C, both of which have been linked to cognitive decline (Derby et al., 2009; Anceline et al., 2014). This is to say, the increased prevalence and risk for AD among women can be explained in part by the abrupt decrease in estrogen production that accompanies the post-menopausal state. Not only does the lack of estrogen decrease concentrations of anti-inflammatory LC-PUFAs and HDL-C in the body, but it also increases TG levels, augmenting secretion of VLDL (very-low density lipoprotein), a lipid carrier known to induce neuroinflammation (Burgess et al., 2006; Chen et al., 2014; Nägga et al., 2018). Additionally,

genetic factors, such as ApoE status, and social determinants, such as education, mental illness, and diet, interact with the post-menopausal state to amplify these detrimental effects, increasing risk of AD.

Lipids and Lifestyle

Diet

Dietary lipids play an integral part in physiological lipid metabolism and, consequently, in the risk of AD and cardiovascular disease. Essential fatty acids like DHA (n-3) and AA (n-6) are largely derived from the dietary consumption of their shorter-chained, slightly less-saturated counterparts ALA (n-3) and LA (n-6), respectively (Schmitz, 2008; Morris and Tangney, 2014). After consumption of these deriving fatty acids, the body is able to anabolize them, creating the LC-PUFAs that contribute to neural processes (Morris and Tangney, 2014). Early on in human existence, our diet consisted of an equal balance of n-6 to n-3 essential fatty acids, but as we have evolved, the n-3 to the n-6 ratio of dietary fatty acids has greatly shifted to one side (Simopoulos, 2006). Today, the Western diet has a ratio of about 17 to 1 n-6 to n-3 fatty acids, meaning most Americans have a lot more LA, AA, and DPA in their bodies, which are able to produce relatively large quantities of inflammatory and oxidative mediators (Simopoulos, 2006). Increased ratios of n-6 to n-3 dietary fatty acids have also been directly associated with increased cognitive decline and risk of AD (Loef and Walach, 2013; MacDonald-Wicks et al., 2019). DHA, on the other hand, an n-3 LC-PUFA usually found in fish and algae, is not largely found in the Western diet. Studies suggest, however, that DHA supplementation may work to combat neuroinflammation, oxidative stress, and cognitive decline. Fish oil supplements containing large amounts of DHA, given to older adults with varying levels of cognition, found that supplementation resulted in decreased brain atrophy and less cognitive decline compared to controls in an *APOE* allele-dependent manner (Daiello et al., 2015). Similarly, Morris et al. observed among subjects over the age of 65 that those who ate fish at least once a week had 60% less risk of AD than those who rarely or never ate fish (Morris et al., 2003). Dietary DHA has also been shown to improve cognition, memory, and brain development from the earliest stages of life through adulthood (Dunstan et al., 2008; McNamara et al., 2010; Muldoonm et al., 2010; Stonehouse et al., 2013; Weiser et al., 2016).

It is important to note that diet can be particularly impacted by race/ethnicity, as well as physical geography, helping to explain differences in AD risk among ethnic groups. According to a global survey of 298 studies, highest levels of DHA and EPA, another n-3 fatty acid, were observed among Japanese, Scandanavian, and indigenous populations, as well as in areas where the Westernized diet had not been fully adopted (Stark et al., 2016). Authors of this survey argue increased consumption of seafood, as dictated by culture or geographical location, greatly impact n-3 LC-PUFA levels in the bloodstream, which offer protective cognitive effects at every stage in life (Joffre et al., 2014; Stark et al., 2016; Weiser et al., 2016).

Genetical Evidence for the Importance of Lipid Metabolism in AD Pathology

Genetic Risk Factors of AD-Related to Lipid Metabolism

Genome-wide Association Studies GWAS and Transcriptome-Wide Association Studies (TWAS) associate AD pathology with several lipid genes (Shi et al., 2010; Hao et al., 2018). While the APOE4 allele carries the greatest risk for AD, other genes and gene-products commonly associated with AD pathology are linked to or interact with lipid metabolism. Several lipid genes associated with AD pathology have recently been reviewed (Tindale et al., 2017). **Table 1** is the list of the major genes from GWAS that are linked with lipid metabolism (Jones et al., 2010).

Genome-wide Association Studies suggest that age-related changes in brain lipid metabolism may be essential to healthy aging and longevity (Tindale et al., 2017). Identification of AD-related genes and how these interact with specific risk factors may provide the rationale for designing effective therapies.

The onset of age related disease can be accelerated with suppression of anti-aging genes, such as Sirtuin 1 (SIRT1). SIRT1 is a histone deacetylase involved with gene expression related to metabolic activity (Grabowska et al., 2017). SIRT1 interacts with lipid metabolism regulation and hepatic oxidative stress and inflammation (Ding et al., 2017). It also regulates circadian rhythms in the liver and brain, maintaining the body's regulation of glucogenesis, fatty acid beta-oxidation, and cholesterol biosynthesis (Bellet et al., 2016). Its involvement in metabolism explains its effects on energy metabolism, neurogenesis, glucose and cholesterol metabolism, and amyloidosis. Sirt 1 also contributes to neuron apoptosis and survival. Downregulation of this anti-aging gene may lead to acceleration of neurodegenerative disease. Nutritional interventions, such as a reduction in overconsumption of carbohydrates, are recommended because they may be associated with preventing cell senescence and maintaining anti-aging gene activity (Martins et al., 2017). SIRT1 expression promotes APP processing on a non-amyloidogenic pathway and clearance of tau from the brain (Herskovits and Guarente, 2014). SIRT1's deacetylase activity increases the activity of lysosome-related genes, facilitating Aβ degradation (Li et al., 2018). SIRT1 is a potential therapeutic target for AD because of its involvement in many amyloid beta and cholesterol pathways.

CONTRIBUTION OF LIPIDS TO AD PATHOLOGY

Although the brain has a very high concentration of long-chain omega-3 and omega-6 fatty acids, there is no conclusive explanation for how these fatty acids participate in various signaling cascades and in AD (Torres et al., 2014; Mohaibes et al., 2017). However, lipodomic studies related to AD pathology have demonstrated a decrease in DHA levels within the brain, predominantly in the hippocampus (Belkouch et al., 2016). Damage to the hippocampus is associated with impaired learning and memory abilities, a symptom of AD onset

TABLE 1 | Lipid metabolism-associated genes with SNP (<0.001) linked with AD from GWAS.

Gene Symbol [***Chromosome location*** (***Mb***)] (Jones et al., 2010)	**The function of the gene product#**	**Changes and known effects on AD pathology**
APOE [*19* (*50*)]	As part of lipoproteins, ApoE is involved in the transport and distribution of lipids into various tissues via plasma and other interstitial fluids (Huang and Mahley, 2014)	Polymorphism of APOE is associated with age of onset (do Couto et al., 1998; Vermunt et al., 2019), cognitive and memory decline (Asada et al., 1996; El Haj et al., 2016), amyloid load (Mecca et al., 2018), cholesterol homeostasis (Leduc et al., 2010), inflammation (Tzioras et al., 2019)
APOC1 [*19* (*50*)]	Involved in HDL and VLDL metabolism, inhibitor cholesteryl ester transfer protein in plasma	Gene polymorphism (Prendecki et al., 2018), oxidative stress (Prendecki et al., 2018), interaction with ApoE (Lucatelli et al., 2011), cognitive impairment (Zhou et al., 2014)
CLU [*8* (*28*)]	Clusterin (ApoJ) is a component of lipoproteins associated with lipids in plasma and CSF	Polymorphism (Shuai et al., 2015; Zhu et al., 2018), interaction with PICALM (Harold et al., 2009; Kamboh et al., 2012), hippocampal function (Erk et al., 2011).
APOC2 [*19* (*50*)]	A component of triglyceride (TG)-rich lipoproteins, including VLDL, HDL), and chylomicrons involved metabolism of these particles; promote VLDL1 secretion, inhibit lipoprotein lipase enzyme activity	Polymorphism associated with AD (Sun et al., 2005), decreased expression associated with increased risk (Lin et al., 2015)
APOC4 [*19* (*50*)]	A lipid-binding lipoprotein thought to play a role in lipid metabolism	Decreased expression associated with increased risk (Lin et al., 2015)
ABCA7 [*19* (*1*)]	Member of the ATP-binding cassette (ABC) superfamily of transporters; catalyzes the translocation of specific phospholipids from the cytoplasmic to the extracellular/lumenal leaflet of the membrane coupled with ATP hydrolysis, lipid homeostasis, binds APOA1, apolipoprotein-mediated phospholipid efflux from cells, cholesterol efflux, lipid raft organization	Polymorphism correlate with memory impairment (Chang et al., 2019), amyloid plaque burden (Zhao Q. F. et al., 2015), cognitive impairment (Chung et al., 2014; Berg et al., 2019)
ABCA1 [*9* (*107*)]	A membrane of the superfamily of ATP-binding cassette (ABC) transporters with cholesterol as its substrate, it functions as a cholesterol efflux pump in the cellular lipid removal pathway	Polymorphism in AD (Chu et al., 2007; Wavrant-De Vrieze et al., 2007; Wang et al., 2013), modulates cholesterol efflux (Shibata et al., 2006; Khalil et al., 2012; Marchi et al., 2019), influences age of onset (Wollmer et al., 2003a)
ABCA12 [*2* (*216*)]	A membrane of the superfamily of ATP-binding cassette (ABC) transporters involved in the transport of molecules across the cellular membrane	SNP with $p < 0.001$ and significantly enriched in AD (Jones et al., 2010)
LIPC [*15* (*57*)]	Hepatic triglyceride lipase is a triglyceride hydrolase and ligand/bridging factor for receptor-mediated lipoprotein uptake	Gene variant might influence AD susceptibility (Xiao et al., 2012)
ATP8A1 [*4* (*42*)]	ATPase Phospholipid Transporting 8A1 catalyzes ATP hydrolysis that is coupled to the transport of aminophospholipids from the outer to the inner leaflet of membranes to maintain their asymmetric distribution	SNP with $p < 0.001$ and significantly enriched in AD (Jones et al., 2010)
ATP8B4 [*15* (*48*)]	Amninophospholipid transport across cell membranes	SNP with $p < 0.001$ and significantly enriched in AD (Jones et al., 2010)
MALL [*2* (*110*)]	Member of the MAL proteolipid family localizes in glycolipid- and cholesterol-enriched membrane (GEM) rafts, and interacts with caveolin-1	SNP with $p < 0.001$ and significantly enriched in AD (Jones et al., 2010)
ATP8A2 [*13* (*25*)]	Involved in flipping phospholipids from the exoplasmic leaflet to the cytosolic leaflet of the cell membrane to generate or maintain membrane lipid asymmetry	SNP with $p < 0.001$ and significantly enriched in AD (Jones et al., 2010)
OSBPL7 [*17* (*43*)] OSBPL9 [*1* (*59*)]	Oxysterol-binding protein (OSBP) family, intracellular lipid receptors Oxysterol-binding protein (OSBP) family, a group of intracellular lipid receptors; cholesterol transfer protein and regulation of Golgi structure and function	Differential expression (BrownIII, Theisler et al., 2004; Herold et al., 2016)
SCARB1 [*12* (*124*)]	Scavenger Receptor Class B Member 1 is a plasma membrane receptor for HDL that also mediates cholesterol transfer to or from HDL	Cholesterol efflux and anti-inflammation (Khalil et al., 2012), endocytosis, transcytosis and Abeta removal (Mackic et al., 1998; Srivastava and Jain, 2002)
VPS4B [*18* (*59*)]	Vacuolar Protein Sorting 4 Homolog B involved in late endosomal multivesicular bodies (MVB) pathway. Degradation of lysosomal enzymes and lipids.	SNP with $p < 0.001$ and significantly enriched in AD (Jones et al., 2010)
ABCG1 [*21* (*42*)]	Coupled to ATP hydrolysis, catalyzes the efflux of sphingomyelin, cholesterol, and oxygenated derivatives like 7-beta-hydroxycholesterol.	Cholesterol efflux (Hirsch-Reinshagen and Wellington, 2007; Wollmer et al., 2007; Marchi et al., 2019)
LIPG [*18* (*43*)]	Diverse class of lipase enzymes includes diacylglycerol lipase (DAGL) and lipoprotein lipase (LPL) and endothelial lipase (LIPG). Hydrolyzes HDL more efficiently than other lipoproteins	Polymorphism and mutation (Baum et al., 1999; Blain et al., 2006), cholesterol homeostasis (Blain and Poirier, 2004; Fidani et al., 2004), stimulation in nucleus basalis and hippocampus (Farooqui et al., 1988)

(Continued)

TABLE 1 | Continued

Gene Symbol [*Chromosome location* (*Mb*)] (Jones et al., 2010)	The function of the gene product[#]	Changes and known effects on AD pathology
PCTP [*17* (*51*)]	Phosphatidylcholine (PC) Transfer Protein; PC synthesis and metabolism, binds single PC molecule and transfers between membranes	Cholesterol transport (Knebl et al., 1994; Demeester et al., 2000)
SLC27A4 [*9* (*130*)]	Family of fatty acid transport proteins; translocation of long-chain fatty acids across the plasma membrane, has acyl-CoA ligase activity for long-chain and very-long-chain fatty acids (VLCFAs)	SNP with $p < 0.001$ and significantly enriched in AD (Jones et al., 2010)
NPC1 [*18* (*19*)]	Intracellular cholesterol transporter which is important in cholesterol removal from endosomal/lysosomal compartment	Increase expression (Kagedal et al., 2010; Maulik et al., 2015), neurocognitive deficit (Johnen et al., 2018)
APOA1 [*11* (*116*)]	Apolipoprotein A-I is the major protein HDL in plasma. It promotes cholesterol efflux from tissues to the liver for excretion and is a cofactor for lecithin cholesterol acyltransferase (LCAT), an enzyme that forms cholesteryl ester	Polymorphism and decreased expression (Helbecque et al., 2008; Smach et al., 2011; Shibata et al., 2013; Lin et al., 2015)
APOC3 [*11* (*116*)]	A component of triglyceride-rich VLDL, and HDL in plasma. Important in triglyceride homeostasis: promotes hepatic VLDL1 assembly and secretion, attenuates hydrolysis and clearance of triglyceride-rich lipoproteins, impairs TRL lipolysis by inhibiting lipoprotein lipase and the hepatic uptake of TRLs by remnant receptors	Polymorphism and decreased expression (Lin et al., 2015; Sun et al., 2005)
APOA4 *[11 (116*)]	Apolipoprotein A4 is a major component of HDL and chylomicrons. Important in chylomicrons and VLDL secretion and catabolism. Required for lipoprotein lipase activation by ApoC-II, a potent activator of LCAT	Decreased expression (Lin et al., 2015), enhanced susceptibility (Papassotiropoulos et al., 2005)
AGTR1 [*3* (*1490*)]	Angiotensin II is a primary regulator of aldosterone secretion	Signal transduction abnormality (Pang et al., 2019)
SOAT1 [*1* (*177*)]	Sterol O-Acyltransferase-1 is an acyltransferase that catalyzes the formation of fatty acid and cholesterol esters, which is important in lipoprotein assembly and dietary cholesterol absorption. It may also act as a ligase	Polymorphism (Lamsa et al., 2007), Cholesterol levels, amyloid load (Wollmer et al., 2003b)

[#]*https://www.genecards.org/*.

(Sarrafpour et al., 2019). With growing evidence that AD is associated with dysregulation of fatty acid metabolism, fatty acid levels may be potential biomarkers of this disease (Fonteh et al., 2014; Wong et al., 2017). In addition to omega fatty acids, the levels of several lipids change with AD pathology (**Table 2**).

Lipid Transport: Apolipoproteins

Brain Lipoproteins and Their Function

Lipoproteins are molecules with a hydrophobic lipid core composed of cholesterol, esters, and triglycerides and a hydrophilic exterior of phospholipids, apolipoproteins, and free cholesterol (Alaupovic, 1996; Hoofnagle and Heinecke, 2009; Braun and Hantke, 2019). Lipoproteins assist with the transport of lipids and amphipathic compounds throughout the body (Feingold and Grunfeld, 2000). However, circulating plasma lipoproteins differ from those within the CNS because only high-density lipoproteins (HDL) can cross the blood-brain barrier (Balazs et al., 2004). The most abundant apolipoproteins, apolipoprotein E (ApoE), and apolipoprotein J (ApoJ) are synthesized by astrocytes and serve as enzyme cofactors and receptor ligands on HDL (Pitas et al., 1987; Feingold and Grunfeld, 2000; Ito et al., 2014).

Apolipoproteins are greatly involved in metabolism, serving as both activators and inhibitors of metabolic enzymes, ligands for lipoprotein receptors, and providing structural support (Feingold and Grunfeld, 2000; Bolanos-Garcia and Miguel, 2003; Filou et al., 2016). They also regulate lipid transport by controlling interactions with receptors, enzymes, and lipid-transport proteins (Bolanos-Garcia and Miguel, 2003; Ramasamy, 2014). Apolipoproteins have receptor binding domains containing low-density lipoprotein (LDL) receptors that direct lipid and substrate delivery to specific brain cells (Clavey et al., 1995; Dehouck et al., 1997; Herz, 2001). Their amphipathic-helices facilitate lipid-binding and lipid transport (Clavey et al., 1995; Prevost and Kocher, 1999; Elliott et al., 2010). LDL receptors also facilitate the clearance of amyloid peptides through the BBB (Shibata et al., 2000).

Contribution of Lipoproteins to AD Pathology

Brain lipoproteins with ApoE are responsible for phospholipid and cholesterol transport (Growdon and Hyman, 2014; Wong et al., 2019). ApoE is mainly expressed in astrocytes and microglia and appears as three major isoforms, ApoE2, ApoE3, and ApoE4, of which ApoE4 is the strongest genetic risk factor for AD (Stone et al., 1997; Ito et al., 2005; Vance and Hayashi, 2010; Chung et al., 2016; Liu et al., 2017; Montoliu-Gaya et al., 2018; Tulloch et al., 2018). ApoE4 demonstrates a lower affinity for lipids than ApoE2 and ApoE3, limiting CNS transport of lipids needed for neuronal remodeling and repair (Bradley and Gianturco, 1986; Barbagallo et al., 1998; Li et al., 2002; Frieden et al., 2017). Furthermore, levels of ApoE LDL receptors directly correlate with Aβ clearance, and promoting

TABLE 2 | Summary of lipids that change in AD.

Lipids	Changes observed in AD
Fatty acids	
Omega-3 fatty acids (#DHA, EPA, DPA, ALA)	DHA decreased in brains, circulation, and CSF of AD individuals (Fonteh et al., 2014, 2020; de Wilde et al., 2017; Snowden et al., 2017; Hosseini et al., 2020). EPA decreased in brain and circulation of AD individuals (Hosseini et al., 2020). DPA increased in livers of AD (Dyall, 2015). ALA increased in plasma and peripheral tissues (Cherubini et al., 2007).
Omega-6 fatty acids (#AA, LA)	AA increased in brains, erythrocytes, and CSF of AD individuals (Thomas et al., 2016; Goozee et al., 2017; Fonteh et al., 2020). LA decreased in AD brain and plasma (Snowden et al., 2017; Cunnane et al., 2012).
Saturated fatty acids (#PA, SA, C15:0)	Increased in the CSF and brains of AD individuals (Fonteh et al., 2014). Odd chain saturated fatty acids derived from microbiome or measures of dairy consumption decreased in CSF of AD (Fonteh et al., 2014; Nasaruddin et al., 2016).
Eicosanoids	Pro-inflammatory eicosanoid pathways are upregulated in AD individuals, while anti-inflammatory eicosanoids are decreased (Biringer, 2019). Prostaglandin and thromboxane B_2 increased in AD brains (Iwamoto et al., 1989). Pro-resolvin mediators, such as lipoxins, are reduced in AD brains (Wang et al., 2015b).
Endocannabinoids	Decreased levels of endocannabinoids and receptors in AD brains (Bedse et al., 2015).
Glycerolipids	
Triglycerides	Total TG lipid levels decreased in the serum of individuals with probable AD (Lepara et al., 2009). Polyunsaturated TG decreased in AD brains (Bernath et al., 2019).
Glycerophospholipids	
Phosphatidylcholine (PC) Phosphatidylethanolamine (PE) Phosphatidylserine (PS)	Total PC lipids decreased in AD brains (Wood, 2012). PC species decreased in CSF of AD individuals #*PC32:0, PC34p:0/34e:1, PC34:1, PC34:0, PC36:1, PC38a:5 (PC-EPA), PC36:0/38p:6, 38a:6 (PC-DHA)* (Fonteh et al., 2013). PC species decreased in plasma of AD individuals. *PC36:5 (PC-EPA), PC38:6 (PC-DHA), PC40:6 (PC-DHA)* (Whiley et al., 2014). PC species increased in the prefrontal cortex of AD individuals. *PC38:6 (PC-DHA)*, *PC40:6 (PC-DHA)* (Igarashi et al., 2011). Total PE lipids decreased in the hippocampus of AD individuals (Prasad et al., 1998). PE species decreased in the hippocampus of AD individuals. *PE-SA*, *PE-OA*, *PE-AA*, *PE-DHA* (Guan et al., 1999). A decrease in PE plasmalogen in AD (Farooqui and Horrocks, 1998). Total PS lipids decreased in the occipital lobe and inferior parietal lobule of AD brains (Farooqui and Horrocks, 1998).
Sphingolipids	
Sphingomyelin (SM) Ceramides (CM) Sulfatides Gangliosides	Total SM lipids lower in CSF of AD individuals (Fonteh et al., 2015). SM species decreased in the CSF of AD individuals #*SM18/14:0, SM18/16:0, SM18/16:1, SM18/17:0* SM species *(SM18/18:0, SM18/18:1)* increased in the CSF of prodromal AD individuals (Kosicek et al., 2012). Total CM lipids increased in AD brains (Filippov et al., 2012). CM species increased in AD brains and plasma *CM16:0 (PA), CM18:0 (SA), CM20:0, CM24:0, CM24:1* (Kim et al., 2017). Total sulfatide levels significantly lower in AD brains in both gray and white matter. The compositional distribution of sulfatide subtypes is unaltered (Han et al., 2002). Ganglioside lipid levels reduced in the temporal lobe of AD brains (Molander-Melin et al., 2005).
Sterols	
Cholesterol Oxysterols Hormones	Cholesterol decreased, and oxysterol/cholesterol precursors increased in MCI and sporadic AD brains (Hascalovici et al., 2009). Total oxidized cholesterol increased in AD brains (Heverin et al., 2004). Oxidized cholesterol species decreased in AD brains, *24S-hydroxycholesterol* (Heverin et al., 2004). Oxidized cholesterol species increased in AD brains, *27-hydroxycholesterol* (Heverin et al., 2004). Lower estrogen increases the risk of AD (Ratnakumar et al., 2019; Uddin et al., 2020). Increased basal cortisol levels in the plasma of demented individuals (Csernansky et al., 2006). Association of cortisol with Aβ deposition (Toledo et al., 2012) and with hypometabolism (Wirth et al., 2019).

#*DHA, Docosahexaenoic acid (C22:6, n-3); EPA, Eicosapentaenoic acid (C20:5, n-3); DPA, Docosapentaenoic acid (C22:5, n-3); ALA, Alpha-linolenic acid (C18:3, n-3); AA, Arachidonic acid (C20:4, n-6); LA, Linoleic acid (C18:2, n-6); PA, Palmitic acid (C16:0); SA, Stearic acid (C18:0); OA, Oleic Acid (C18:1, n-9); PC, phosphatidylcholine; PCxxa:#, PC specie with xx carbon number and acyl-linked fatty acid at the sn-1 position containing # (number) double bonds; PC**xx**p:**#**, PC specie with xx carbon number and alk-1-enyl (plasmalogen)-linked fatty acid at the sn-1 position containing # (number) double bonds PC**xx**e:**#** PC specie with xx carbon number and alkyl (ether)-linked fatty acid at the sn-1 position containing # (number) double bond; PE, phosphatidylethanolamine; PS, phosphatidylserine; SM, sphingomyelin; CM, ceramide; TG, triglyceride.*

the expression of these receptors are potential therapeutic targets for AD treatment (Zhao et al., 2018). ApoJ, also known as clusterin, is expressed in astrocytes, neurons, and ependymal cells (Nuutinen et al., 2005, 2007). This neuroprotectant initiates a defense response to neuronal damage and clears Aβ across the BBB via LDLR-2 (Merino-Zamorano et al., 2016; Nelson et al., 2017; Zandl-Lang et al., 2018). ApoJ's role in Aβ accumulation and toxicity is still undetermined because variability under different contexts and environments confound results (Foster et al., 2019).

Lipids and the Blood-Brain Barrier

The Blood-Brain Barrier

The blood-brain barrier (BBB) is a semipermeable membrane that carefully regulates the exchange of solutes between blood and brain to maintain CNS homeostasis and block entry of toxins and pathogens into the CNS (Bradbury, 1984; Abbott et al., 2010; Betsholtz, 2014; Daneman and Prat, 2015; Ferreira, 2019; Moura et al., 2019). The integrity of the BBB is largely dependent on its tight junctions (Brown and Davis, 2002; Castro Dias et al., 2019), adherens junction proteins, and ability to control the vesicular movement of macromolecules through transcytosis and pinocytosis (Dehouck et al., 1997; Baldo et al., 2014). The BBB permits free diffusion of gases, such as oxygen and carbon dioxide, but small solutes such as lipophilic molecules and ions enter through receptor-mediated transcytosis or via channels (Fishman et al., 1987; Zlokovic, 2008; Preston et al., 2014; Andreone et al., 2017; Villasenor et al., 2017; Ayloo and Gu, 2019). The BBB is critical in linking multiple major organ systems, and any dysfunction in the lipid bilayer's ability

to act as a barrier may lead to neuronal degeneration (Zhao Z. et al., 2015; Halliday et al., 2016; Muszynski et al., 2017; Nation et al., 2019).

Importance of Lipids in BBB Function

In addition to composing the BBB lipid bilayer, lipids, including phospholipids, sphingolipids, and cholesterol, also compose the plasma membrane of vesicles involved with receptor-mediated transcytosis within the CNS (Kramer et al., 2002; Dodelet-Devillers et al., 2009; Campbell et al., 2014; Andreone et al., 2017). The formation and function of vesicles required to transport essential macromolecules across the BBB may be affected by the plasma membrane lipid composition (Lingwood et al., 2009; Lingwood and Simons, 2010; Kaiser et al., 2011). In particular, DHA disrupts the membrane domains necessary to form such transport vesicles and therefore contributes to BBB integrity and suppression of transcytosis (Ouellet et al., 2009; Freund Levi et al., 2014; Pan et al., 2015, 2016; Belayev et al., 2018). There is also recent evidence that the membrane transport protein, Mfsd2a, controls lipid exchange and plays a key role in the transport of DHA into the brain, though this pathway is largely undetermined (Segi-Nishida, 2014; Zhao and Zlokovic, 2014; Keaney and Campbell, 2015; Andreone et al., 2017). Loss of Msfd2a transport function resulted in decreased DHA transport and increased activity levels of transcytosis within CNS endothelial cells (Andreone et al., 2017). A leaky barrier increases the brain's susceptibility to toxins and pathogens and homeostasis disruption, and ultimately, neuronal dysfunction (Abbott, 2000; Hutchinson, 2010; Ikeshima-Kataoka and Yasui, 2016; Block, 2019).

The Contribution of the BBB to AD Pathology

Loss of BBB function may contribute to neurodegenerative diseases, including AD (Banks, 1999; Gilgun-Sherki et al., 2001; Zlokovic, 2008; Carvey et al., 2009; Karamanos et al., 2014; Sweeney et al., 2018; Katt et al., 2019). According to multiple independent studies, BBB breakdown in AD is demonstrated by decreased integrity of BBB tight junctions, pericyte and endothelial degeneration, RBC extravasation, and brain capillary leakages (Zlokovic, 2008; Carvey et al., 2009; de Vries et al., 2012; Nelson et al., 2016; Sweeney et al., 2018). A buildup of blood proteins and macromolecules due to barrier leakiness may damage vasculature and brain parenchyma, which induces neuronal degeneration. Studies have also indicated that AD pathology includes reduced expression of glucose transporters in the BBB (Kalaria and Harik, 1989; Harik and Kalaria, 1991; Guo et al., 2005; Agrawal et al., 2017; Block, 2019). This may exacerbate AD cerebrovascular degeneration and cognitive function, considering that the brain requires a continuous supply of glucose and utilizes the most glucose of the major organs (Benton et al., 1996; Dienel et al., 1997; Benton, 2001; Gong et al., 2006). The BBB contains a wide variety of structural components to regulate the brain's health and function, but a loss of function in any such component may lead to dyshomeostasis and a rapid cascade of dysfunctions in other structures within the brain.

Lipids Contribute to Amyloid Precursor Protein Processing

Amyloid Precursor Protein Processing

Amyloid precursor protein (APP) is a type I transmembrane protein that is cleaved into amyloid β-peptide (Aβ) by β- and γ-secretases (Nunan and Small, 2000; Hartmann, 2012). APP is synthesized in the endoplasmic reticulum and is found in the highest concentrations in neuron's *trans*-Golgi-network, suggesting that APP is associated with secretory pathways (Palacios et al., 1992; Stephens and Austen, 1996; Kitazume et al., 2001; Tam et al., 2014; Toh et al., 2017; Liu et al., 2019). There are two accepted proteolytic pathways for APP processing — non-amyloidogenic and amyloidogenic (Ishiura, 1991; Kojima and Omori, 1992; Sisodia, 1992; Roberts et al., 1994; Mills and Reiner, 1999; Soriano et al., 2001; Irizarry et al., 2004; Song et al., 2004; Chow et al., 2010; Wang et al., 2010; Tomita and Wong, 2011). The non-amyloidogenic pathway involves cleavage of APP by α-secretase at the plasma membrane, releasing soluble APPα (sAPPα) fragments into the extracellular environment, and normalizes AG genes and memory (Volmar et al., 2017). The amyloidogenic pathway involves cleavage of APP by β-secretase in early endosomes, releasing sAPPβ fragments in the endosomal lumen, and increasing susceptibility to Aβ plaques that are relevant to AD pathology (Estus et al., 1992; Golde et al., 1992; Saftig et al., 1996; Ehehalt et al., 2003; Andrew et al., 2016; Grimm et al., 2016).

The Role of Lipids in APP Processing

The β-site APP-cleaving enzyme 1 (BACE-1) is the major β-secretase that targets endosomes with APP in transit to endocytosis sites on the plasma membrane (Shimokawa et al., 1993; O'Brien and Wong, 2011; Chun et al., 2015; Audagnotto et al., 2018). Both APP and BACE-1 are associated with lipid rafts, which are membrane domains enriched with cholesterol, sphingolipids, and gangliosides that are crucial to vesicle trafficking and intracellular transport (Ehehalt et al., 2003; Yoon et al., 2007; Marquer et al., 2011; Bhattacharyya et al., 2013). Recent studies have proposed that BACE-1 in cholesterol depleted environments displayed inhibited β-secretase activity, suggesting that cholesterol and lipid composition of the intracellular environment may be a large determinant of whether BACE-1 can access APP endosomes (Dash and Moore, 1993; Cheng et al., 2014; Mukadam et al., 2018). However, other studies suggest that both homeostasis of lipid composition and oxidation state of lipids, including DHA, are critical to APP processing (Grimm et al., 2012; Bhattacharyya et al., 2013; **Figure 2**). Under conditions with high concentrations of oxidized lipids, levels of sAPPα fragments decreased while sAPPβ levels increased (Grimm et al., 2016). A novel mechanism of proteolytic activity regulation of secretases involves a separating lipid boundary with their substrates, APP (Kaether and Haass, 2004). Lipid mediators of inflammation also interact with APP processing at the level of O-GlcNAcylation (Sastre et al., 2008; Jean-Louis et al., 2018). Thus, oxidized or inflammatory lipids may shift APP processing from the non-amyloidogenic to an amyloidogenic pathway (**Figure 2**).

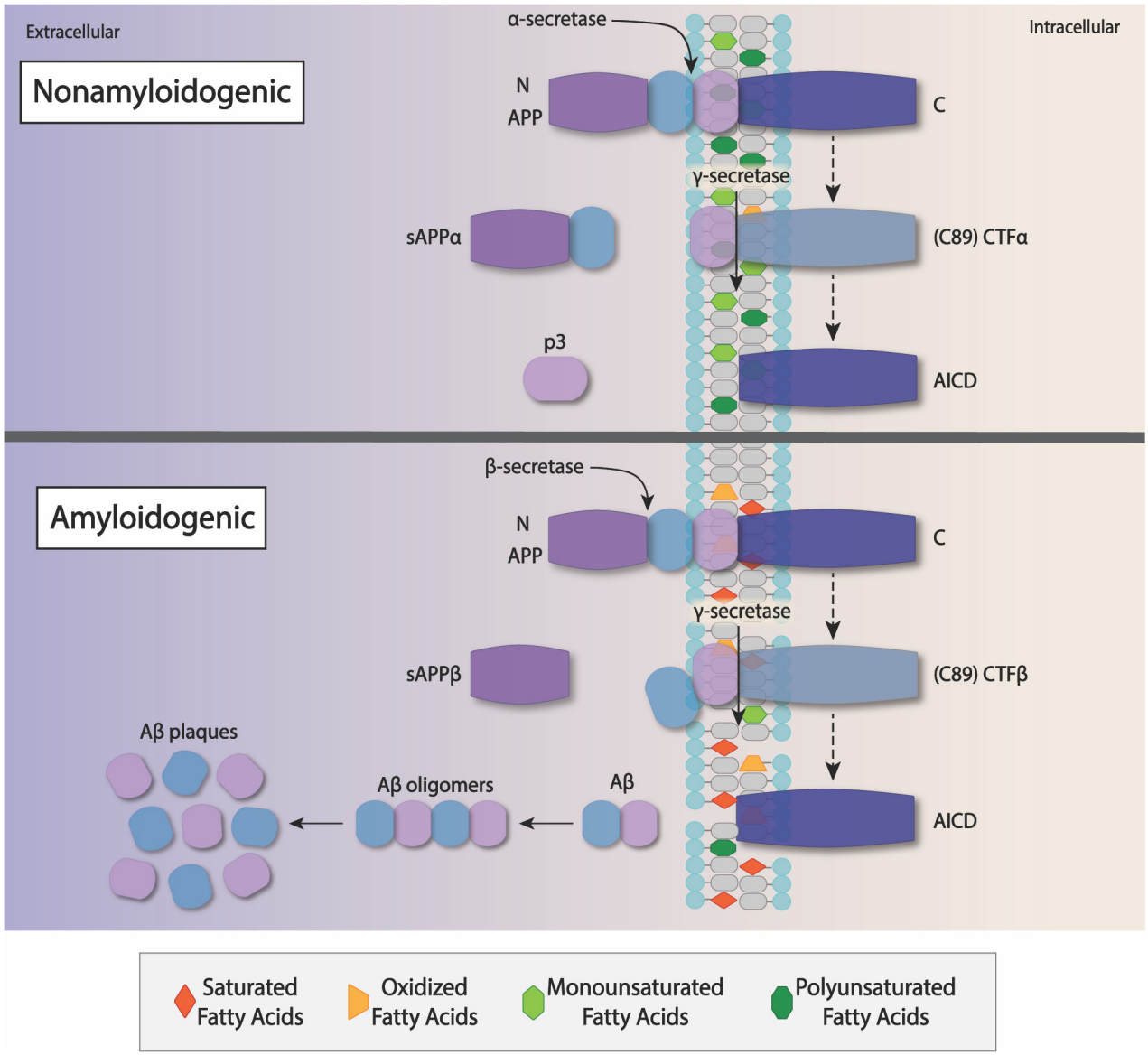

FIGURE 2 | The importance of Lipids on APP processing – APP is a transmembrane protein that is cleaved by several proteases: α-secretase, β-secretases, and γ-secretases. *Non-amyloidogenic processing of APP*– In a cell with a membrane containing normal or high amounts of unsaturated fatty acids, especially DHA, preference is given to cleavage by α-secretase In this case, a well-structured membrane holds onto an intact APP as it is cleaved by the α-secretase and subsequently the α-secretase releasing the secreted ectodomain sAPPα, along with a small protein fragment, p3, and APP intracellular C-terminal domain (AICD) peptide in the extracellular space. sAPPα and p3 do not form neurotoxic fibrils and plaques, and so this process is referred to as non-amyloidogenic APP processing. *Amyloidogenic processing of APP* – In contrast, PUFA enriched structure of healthy neurons, the presence of saturated and oxidized fatty acids results in the disruption of the cell membrane structure, and this favors β-secretase activation. APP is cleaved at its' N-terminus by β-secretase, releasing a soluble ectodomain sAPPβ into the extracellular space. γ-secretase subsequently cleaves the cell-associated C-terminus releasing and Aβ peptides of varying lengths into the extracellular space. Insoluble Aβ fibrils aggregate as oligomers that ultimately clump to form plaques within the brain. These plaques contribute to oxidative stress, neuroinflammation, and eventually decreased brain function.

The Intersection of Lipids, APP Processing, and AD Pathology

The Aβ fragments of APP is the major component of AD amyloid plaques, and such dysregulation of APP trafficking and processing are relevant to understanding AD pathology (Caporaso et al., 1994; Thinakaran and Koo, 2008; Zhang et al., 2011; Tan and Gleeson, 2019; Yuksel and Tacal, 2019). Intracellular Aβ accumulation in neurons of patients with AD and metabolic analysis of brain function indicate a possible dysfunction in Aβ transport exiting the brain (O'Brien and Wong, 2011; Yuksel and Tacal, 2019). Lipids rafts play important roles in APP trafficking (Yoon et al., 2007; Yang

et al., 2013). Moreover, palmitoylation dictates how APP is processed (Bhattacharyya et al., 2013). *Trans* fatty acids influence amylogenic APP processing, while the level of fatty acid unsaturation determines the activity of secretases (Yang et al., 2011; Grimm et al., 2012). Future research relating to changes in brain lipid composition in pre-symptomatic AD may provide a link with early disease onset, dysregulation of lipid metabolism, and APP processing.

The Intersection of Lipid Rafts, APP Processing, and AD Pathology

Lipid rafts are dynamic clusters of membrane lipids that interact with protein complexes to promote intracellular signal transduction (Mesa-Herrera et al., 2019). Normal aging is associated with gradual reductions in cholesterol and polyunsaturated fatty acids (PUFAs) in lipid rafts. With age-related changes lipid rafts composition, alterations in intracellular communication may be associated with age-associated reductions in synaptic plasticity. In neurodegenerative diseases, the composition of lipid rafts changes more rapidly, most notably in n-3 and n-6 PUFAs (Li et al., 2018). Lipid raft aging appears to be exacerbated in Alzheimer's Disease, which may serve as the underlying contribution to disrupted signal transduction, increased APP processing, and rapid formation of AB aggregates (Grassi et al., 2019). Normal APP signal transduction involves cleaving APP into AB into the extracellular environment. However, if APP interacts with ApoE and tau on a lipid raft with an atypical lipid composition, signal transduction may be disrupted, promoting the formation of AB aggregates. Other alterations include reductions in unsaturation of FA in AD patients, as compared to controls (Kao et al., 2020). Lipid raft aging also appears to exhibit gender differences, such that women had more severe changes in lipid raft composition as compared to men. This may serve as supportive evidence for the finding that postmenopausal women are more likely to progress from MCI to AD than age-matchd men (Herrera). Considering that lipid raft function is sensitive to aging, further characterization of composition changes in lipid rafts within the brain may be useful as a biomarker of neurodegenerative stages.

Lipids and Cellular Remodeling

Role of Lipid Remodeling in Synaptogenesis

Lipid bodies (LBs) are spherical lipid-rich organelles associated with lipid storage, metabolism, cell signaling, and inflammation (Schmitz and Muller, 1991; Melo et al., 2011). At regulated levels, LBs maintain lipid homeostasis and cellular function, but in response to brain inflammation and increased neuronal oxidative stress, these LBs grow in size and accumulate within microglial cells (Tremblay et al., 2016; Hu et al., 2017). Though the pathway is still largely undiscovered, LBs in microglia appear to communicate with organelles such as the mitochondria, which control cell-death mechanisms (Tyurina et al., 2014). When exposed to lipopolysaccharides, LBs contact to mitochondria was disrupted, but DHA treatment reduced such effects. DHA may be a key factor in preserving mitochondrial health and regulation of microglial activity (Tremblay et al., 2016; Maysinger et al., 2018). When regulated in rodent models of AD, microglia slows the accumulation of Aβ plaques, but a proliferation of microglia activity may result in brain inflammation and degradation of neuronal synapses (Lim et al., 2000; Stahl et al., 2006; McClean et al., 2015). Microglial dysfunction has been implicated as a contributor to AD pathogenesis (Hansen et al., 2018). Microglia cells in the brain contribute to the reorganization of neuronal circuits by phagocytosing dead neurons and their dendritic spines and axon terminals. These immune cells contribute to neural plasticity (Wu and Zhuo, 2008; Yates, 2014; Yang et al., 2019), which refers to the brain's ability to maintain, modify, and strengthen these synapses in order to permit neuronal communication (Tremblay et al., 2011).

Importance of Lipid Remodeling/Synaptogenesis in AD Pathology

Synaptogenesis is the formation of nerve synapses involving the reorganization of cell structural components (Aoki et al., 2003; Kelsch et al., 2010). Several studies suggest that presynaptic and postsynaptic development is initiated by signaling pathways involving cholesterol (Mauch et al., 2001; Fester et al., 2009). Changes in fatty acid content occur prior to synaptogenesis in cones (Martin and Bazan, 1992). Studies have shown that neurons deprived of lipid rafts underwent a cascade of effects inhibiting synaptic growth and development (Bazan, 2005; Welberg, 2014; Mochel, 2018). Depletion of lipid rafts decreased dendritic density and increased the synapse, disrupting neuronal communication (Martin, 2000; Hering et al., 2003; Sebastiao et al., 2013; Wang, 2014). The transport protein, apolipoprotein E (apoE), monitors cholesterol transport from glial cells to neurons, and impaired ApoE is implicated in deficits in synaptic plasticity and cognitive function (Periyasamy et al., 2017). Of the three isoforms of ApoE, ApoE4 is a prevalent risk factor that is synergistic with obesity and age for AD (Butler, 1994; Riedel et al., 2016; Jones and Rebeck, 2018; O'Donoghue et al., 2018; Glorioso et al., 2019). ApoE4 binds fewer lipids and is most likely involved in changes in cholesterol flux and metabolism (de Chaves and Narayanaswami, 2008; van den Kommer et al., 2012; Mahley, 2016; Nunes et al., 2018), accounting for altered synaptogenesis and neural plasticity.

Lipids and Myelination

The Importance of Myelination

Action potentials propagate along axons through rapid saltatory conduction. Synthesized by oligodendrocytes in the CNS and Schwann glial cells in the PNS, myelin membranes act as electrical insulators, permitting higher nerve conduction velocities and greater neuronal communication efficiency (Almeida and Lyons, 2014; Almeida and Lyons, 2017). Without myelin, axons would require more energy to depolarize its membrane (Stassart et al., 2018). Myelin is composed of several lipids and protein layers that wrap around most of the axon, except at nodes of Ranvier, which are regions highly concentrated with sodium ion channels (Finean and Robertson, 1958; Davison, 1972; Burgisser et al., 1986; Wender et al., 1988; Ando et al., 2003; Schmitt et al., 2015; Montani and Suter, 2018). Myelination of axons is a dynamic process through development and adulthood, and this process, in addition to myelin sheath modification and myelin

repair, contributes to synaptic remodeling and neural plasticity (Zatorre et al., 2012).

The Role of Lipids in Myelination

The myelin membrane consists of myelin-specific proteins and high-level synthesis of lipids representative of all major classes, such as cholesterol, glycosphingolipids, glycerophospholipids, and galactolipids (Chrast et al., 2011). Lipids comprise approximately 80% of myelin's dry weight, accounting for glia's high demand for fatty acids, which are fundamental building blocks of its lipid structure (Dimas et al., 2019). Myelin accounts for a majority of the white matter in the brain, which is consistent with reported reduced myelin density associated with AD white matter changes in the brain (Nasrabady et al., 2018).

Brain Myelination and AD Pathology

Reduced number and activity of oligodendrocytes and precursor cells can damage myelin integrity, contributing to AD pathology's characteristic neuronal loss (Bartzokis, 2011). Oligodendrocytes support and regulate neurons, but they are primarily responsible for myelin production (Simons and Nave, 2015). Myelinating oligodendrocytes are sensitive to lipid peroxidation because oxidative stress inhibits expression of genes that promote oligodendrocyte differentiation (French et al., 2009). This implies that disruption of myelin synthesis may be a central feature of AD pathology, and can be exploited for therapy (Desai et al., 2010). Dysfunction in these processes may be linked to white matter abnormalities and cognitive impairment associated with AD due to damaged signal conductivity and synchronicity needed for information processing between neurons (Ihara et al., 2010; Alexander, 2017; Nasrabady et al., 2018). The causal relationship between myelination and AD has not been elucidated, but white matter changes arising from myelination dysfunctions have been described in AD brains (Kohama et al., 2012). Additional evidence for the contribution of myelin breakdown on AD pathology comes from studies showing that the rate and severity of myelin breakdown in healthy seniors are associated with APOE status, a major risk factor of AD (Bartzokis et al., 2006).

Lipids and Receptor-Mediated Signaling

Neuronal Receptor Signaling Pathways

Neurons communicate via electrochemical signals and neurotransmitters across gaps called synapses associated with several integrated networks (Mayer, 1993; Laughlin and Sejnowski, 2003; Salinas, 2009; Hahn et al., 2019). The presynaptic neuron releases neurotransmitters through exocytosis, and those chemicals bind to the postsynaptic neuron's neurotransmitter receptors to alter postsynaptic neuronal activity (Kennedy, 2013). One class of neurotransmitter receptors, called ligand-gated ion channel receptors, opens an ion pore through the membrane upon ligand binding. Ions cannot travel through the hydrophobic lipid membrane and, therefore, can only pass through channels controlled by these receptors. Ions entering the ligand-gated channel can initiate excitatory or inhibitory signals, but both rapidly influence neuronal function (Cantor, 2018). Another class of neurotransmitter receptors, G-protein-coupled receptors (GPCRs), bind to the ligand and initiate an intracellular mechanism in which its G-proteins alter cAMP levels to stimulate or inhibit the neuron, and may involve lipid agonists (Hansen, 2015). Unlike ligand-gated ion channel receptors, GPCRs are slower but longer-lasting in affecting neuronal activity (Lovinger, 2008).

Role of Lipids in Neuronal Signaling

While cascades of protein kinases and phosphatases have been largely studied, there is an increasing interest in lipid-based pathways involving lipid kinases and phosphatases. Lipids are versatile in signal transduction pathways and act as hormones, ligands, substrates, and mediators (Eyster, 2007; Piomelli et al., 2007; Piomelli, 2012). Sphingolipids and cholesterol comprise lipid rafts, which are regions in the plasma membrane that organize signaling molecules, amplify intracellular signaling cascades, and regulate both neurotransmission and membrane protein trafficking (Levental and Veatch, 2016). Additionally, lipids are integral to GPCR signaling cascades. Following GPCR binding, phospholipase C (PLC) cleaves the polar phosphate head of phospholipids and forms diacylglycerol (DAG), a lipid second messenger (Black et al., 2016). Fatty acids (FAs), especially those belonging to the omega-3 and omega-6 classes, act as ligands for membrane receptors in a variety of pathways (Mobraten et al., 2013). The wide diversity of lipids and their structures contributes to AD, and their multiple roles in signal transduction may influence AD pathology.

Signaling Lipids Contribute to AD Pathology

Endocannabinoid signaling is responsible for inhibition and excitation in modulating synaptic strength, implicating its possible role in AD and associated inflammatory pathology (Skaper and Di Marzo, 2012). Although the mechanism has not been elucidated yet, free radicals and oxidative stress increase GPCR cannabinoid 2 receptors (CB2) expression in AD microglial cells, increasing neuroinflammation (Paloczi et al., 2018). Inflammation protects the brain against neurotoxins, but excessive inflammation may contribute to neurodegeneration. Another study suggested that monoacylglycerol lipase (MAGL) produces neuroinflammatory prostaglandins through the hydrolysis of endocannabinoids (Piro et al., 2012). Inhibiting MAGL activity is a potential AD therapeutic target because it is reported to prevent neuroinflammation, neurodegeneration, and impaired synaptic plasticity (Chen et al., 2012). Dysregulation in neuronal signaling cascades may contribute to increased susceptibility to neuronal dysfunction and are, therefore, important in studying its effects and relation to AD.

Lipids and Inflammation

The Importance of Inflammation

Inflammation is a defense mechanism initiated by the immune system in response to pathogens, injured cells, infections, and other toxic stimuli. A signaling cascade results in leukocyte migration to damaged sites, in which released cytokines recruit other immune cells to heal injured tissue (Robinson et al., 2018). Specifically, within the CNS, activation of microglia and its associated cytokine production are primarily responsible for the inflammatory responses (Frank et al., 2007; Ghosh et al., 2012;

Zhu et al., 2019). However, unregulated inflammation, excessive cytokine production, and failure to resolve inflammatory responses all contribute to chronic neuroinflammation, a biomarker of many neurodegenerative diseases, including AD (Wang et al., 2015a).

Lipids and Inflammation

Several studies implicate the role of lipids and lipid metabolism in inflammatory responses (Janciauskiene and Wright, 1998; Kang and Rivest, 2012; Zhang et al., 2018; Ntambi, 2019). Eicosanoids are a class of lipid mediators inflammation produced by innate immune cells that contribute to acute inflammation, resulting in pain, loss of function, heat, and swelling (Higgs et al., 1984; Williams and Higgs, 1988; Hedqvist et al., 1991; Umamaheswaran et al., 2018). Following the elimination of toxic stimuli, innate immune cells cease the production of eicosanoids and begin production of specialized pro-resolving lipid mediators (SPMs) to resolve inflammation (Serhan, 2010; Chandrasekharan and Sharma-Walia, 2015; Chiurchiu et al., 2018; Maclean et al., 2018). Synthesized from omega-3 fatty acids, docosahexaenoic acid (DHA), and eicosapentaenoic acid (EPA), SPMs resolve inflammation by inhibiting polymorphonuclear leukocytes (PMN) and lowering vascular permeability This process may be impaired in AD (Whittington et al., 2017).

Inflammatory Lipids and AD Pathology

A disproportionate level of inflammation can disrupt the balance between eicosanoids and SPMs, overwhelming the brain's ability to return to a non-inflammatory state. This suggests the brain's dependence on SPMs and its omega-3 precursors, DHA, and EPA (Serhan et al., 2018). AD pathology includes decreased DHA levels (Fonteh et al., 2014; Yassine et al., 2017), which may account for heightened brain inflammation that leads to declining cognitive health. Moreover, many studies have reported alterations to the eicosanoid pathway in AD (Biringer, 2019), further heightening research interest in the balance between eicosanoids and SPMs (Serhan et al., 2015). AD is also associated with elevated microglia-induced neuroinflammation, increases in proinflammatory cytokines, and upregulated expression of phagocytic receptors in white matter microglia (Zheng et al., 2016). One receptor, CD36, promotes both pro-inflammatory and oxidative pathways upon binding to ligands, including lipids and Aβ (Doens et al., 2017). Overexpression may lead to dysregulated inflammation and increased oxidative stress, a biomarker of the inflammatory response, and AD (Park et al., 2014; Koizumi et al., 2016). White matter is critical to neuronal connectivity and processing speed, and such white matter inflammation may result in neurodegeneration and, therefore, the cognitive decline (Raj et al., 2017). Further studies aim to determine if inflammation contributes to the onset of AD or exacerbates already-existing neuropathology (Heppner et al., 2015).

Lipids and Oxidative Stress

Oxidative Stress

Oxidative stress is defined as a disruption in homeostasis between antioxidants and oxidants, and more specifically, an accumulation of reactive oxidative species (ROS) and reactive nitrogen species (RNS) (Apak et al., 2016; Hameister et al., 2020). ROS belongs to a family of compounds containing partially reduced oxygen species, such as O_2– and HO-, that are generated primarily by the electron transport chain during aerobic respiration (Zhao et al., 2019). ROS are involved in many redox-dependent processes, including cell signaling, homeostasis, immune system responses, energy metabolism, and tissue remodeling. However, an excess of ROS or impaired control of the balance between antioxidants and oxidants leads to oxidative stress, which is implicated in the progression of neurodegenerative diseases (Cheignon et al., 2017). Because the brain consumes approximately 25% of the body's glucose, its high energy consumption increases neurons' susceptibility to oxidative stress and overproduction of ROS (Wezyk et al., 2018).

Membrane Lipids Are Damaged During Oxidative Stress

Excess ROS can lead to increased lipid peroxidation within the brain, altering membrane permeability and activity of membrane receptors and their associated enzymes (Birben et al., 2012). Lipid peroxidation produces reactive aldehydes, including malondialdehyde (MDA) and 4-hydroxynonenal (HNE), that modify and bind to proteins involved in metabolism, antioxidant defense systems, and axonal growth. By modifying Tau protein, 4-HNE can indirectly lead to an increase in neurofibrillary tangles, which is consistent with proteomic reports of increased 4-HNE in AD hippocampal tissue and neurofibrillary tangles (Cheignon et al., 2018). Moreover, low-density lipid lipoprotein receptor-related protein (LRP1) is involved in Aβ peptide removal. LRP1 is oxidized by Aβ, inhibiting its ability to clear Aβ and therefore leading to Aβ accumulation in the brain (Shinohara et al., 2017). LRP1 is another protein that is covalently modified by 4-HNE, further supporting that unrestrained lipid peroxidation produces excess reactive products that initiate a cascade of dysregulations within pathways necessary to neuronal health (Butterfield et al., 2002). Oxidant/antioxidant imbalance forms blood-based biomarkers that can be used for early, non-invasive diagnosis (Wojsiat et al., 2018), or for AD therapies (Yatin et al., 2000; Sultana et al., 2004).

Oxidative Stress and AD Pathology

Many trials seek to assess different antioxidant therapeutic approaches to alleviate oxidative stress, a key biomarker of AD. CoQ_{10}, creatine, idebenone, latrepirdine, triterpenoids, omega-3 PUFAs, vitamin E, and vitamin C are just a few antioxidants that are extensively studied in their treatment of neurodegenerative diseases (Yatin et al., 2000; Kumar and Singh, 2015).

Lipids and Immune Response

The Immune System

The immune system, which is divided into the innate and adaptive immune system, is critical to defending the body against infectious and toxic stimuli (Simon et al., 2015). The innate immune system utilizes cytokine production and modulation to mount a quick but sufficient response to pathogens, including viruses, bacteria, and parasites. The innate immune system is also

responsible for activating the adaptive immune system, which is slower due to the lengthy production of specific antibodies to the foreign antigen (Iwasaki and Medzhitov, 2015). Studies in the past 20 years have refuted the notion of the brain as being "immunologically privileged" in relying largely on innate immune system mechanisms within the CNS. While it was thought that the CNS and immune system were separate due to the blood-brain barrier, the detection of lymphatic vessels connecting T-cells in lymph nodes to cerebrospinal fluid (CSF) in the meninges provided evidence for the brain's semi-dependence on the adaptive immune system (Louveau et al., 2015). Neuroimmune processes are activated by vagal nerve signaling, immune signals, and complement proteins, resulting in increased activity of microglia and astrocytes (Tchessalova et al., 2018).

Lipids and Immunity

Studies reported increased levels of platelets and vascular lesions in AD patients outside of the brain, contributing to cerebral amyloid angiopathy, a biomarker of AD that shows increased amyloid protein in the brain arteries (Kniewallner et al., 2015). Although platelets combat vascular injury, they are also involved in APP processing, and transitively, the formation of Aβ plaques (Evin et al., 2003; Evin and Li, 2012). The balance of omega-3 and omega-6 PUFAs may affect platelet levels, as membrane essential fatty acids (EFAs), primarily DHA and EPA, form prostaglandins PGE_1, PGE_2, and PGE_3, all of which participate in a variety of immunological and signaling pathways in the brain (Chang et al., 2009). PGE_1 has anti-inflammatory properties, and conversely, PGE_2 strongly promotes inflammation by acting on different receptors (Iyu et al., 2011). PGE_3 is responsible for regulating PGE_2's inflammatory effects by competing with its formation from precursor EFAs (Chang et al., 2009). Imbalances in the omega-6 to omega-3 PUFA ratios disrupt the formation of PGE_3, which minimizes the regulation of PGE_2 induced inflammation. Moreover, this imbalance of PUFAs is associated with changes in neuronal brain composition that, in combination with drug therapies, can reduce the risks and slow the progression of AD (Giulietti et al., 2016).

Immunity and AD Pathology

An impaired BBB is implicated with the onset of AD, which may increase the BBB's permeability to pathogens and immune cells (Veerhuis et al., 2011). Levels of cytotoxic and helper T-cells are upregulated in brain parenchyma of AD patients (Oberstein et al., 2018). Helper T cells and pro-inflammatory cytokines target neurofibrillary tangles and plaques composed of Aβ and Tau and activate microglia at these sites, further exacerbating neuroinflammation (Gold et al., 2014; Martinez-Frailes et al., 2019). One class of cytokines, called chemokines, stimulates leukocyte migration from blood to tissues. CCL5 is a chemokine that is amplified in response to reactive oxygen species and oxidative stress within the brain's endothelial cells, promoting even more T cell migration across the leaky BBB. These inflammatory mediators are elevated in the CSF and blood and are possible biomarkers for detecting AD and its progression (Mietelska-Porowska and Wojda, 2017).

Lipids and Energy Regulation

Sources of Brain Energy

Although the human brain comprises only 2% of the body weight, it consumes approximately 20% of glucose, demonstrating its disproportionately high energy demand (Mergenthaler et al., 2013). The majority of the energy utilized by the brain is dedicated to returning neurons to their resting states after depolarization, and the remaining 20−25% of energy is allocated toward synthesizing vesicles and neurotransmitters (Harris et al., 2012). The brain relies on a constant flow of glucose and oxygen, which are delivered through the blood. However, during fasting periods, when glucose levels are decreased, the liver can supply ketone bodies to support metabolism within the brain (Patel et al., 1975; Hawkins and Biebuyck, 1979; Nehlig, 2004). These delivered ketone bodies are primarily utilized by astrocytes, and upon arrival, ketolysis of the ketone bodies generates acetyl CoA, an important substrate for the tricarboxylic acid (TCA) cycle and therefore, ATP production. Although the brain has a large ATP requirement, it does not utilize these ketone bodies or fatty acids as a significant source of energy like in other organs, such as the liver. It is hypothesized that evolution selected against this pathway because it produces ROS and therefore, contributes to oxidative stress that contributes to neurodegeneration (Schonfeld and Reiser, 2017).

Role of Brain Energy Regulation in AD Pathology

Transport and utilization of glucose within the brain are tightly regulated, but mitochondrial dysfunction and decreased expression of glucose transporters (GLUT) are potential contributors to AD (Yin et al., 2016). Highly concentrated in the BBB, GLUT1 transports glucose across the endothelium and into astrocytes, whereas GLUT3 is predominantly found in axons and dendrites, underscoring its role in neuronal glucose transport and distribution (Vannucci et al., 1998). Reduced GLUTs expression at the BBB and within neurons is associated with AD, which may explain overall decreased glucose metabolism in AD pathology (Yin et al., 2016).

Mitochondrial Dysfunction and AD Pathology

Mitochondria are organelles central to brain energy processes, and altering glucose availability or dysregulating oxidative phosphorylation can have direct effects on neuronal function and cognitive health (Picard and McEwen, 2014; Anderson, 2018). Recent reports have hypothesized that Aβ may initiate mitochondrial dysfunction, and one theory proposes that Aβ raises cytosolic calcium levels, inhibiting oxidative phosphorylation and, therefore, ATP production (Cardoso et al., 2001; Eckert et al., 2010; Spuch et al., 2012; Kaminsky et al., 2015; Brewer et al., 2020). Moreover, mitochondria delivery to needed brain regions is dependent on tau, a protein related to microtubules (Quintanilla et al., 2012; Amadoro et al., 2014). Mitochondria are observed to be differentially localized in AD brains, suggesting that mitochondria trafficking is affected (Nicholls and Budd, 2000; Duchen, 2012; Devine and Kittler, 2018; Son and Han, 2018; Rangaraju et al., 2019), and provides further support for mitochondrial-based contributors to neurodegeneration.

TABLE 3 | Lipid diets and their effects on AD.

Lipid diet interventions	Effects on AD
Algal DHA[#]; 2 g daily for 18 months	Supplementation with DHA compared with placebo did not slow the rate of cognitive and functional decline in patients with mild to moderate Alzheimer's disease (Quinn et al., 2010).
Consumption of fish once or more per week	In adults above the age of 65, participants who consumed fish once or more per week had 60% less risk of developing Alzheimer's compared to participants who rarely or never ate fish (Morris et al., 2003).
Omega-3 PUFA; 600 mg EPA and 625 mg DHA daily for 4 months	In adults with mild cognitive impairment and probable AD, omega-3 supplementation had negligible effects on cognition or mood (Phillips et al., 2015).
EPA-DHA for 26 weeks; stratified into high-dose (180 mg EPA-DHA daily) and low-dose (400 mg daily)	In cognitively healthy adults over 65 years old, there were no significant differential changes in any of the cognitive domains for either low-dose or high-dose fish oil supplementation compared with placebo (van de Rest et al., 2008).
Study participants are postmenopausal women (60–84 years); 1g DHA, 160 mg EPA, 240 mg Ginkgo biloba, 60 mg PS, 20 mg per day for 6 months	In a randomized, double-blind study, a high dose of omega-3 nutrients has cognition and mobility benefits to older women (Strike et al., 2016).
DHA-EPA; 1.7 g DHA and 0.6 g EPA daily for 6 months (OmegAD Study)	Omega-3 fatty acids did not delay the rate of cognitive decline, nor did it have marked effects on neuropsychiatric symptoms except for possible positive effects on depressive symptoms in non-APOE4 carriers and agitation symptoms in APOE4 carriers (Freund-Levi et al., 2006; Freund-Levi et al., 2008). Plasma levels of AA decreased while DHA and EPA levels increased at 6 months. Specialized pro-resolving mediators (SPMs) do not change in the omega-3 group but a decrease in the placebo group. SPM changes associate with cognitive changes in AD (Lopez et al., 2011).
Omega-3 PUFAs; 1.8 g daily for 24 weeks	The omega-3 supplementation treatment group showed significant improvement in the Alzheimer's Disease Assessment Scale compared to the placebo group in participants with mild cognitive impairment. However, there was no significant improvement in Alzheimer's disease study participants (Chiu et al., 2008).
Supplementation with omega-3 fatty acids alone or omega-3 plus alpha-lipoic acid; 675 mg DHA and 975 mg EPA or 675 mg DHA and 975 mg EPA plus 600 mg lipoic acid daily for 12 months	Combining omega-3 fatty acids with lipoic acid slowed both cognitive and functional decline in mild to moderately impaired AD participants over 12 months compared to placebo (Shinto et al., 2014).
3 DHA exposure variables used in separate analyses; plasma DHA, dietary DHA, and consumption of cold-water fish	Plasma and dietary DHA were associated with a decreased risk of dementia and AD (Lopez et al., 2011).
Arachidonic acid and DHA supplementation;240 mg of AA and DHA daily for 90 days	Participants with mild cognitive impairment showed a significant improvement in the immediate memory and attention score compared to placebo, but there was no significant improvement in participants with AD (Kotani et al., 2006).
Docosahexaenoic acid-concentrated fish oil supplementation; 430 mg of DHA and 150 mg of EPA daily for 12 months	In participants with mild cognitive impairment, supplementation resulted in a significant improvement in short-term memory, working memory, immediate verbal memory, and delayed recall capability (Lee et al., 2013).
Fortasyn Connect supplementation; 125 mL once-a-day drink containing Fortasyn Connect for 24 months (LipiDiDiet Trial)	In individuals with prodromal AD, Fortasyn Connect supplementation had no significant effect on neuropsychological test battery results (Soininen et al., 2017).
FINGER Study – Dietary intervention using a diet with 10–20% of daily energy (E%) from proteins, 25–35% from fat (less than 10E% from SAFA, 10–20% from MUFA, 5–10% from PUFA (including 2,5–3 g/day n-3 fatty acids); 45–55% from carbohydrates (less than 10% refined sugar); 25–35 g/day dietary fiber; less than 5 g/day salt; and less than 5 E% from alcohol for 2 years	In adults over 60 years old, there was a significant beneficial intervention effect on overall cognitive performance, including memory, executive function, and psychomotor speed (Rosenberg et al., 2020).

[#]*DHA, Docosahexaenoic acid (C22:6, n-3); EPA, Eicosapentaenoic acid (C20:5, n-3); MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SAFA, saturated fatty acids.*

POTENTIAL AD THERAPIES TARGETING LIPID METABOLISM

Dietary Modification Studies

With the realization that lipids are altered in AD pathology, several studies have identified specific lipids that may be used as dietary supplements to alleviate AD symptoms (**Table 3**). The major lipids include omega-3 fatty acids (DHA, EPA), choline-containing lipids, cholesterol, and lipids with antioxidant properties (CoQ_{10}, Vitamin K).

Several dietary intervention studies using DHA have yielded mixed effects on AD symptoms. A likely reason for these mixed results is that different disease severity, different formulations, and variable endpoint and time of interventions were studied (Fonteh, 2018). Recent studies indicate that the best form of DHA delivery into the brain is through the Msf2a LPC receptors (Sugasini et al., 2019). A better understanding of the right formulation and optimal concentrations of DHA probably supplemented at the prodromal phase of AD will likely yield beneficial outcomes.

Modification of Lipid Metabolism

Metabolism of lipids can be altered to prevent depletion of their levels in the AD by targeting pathways that transport or catabolize these lipids in the brain.

Lipid Transport Into the Brain

Several lipoproteins and their receptor complexes are the major form by which lipids bypass the BBB to be delivered into the brain. Several of these lipoprotein genes are linked to AD pathology (**Table 1**). Some lipoproteins have protective effects, while others have AD enhancing properties. For example, HDL has been shown to be protective by improving Aβ clearance, delaying Aβ fibrillization, suppressing vascular inflammation, and inducing endothelial nitric oxide production (Button et al., 2019).

Cholesterol Metabolism

Since cholesterol metabolism altered at several stages of AD, modulation of its metabolism may have beneficial effects on disease progression. Modification of cholesterol homeostasis can be influenced during its consumption, at the level of its biosynthesis, and during its transport into the brain. The use of statin to alter cholesterol biosynthesis is proposed to be insightful in AD pathophysiology and therapy (Wolozin et al., 2004; Hoglund et al., 2005; Biondi, 2007; Evans et al., 2009). Gene therapy targeting cholesterol 24-hydroxylase reduces the amyloid pathology before or after the onset of amyloid plaques in mouse AD models (Hudry et al., 2010). Studies in mouse models show that blocking the conversion of cholesterol to cholesterol esters has beneficial effects on AD (Shibuya et al., 2015). The relationship between hypercholesterolemia, cholesterol-lowering therapies, and the role of oxysterols in AD pathology have led to the proposition that cholesterol metabolites are valuable targets for alternative AD treatments or prevention (Loera-Valencia et al., 2019). Neuroinflammatory pathways mediated by toll-like receptor 4 (TLR4)-mediated signaling can aggravate AD symptoms. In a rodent AD model, treatment with an anti-inflammatory steroid (atorvastatin) regulates this inflammatory process and results in the amelioration of cognitive deficits (Wang et al., 2018).

Lipolytic Enzymes

The activity or expression of several lipolytic enzymes are altered in AD. Phospholipase A_2 (PLA_2) is associated with amyloid plaques, and reduction of its activity and expression ameliorates AD. Plasmalogen selective PLA_2 is also altered in AD. Our studies show an increase in PLA_2 activity of CSF of AD participants accompanied by an increase in lysophosphatidylcholine (LPC). LPC is known to disrupt the BBB, and changes in PLA_2 are associated with inflammation. The association of PLA_2 with AD pathology suggests that inhibitors of PLA_2 activity or expression may be an effective means of preventing AD. Ong et al. (2015) reviewed the importance of several natural and synthetic PLA_2 inhibitors on the treatment of neurological disorders. Since PLA_2 isoforms may have divergent effects on membrane remodeling and function, there is a need for isoform-specific inhibitors in order to avoid toxicity encountered with non-selective inhibitors. In addition to PLA_2, phospholipase D (PLD) and phospholipase C (PLC) expression and activities are associated with AD pathology. These lipases that are linked with neurite growth and signaling, respectively, offer other avenues for exploring AD treatments.

Lipid Oxidation Inhibitors

There is convincing evidence for the importance of oxidative stress on AD pathology (Sun et al., 2001; Bassett and Montine, 2003; Bacchetti et al., 2015). The most important brain fatty acid, DHA, is a polyunsaturated fatty that is easily susceptible to oxidative damage. While HDL is protective against oxidative damage, VLDL is easily oxidized. Oxidatively damaged lipids contribute to AD pathology by their highly neurotoxic properties (Bassett et al., 1999). Approaches that reduce oxidation are expected to reduce AD progression. These include the use of natural antioxidants, carnosine, lipoic acid, Ginkgo biloba flavonoids, soybean isoflavones, vitamin K, homocysteine, curcumin (Rutten et al., 2002; Vina et al., 2004; Frank and Gupta, 2005; Mancuso et al., 2007; Zhao, 2009; Cankurtaran et al., 2013). A limitation of natural antioxidant is the lack of demonstration of efficacy. Given that oxidative stress destroys mitochondrial function, an objective measure of any antioxidant can be their ability to restore mitochondrial function (Kumar and Singh, 2015; Kwon et al., 2016; Yu et al., 2016). The role of endogenous lipids in oxidative stress can be exploited when there is an uncontrolled formation of ROS and RNS or when the antioxidants contribute to disease pathology (Leuti et al., 2019). Also, the source of ROS determines the effects on cellular physiology and manipulation of the ubiquinone redox state is proposed to be a viable approach of delaying aging and therapy (Scialo et al., 2016; Wojsiat et al., 2018).

CONCLUDING REMARKS

Biochemical, physiological, and genetic analyses show that lipid metabolism interphases with all the major facets of AD pathology (**Figure 1**). In normal aging, lipid metabolic homeostasis ensures that the basic functions of the brain are met. In AD, there is dyshomeostasis of lipid metabolism, and this results in abnormal functions of the brain that characterize disease progression. This underscores the need for detailed analyses of brain lipid homeostasis in order to unravel more comprehensive mechanisms, specific biomarkers, and novel therapies of AD.

AUTHOR CONTRIBUTIONS

AF contributed to the conceptualization and study design, supervised the data, and carried out the project administration and funding acquisition. HC, VS, and AF contributed to writing of the original draft and manuscript preparation. HC and AF contributed to the manuscript review and editing. VS and AF prepared the figures and illustrations.

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REVIEW
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Lipid Droplets in Neurodegenerative Disorders

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Knowledge of lipid droplets (LDs) has evolved from simple depots of lipid storage to dynamic and functionally active organelles involved in a variety of cellular functions. Studies have now informed significant roles for LDs in cellular signaling, metabolic disease, and inflammation. While lipid droplet biology has been well explored in peripheral organs such as the liver and heart, LDs within the brain are relatively understudied. The presence and function of these dynamic organelles in the central nervous system has recently gained attention, especially in the context of neurodegeneration. In this review, we summarize the current understanding of LDs within the brain, with an emphasis on their relevance in neurodegenerative diseases.

Keywords: lipid droplet, CNS, astrocytes, microglia, fatty acids, neurodegeneration, brain

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INTRODUCTION

Lipid droplets (LDs) are spherical organelles that store intracellular neutral lipid such as triacylglycerols (TAGs) and cholesteryl esters (CEs; Welte, 2015; Cohen, 2018). LDs serve as lipid reservoirs for cells by providing substrates for membrane formation and energy metabolism (Walther and Farese, 2012). Adipose tissue is the most LD-enriched tissue in the body, where fatty acids are stored in times of nutrient excess and then mobilized with increased energy demand (Missaglia et al., 2019). LDs also affect physiological processes in the periphery beyond simple fatty acid storage and supply, such as in inflammation and insulin signaling. For example, LDs in various immune cell types contain a large pool of intracellular arachidonic acid (AA), which provides a reserve of precursors for eicosanoid synthesis (Bozza and Viola, 2010; Saka and Valdivia, 2012; Dichlberger et al., 2016). Enzymes involved in AA processing have been demonstrated to LDs, indicating that these organelles serve as a supply site for inflammation (Bozza et al., 2011). Additionally, LDs have been linked to peripheral metabolic dysfunction such as ectopic lipid accumulation (Puri et al., 2007) and insulin resistance (Gemmink et al., 2017). Overexpression of LD-associated proteins such as Cidea increase fat accumulation in mice, and human expression of LD proteins in adipose correlates with clinical insulin resistance (Puri et al., 2008). These studies therefore suggest a role for LDs in obesity-driven metabolic dysregulation.

Lipid droplets may also affect cellular physiology and function in the central nervous system (CNS). The brain is the second most lipid-rich organ (Hamilton et al., 2007), storing 20% of the body's total cholesterol (Zhang and Liu, 2015). Alteration in the lipid composition of CNS cells has been shown to affect cell function and normal neural activity (Puchkov and Haucke, 2013; Bruce et al., 2017). Notably, neurodegenerative diseases, including Alzheimer's disease (AD), and Parkinson's disease (PD), share lipid dysregulation as a metabolic feature in disease pathology. In this review, we discuss evolving knowledge and recent advances in understanding the contribution of LDs to pathogenesis of neurodegenerative diseases. Growing knowledge of LDs in the CNS

is important to the advancement of the field, as these dynamic organelles may reveal common mechanisms and potential therapeutic targets to neurodegenerative disease.

LDS STRUCTURE, COMPOSITION, AND BIOGENESIS

Hydrophobic molecules such as TAGs, CEs, and retinyl esters constitute the core of a lipid droplet (Meyers et al., 2017), while the outer surface is formed by an amphipathic lipid monolayer embedded with LD-associated proteins (The general structure of an LD is illustrated in **Figure 1**). Additionally, proteins can reside in the LD core depending on the cell type. This unique monolayer distinguishes LDs from organelles of similar size, such as lysosomes and endosomes, as the latter exhibit a lipid bilayer. Of the many LD-associated proteins, members of the perilipin family have been well described for their essential roles in LD metabolic regulation (Sztalryd and Brasaemle, 2017). The exterior protein components of LDs allow for a variety of unique interactions that may explain the myriad of cellular roles accomplished via LDs in energy homeostasis, cellular communication, and disease.

Lipid droplets arise from the endoplasmic reticulum (ER) by budding off the cytoplasmic leaflet of the ER membrane (Walther et al., 2017). They are comprised of acyl-glycerols that are synthesized through the action of diacylglycerol transferases (DGATs), which convert acyl-CoA-bound fatty acids, and diacylglycerols (DAGs) into the TAGs that fill the LD core (Harris et al., 2011). Cholesterol acyltransferases synthesize CEs which are also incorporated into the core of nascent LDs (Zhu et al., 2018). Once separated from the ER membrane, LDs may continue to grow via LD fusion and further TAG incorporation. Fusion of LDs with the aid of members of Cell death-inducing DFF45-like effector (CIDE) family proteins (Gao et al., 2017) coalesce smaller LDs into larger LDs. Re-localization of TAG synthesis enzymes like DGAT2 and GPAT4 from the ER to the LD surface allows direct synthesis of TAGs from cellular lipid sources (Wilfling et al., 2013), such as fatty acids derived from autophagic phospholipid breakdown (Nguyen et al., 2017). The incorporation of cellular debris into LDs is commonly seen during periods of stress and starvation and are thought to protect the cells from lipotoxicity (Rambold et al., 2015). LDs exhibit a variety of protein and lipid signatures, and these various compositions can help determine LD localization and utilization. For example, perilipin 2 (PLIN2) has relatively low control over lipolysis, so LDs that contain PLIN2 may be more easily broken down. Conversely, PLIN1 and PLIN5 actively promote lipolysis when activated. PLIN1 acts through its release of CGI-58, a co-activator of adipose triglyceride lipase (ATGL; Lass et al., 2006), while PLIN5 binds directly to ATGL to promote lipolysis (Wang et al., 2011). Therefore, LDs with varying PLIN proteins will behave differently across various tissues and environmental conditions (Sztalryd and Brasaemle, 2017).

Lipid homeostasis is necessary for maintaining neuronal function and synaptic plasticity (Montesinos et al., 2020), and dynamic interaction between perilipins and lipases on the LD surface regulate cellular lipid storage, breakdown, and metabolism (Olzmann and Carvalho, 2019). In most cells, the bulk of LD breakdown is accomplished through ATGL, another LD outer layer protein (Etschmaier et al., 2011). Lipophagy is also a recognized LD breakdown process, in which an LD is taken up into an autophagosome and subsequently fuses with a lysosome to breakdown LD contents mainly through lipid acid lipases (Cingolani and Czaja, 2016). During times of metabolic stress, lipases cleave triglycerides into FAs, which are then processed in the mitochondria to liberate the energy stored in droplets via beta-oxidation into acetyl-CoA and subsequent TCA cycle activity and oxidative phosphorylation (Zechner et al., 2012). LDs have been shown to provide energy substrates (Cabodevilla et al., 2013; Farmer et al., 2019), lipid signaling molecules (Arrese et al., 2014), and membrane infrastructure materials (Zehmer et al., 2009) for various cell types.

LDS FORMATION IN CELLS OF THE BRAIN

Essentially all brain cell types have been shown to form LDs (**Table 1** and **Figure 2**). A recent study claimed that the majority of brain LDs (stained with the fluorescent neutral lipid probe BODIPY) were co-localized with ionized calcium binding adaptor molecule 1 (Iba1), a microglia/macrophage-specific protein. This finding implicates microglia as a main harbor of LDs (Marschallinger et al., 2020). These lipid-associated microglia had a unique transcriptomic signature compared to non-LD-laden microglia, suggesting that LDs in microglia are either a cause or result of substantial transcriptional modulations. However, subsequent co-localization using other brain cell-specific markers was not reported in this study, leaving the door open for other cell types to be involved. For example, ependymal cells that line the cerebral ventricular system have been shown to accumulate LDs, along with Glial fibrillary acidic protein (GFAP) positive cells that are closely associated in the ependymal niche (GFAP is a common marker for astrocytes and ependymal cells; Hamilton and Fernandes, 2018). Lesions to the CNS have also induced LDs in neurons and astrocytes (Ioannou et al., 2019), and glial cells have been shown to form LDs from phagocytosed myelin fragments (Lee et al., 1990). Finally, a thorough immunofluorescence study examining cell types that harbor PLIN positive droplets found Iba1+, GFAP+, NeuN+ (a neuron specific antibody), and S100β+ (a calcium binding protein that is localized in astrocytes) to colocalize with droplets (Shimabukuro et al., 2016). Together, these studies and others demonstrate that multiple cell types in the brain are capable of forming LDs.

ANATOMICAL STRUCTURES ASSOCIATED WITH LDS IN THE BRAIN

The subventricular zone (SVZ) has surfaced as a key region for LDs in the brain. The SVZ lines the wall of the lateral ventricles and is composed of neural stem cells that are capable

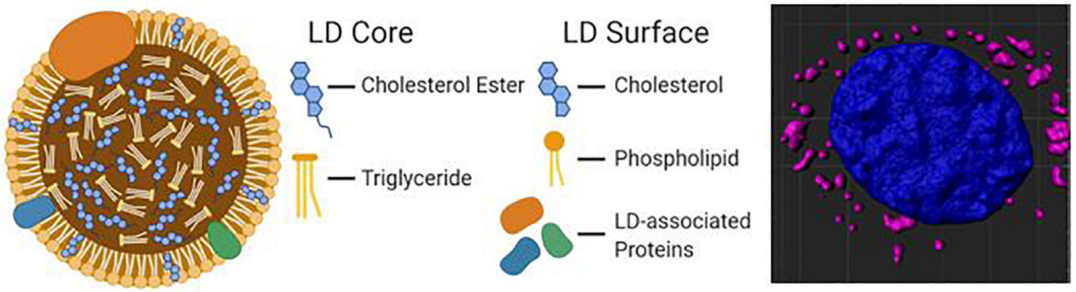

FIGURE 1 | A diagram of the molecular structure of a standard lipid droplet (LD) along with a software reconstruction of a microscopic image of astrocytes *in vitro* containing lipid droplets (highlighted in pink; LipidSpot) surrounding a nucleus (in blue; DAPI).

of differentiating into various CNS lineages (Doetsch et al., 1999). This highly active and heterogeneous cellular region is an energetically needy zone, and it is reasonable to expect ample energy stores are on board in order to meet its energetic needs (Stoll et al., 2015). It was first shown that large LDs were found in the ependyma of the SVZ (Doetsch et al., 1997). This was accomplished using an electron microscopy approach that detailed the associations of various SVZ cell types with confirmatory immunostaining.

Another group found that the LD lipase ATGL, which is encoded by the patatin-like phospholipase domain-containing protein 2 (PNPLA2) gene, is highly active in the SVZ and choroid plexus. Furthermore, mutating PNPLA2 led to a significant increase in LD formation in both areas (Etschmaier et al., 2011). This study was the first to report a function of PNPLA2 in the brain and describe its regional role in maintaining cerebral lipid metabolism. Additionally, knock out of the GTPase regulator associated with focal adhesion kinase-1 (GRAF1) induced LDs in the brains of post-natal day seven pups (Lucken-Ardjomande Hasler et al., 2014). Apart from studies of LDs in the brain arising from genetic alterations, it was found that LDs accumulate in the SVZ progressively with age; 12-month-old mice showed a nearly two-fold increase in LDs in the SVZ compared to 3-month-old mice (Bouab et al., 2011).

The SVZ has also been shown to harbor LDs in the context of AD. Hamilton and colleagues showed that both an AD mouse model and AD human post-mortem tissue accumulate LDs along the lateral ventricle (Hamilton et al., 2015). Using tandem mass spectrometry, the LD contents were identified, revealing high concentrations of oleic acid-enriched TAGs (Hamilton et al., 2015). Interestingly, direct infusion of oleic acid into the lateral ventricle was sufficient to induce LD formation along the SVZ, but insufficient to impair neurogenesis (Hamilton et al., 2015). Another group found the choroid plexus to have more LDs as AD progressed in human post mortem tissue (Yin et al., 2019). Further studies are needed to clarify the role of LDs in the SVZ in normal aging and neurodegeneration.

Although the SVZ is the most extensively studied brain region with regards to LD formation, other structures such as the frontal cortex, hippocampus, olfactory bulbs, and hypothalamus have been shown to accumulate LDs (**Table 1**). In fact, prior studies on the hypothalamus indicate that LDs may affect certain processes such as satiety (Kaushik et al., 2011). In this study, Kaushik demonstrates that autophagy during starvation leads to the mobilization of neuronal lipids which can then increase food intake though the upregulation of agouti-regulated peptide. This is just one of many studies which explore the wide variety of brain regions and biological processes which LDs can affect (**Table 1**).

WHAT CAUSES LIPID DROPLETS TO FORM IN THE BRAIN?

Aging

Lipid droplets appear to accumulate in the brain during the normal process of aging. For example, analysis of microglia from 20-month-old mice revealed an abundance of BODIPY+ cells in comparison to a matched 3-month-old cohort (Marschallinger et al., 2020). Analyses of human tissue (postmortem) also revealed that PLIN2+ Iba1+ microglia were more frequent in an aged (67-years-old) individual than in a young (22-years-old) individual (Marschallinger et al., 2020). A significant increase in LDs has also been observed in the pia mater, cortex, and striatum in 18-month-old mice as compared to middle aged mice (Shimabukuro et al., 2016). Furthermore, an electron microscopy analysis of the basement membrane of the blood brain barrier (BBB) in 6-month-old versus 24-month-old mice showed an age-dependent accumulation of LDs which caused significant thickening of the basement membrane (Ceafalan et al., 2019). On the contrary, LDs are more commonly found in perivascular cells in middle age and then seem to shift toward the parenchyma in old age (Shimabukuro et al., 2016). Given these findings, age appears to regulate LD accumulation and regional deposition.

Inflammation

From *in vitro* studies of LDs to *ex vivo* brain imaging, inflammation has repeatedly been associated with LD formation as both a cause and as an effect (Bozza and Viola, 2010). Lipopolysaccharide (LPS), a commonly employed pro-inflammatory stimulus, has been shown to increase the number and size of LDs in microglia (Khatchadourian et al., 2012). PLIN2 was shown to colocalize with these droplets, providing more evidence that PLIN2 is an LD-associated protein that can be considered a marker for both LDs and inflammation in

TABLE 1 | Lipid droplet-related literature pertaining to the brain.

Model organism	Area of interest	Cell type	Author and year of publication	Summary of findings
Human	Frontal cortex	Neuron	Paula-Barbosa et al., 1980	LD-like vesicles visible in cortical dendrites that had abundant degeneration.
	Frontal lobe	Astrocyte	Miyazu et al., 1991	Sudanophilic LDs observed in the thalamus of a patient with Nasu-Hakola disease.
	Medial temporal	Various	Derk et al., 2018	DIAPH1 colocalize with LD accumulation in myeloid cells.
	Choroid plexus	Adrenal Cortical	Eriksson and Westermark, 1990	Amyloid inclusions associated with LDs in close contact to fibril bundles.
	Whole brain	Neuron	Hulette et al., 1992	Brain biopsy found ballooned neurons filled with oligolamellar cytosomes and LDs.
			Ozsvar et al., 2018	Demyelination debris contribute to LD formation; volume highest in corpus callosum
Rat	Cerebral cortex	Neuron	Smialek et al., 1997	Injection of squalene led to LD accumulation in myelin sheaths of neurons.
	Hippo-campus	Neuron	Ahdab-Barmada et al., 1986	Excess oxygen caused neuronal necrosis. Neurons accumulate electron dense LD.
			Cole et al., 2002	The protein α-synuclein was less effective at regulating TAG turnover and showed variable distribution on LDs.
	Median eminence	Tanycyte	Brawer and Walsh, 1982	The number and size of LDs increased with age.
	Olfactory bulbs	Neurons	de Estable-Puig and Estable-Puig, 1973	LDs are manifestations of cell response to injury.
	Perineurium	Perineurial glia	Benstead et al., 1989	LD formation was found to be an early reactive change to ischemia in perineurial, endothelial, and Schwann cells.
	Pineal gland	Pinealocyte	Johnson, 1980	Removal of the hypophysis led to significant loss of LDs in the pineal gland.
	Pituitary gland	Folliculostellate	Stokreef et al., 1986	Folliculostellate cells became packed with LDs after estrogen withdrawal.
		Neuron	Gajkowska and Zareba-Kowalska, 1989	Supraopticus and paraventricularis neurons show increased LDs post-ischemia.
	Striatum	Neuron	Marasigan et al., 1986	LDs formed in neurons of rats injected with kainic acid.
	Whole brain	Glia	Kamada et al., 2003	Macrophages and astrocytes play roles in lipid metabolism.
			Kamada et al., 2002	LDs localized w/in microglia in ischemic core and astrocytes in penumbra.
	Choroid plexus	Astrocyte	Ueno et al., 2001	LD frequently found in several brain regions of senescence-accelerated mice.
Mouse	Frontal lobe	Macrophage	Sturrock, 1983	About half of macrophages in the brains studied were distended due to excess LDs or foamy aggregations.
	Medial temporal	Various	Sturrock, 1988	LDs appeared in the choroid plexus with increased age.
	Cortex	Astrocyte	Nakajima et al., 2019	Inhibition of DAG acyltransferase blocks LD formation and lipotoxic cell death
		Autolysosome	Yang et al., 2014	Lipids impeded macroautophagy and clearance in an AD mouse model.
	Hippo-campus	Neuron, glia	Chali et al., 2019	Neuronal loss and glial cell proliferation associated with changes in lipid related transcripts.
			Ioannou et al., 2019	Neurons expelled fatty and nearby astrocytes engulfed and stored them as LDs.
		Microglia	Marschallinger et al., 2020	LD-accumulating microglia were defective in phagocytosis, produced high levels of ROS, and secreted pro-inflammatory cytokines.
	Hypothalamus	Astrocyte	Kwon et al., 2017	Hypothalamic astrocytes accumulated LDs and had increased cytokines.
		Tanycyte	Rawish et al., 2020	There was high LD signal in mice fed a high at diet, which returned to normal under telmisartan treatment.

(Continued)

TABLE 1 | Continued

Model organism	Area of interest	Cell type	Author and year of publication	Summary of findings
			Kim et al., 2020	A high fat diet increased the number and size of LDs.
		Neuron	Crespo et al., 1995	Neurons stimulated with CDP-choline displayed LDs in their cytoplasm.
	Mesencephalon	Neuron	Han et al., 2018	Lipid dysregulation in PD involved upregulated expression of Plin4, increased LD deposition, and loss of neurons.
	Neo-striatum	Various	Sturrock, 1980	Pericytes contained LDs in the neostriatum, indusium griseum, and anterior commissure at various ages.
	SVZ	Neuronal stem	Bouab et al., 2011	Cells with increased numbers of large LDs showed heightened signs of quiescence and metabolic disturbance.
			Hamilton et al., 2015	Impaired FA metabolism suppressed neural stem cell activity
	White matter	Various	Liberski et al., 1989	Macrophages in mice with Creutzfeldt-Jakob disease were filled with LDs.
	Whole brain	Glia	Ogrodnik et al., 2019	Mice fed high fat diets had increased LDs and cells with more LDs were more likely to be senescent.
		Neuron	Hamilton et al., 2010	Postmortem AD brains and 3xTg mice were shown to accumulate neutral lipids in ependymal cells.
		Various	Shimabukuro et al., 2016	Lipid-loaded cells displayed a variety of distinct phenotypes based on their location and numbers increased with age.
	Cortex	Glia	Cabirol-Pol et al., 2018	ND23 knockdown in glial cells created massive LD accumulation and induced brain degeneration.
			Kis et al., 2015	LDs were localized in glia and enriched in the cortex.
Fly	Whole brain	Neuronal stem	Bailey et al., 2015	LDs played an antioxidant role in neural stem cells by reducing ROS and protecting against peroxidation.
	Hypothalamus	Neuron	Nakai et al., 1979	Large LDs were found in CSF contacting neurons.
	Various	HeLa	Papadopoulos et al., 2015	LD targeting may contribute to HSP pathogenesis.
		Glia	Meng et al., 2015	ROS and neuronal mitochondrial dysfunction contributed to LD accumulation prior to neurodegeneration onset.
		Neuron	Calderon-Garciduenas et al., 2002	Air pollution caused ApoE-positive LDs to be deposited in SMC and pericytes
Other	Zebra-fish	Embryo	Arribat et al., 2020	Loss of spastin resulted in a higher number of smaller LDs.
	In vitro	Glia	Lucken-Ardjomande Hasler et al., 2014	GRAF1α was found on LDs in primary glial cells that were fed oleic acid. Overexpression of GRAF1a promoted LD clustering and perturbed lipolysis.
In vitro	*In vitro*	Glia	Khatchadourian et al., 2012	LPS treated microglia accumulated LDs and Plin2 colocalized with droplets.
			Lee et al., 2017	An increased BBB Ki induced LD formation, activated stress pathways, and increased inflammatory cytokines.
			Farmer et al., 2019	E4 astrocytes have increased lipid content compared to E3
		HeLa	Edwards et al., 2009	Spartin may be recruited to LDs.
			Hooper et al., 2010	Lack of spartin expression contributes to Troyer syndrome.
			Cole et al., 2002	PD mutations in α-syn showed less variable LD distribution and less TG turnover. α-syn formed oligomers within cells and associated with LD.
		N41	Libby et al., 2015	Cells treated with LPL accumulated lipid into droplets.

These papers have been classified by the model organism that was used, area of the brain that was studied, and cell type of focus.

the brain. This was repeated recently in microglia-derived BV2 cells and expanded into an *in vivo* model of LPS treatment. That study by Marschallinger and colleagues found that more microglia contained LDs in LPS-treated mice when compared to non-treated controls (Marschallinger et al., 2020). To assess how the vasculature might affect LDs in the brain, Lee and colleagues

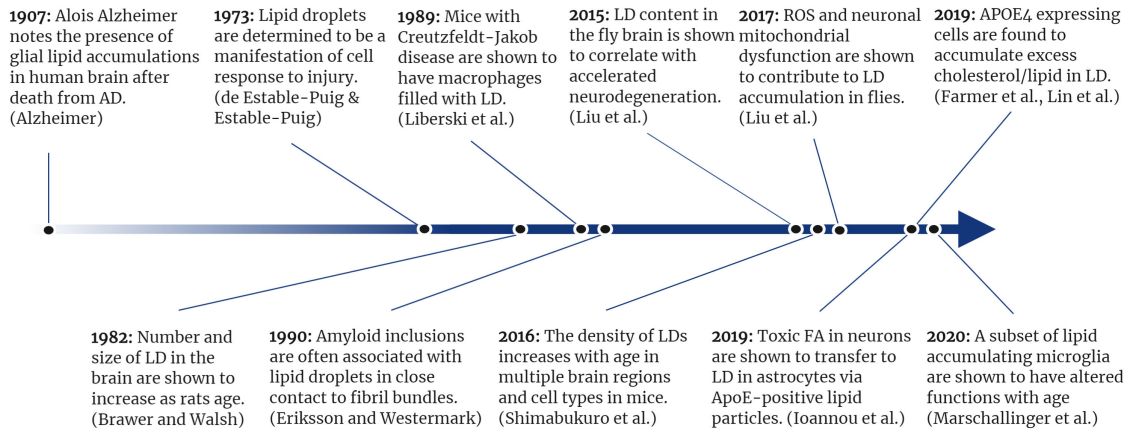

FIGURE 2 | A timeline of selected lipid droplet discoveries as they relate to neurodegenerative disease.

i.v. infused triglyceride-rich lipoproteins (TGRL) and lipoprotein lipase into mice and found increased BBB permeability, therefore indicating that hyperlipidemia may increase lipid spill-over into the brain. To test how this treatment affected resident brain cells, they treated normal human astrocytes with the TGRL lipolysis products and found increased LD formation (Lee et al., 2017). Another study found that palmitate treatment of isolated primary astrocytes increased inflammatory markers including TNF-alpha, IL-1 beta, IL-6, and MCP-1 in addition to Oil Red O (a fat-soluble dye) staining and PLIN1 and PLIN2 transcription. Interestingly, treatment of microglia with conditioned media from lipid-loaded astrocytes enhanced microglial chemotaxis through a CCR2-MCP1 mechanism (Kwon et al., 2017). These data suggest that LD-associated astrocyte inflammation may subsequently signal to microglia to augment the inflammatory response. However, it remains to be seen whether inflammation causes LDs, LDs cause inflammation, or both.

Oxidative Stress

Intracellular reactive oxygen species (ROS), as well as ectopic treatment with oxidative stressors such as hydrogen peroxide, induce LD formation in various cell types in the periphery (Lee et al., 2013, 2015; Jin et al., 2018). Similarly, increased oxidative stress in the brain appears to drive LD accumulation in a cell-specific manner. For example, neuronal hyperactivity from trauma or chemogenetic activation increases glial LD accumulation (Ioannou et al., 2019). These LD-laden glia upregulate genes to neutralize the peroxidated lipids generated by activated neurons. Astrocytes in particular appear to be uniquely suited for ROS management due at least in part to fatty acid binding protein 7 expression (Islam et al., 2019). Liu and colleagues first proposed this neuron-astrocyte metabolic coupling model in which neurons under stress export oxidized lipids to astrocytes as a means of neuroprotection (Meng et al., 2015; Liu et al., 2017). LDs in glia may then be viewed as indirect indicators of neuronal damage from oxidative stress. Protective LD formation in glia has also been observed in the SVZ niche, where glia protect neuroblasts from peroxidation and thereby promote neural stem cell proliferation (Bailey et al., 2015). Therefore, oxidative stress appears to be a driver of LD formation in the brain both under formative physiological processes during neuronal development, as well as in diseases associated with increased neuronal oxidative stress.

WHAT NEURODEGENERATIVE DISORDERS HAVE BEEN LINKED TO LIPID DROPLETS?

Amyotrophic Lateral Sclerosis

Amyotrophic lateral sclerosis (ALS) is the most common form of motor neuron disease (Tiryaki and Horak, 2014). ALS is characterized by the progressive degradation of motor neurons in the CNS, leading to the inability to both initiate and control muscle movement. In addition to genetic mutations, altered metabolic function in ALS has been observed in cellular processes implicated in ALS pathology such as cell stress and energy homeostasis (Vandoorne et al., 2018).

Recent studies interested in lipid metabolism have shed light on connections between LDs and ALS pathology (Pennetta and Welte, 2018). For example, it is known that mutations in the human VAMP-associated protein B (hVAPB) cause ALS, although the disease-causing mechanism itself remains unclear (Sanhueza et al., 2015). Sanhueza and colleagues performed a genome-wide screen in *Drosophila* to identify pathways involved in hVAPB-induced neurotoxicity and found that the list of modifiers was mostly enriched for proteins linked to LD dynamics. One modifier highlighted in this study was acyl-CoA synthetase long-chain (Acsl). Acsl promotes LD biogenesis and its downregulation reduces LD nucleation which decreases the size and number of mature LDs (Kassan et al., 2013). Furthermore, another group showed that gain of function mutations in the LD protein seipin contributed to motor neuron disease symptoms in mice (Yagi et al., 2011; Sanhueza et al., 2015). Together these studies suggest that impaired LD biogenesis may be an important pathological aspect to hVAPB-mediated ALS.

A mutation that contributes to an early onset form of ALS occurs in the gene SPG11 and affects lysosome recycling (Branchu et al., 2017). Branchu and colleagues observed intracellular lipid accumulation followed by lipid clearance from lysosomes into droplets in wild-type (WT) mice. However, in Spg11 knockout mice, there was a significantly slower rate of lipid clearance and a decrease in LD size and number. Another study that implicates aberrant lysosomal function as a contributor to ALS reported that the C9orf72 gene plays a key role in metabolic flexibility in times of stress/starvation (Liu et al., 2018). They found that loss of C9orf72 led to an increase in LDs, and that starvation-induced changes in lipid metabolism were mediated by coactivator-associated arginine methyltransferase (CARM-1). Since CARM-1 regulates lysosomal function and lipid metabolism, these results suggest that the dysregulation of lipid metabolism, including the aberrant accumulation of LDs, could contribute to ALS pathology.

Similar to studies linking LDs and peroxidation to AD, there also appears to be a connection between LDs and cellular stress in ALS. Bailey et al. showed in *Drosophila* that ROS accumulation increased glial LD content, and that when glia were unable to produce LDs, neuroblasts experienced peroxidative damage (Bailey et al., 2015). Additional work by Simpson et al. provided a link between LDs, peroxidation, and ALS by showing a positive correlation between lipid peroxidation markers in ALS patient cerebrospinal fluid and disease burden (Simpson et al., 2004). Thus, LD dynamics may contribute to ALS, potentially through a mechanism in which glia are unable to protect neurons through normal lipid accumulation and storage mechanisms (Pennetta and Welte, 2018).

Huntington's Disease

Huntington's disease (HD) is a hereditary neurodegenerative disease caused by a mutation in the huntingtin gene (Htt) that then codes for the Htt protein (McColgan and Tabrizi, 2018). Mutant Htt contains an expanse of repeating glutamines at the N terminus, thus causing Htt oligomerization and aggregation that leads to neuronal death. A wide range of metabolic abnormalities characterizes HD, including alterations in autophagy (Croce and Yamamoto, 2019). Martinez-Vicente et al. (2010) found that macroautophagy is compromised in cellular and mouse HD models and in HD patient-derived tissues, as evidenced by the inability to recognize and properly sequester unneeded cellular components. Autophagosomes in HD models failed to recognize and load excess cargo, thus causing autophagic cytosolic components to have a slower turnover leading to toxic accumulation of lipid in cells. They also observed an increase in LD number and area in fibroblasts, hepatocytes, striatal cells, and primary neurons from a HD mouse model (Qhtt mice). Furthermore, striatal tissue from advanced-stage HD patients had increased Oil Red O staining density when compared to age-matched controls (Martinez-Vicente et al., 2010). The authors hypothesized that increased LD content in HD cells could be due to their reduced ability to recognize and degrade excess lipid by macroautophagy.

Aditi et al. (2016) used transgenic *Drosophila* expressing the mutated form of human Htt in neurons to better understand HD energetics. They found that when compared to controls, diseased flies exhibited a characteristic pattern of weight change that is correlated with HD progression, as evidenced by altered lipid concentrations over time. Interestingly, and in contrast to the above study by Martinez-Vicente's group, flies had high levels of lipid at disease onset and low levels at the terminal stage in neurons. This finding was also true for abdominal body fat cells, despite only expressing mutant Htt in neurons. LD size mirrored the overall lipid levels, with large LDs being found in 3-7-day-old diseased adults and small LDs forming by days 11-13 in diseased flies. These findings suggest that mutant Htt leads to dysregulated lipid metabolism in addition to neurodegeneration. However, further studies in both mammalian and fly models are needed to fully elucidate the role LDs may play in the onset of HD.

Parkinson's Disease

Parkinson's disease is caused by a loss of dopaminergic neurons and leads to abnormal brain activity and symptoms such as tremors, bradykinesia, and limb rigidity (Kalia and Lang, 2015). Lewy bodies are pathological hallmarks of PD that are found in pre-synaptic terminals of neurons. These Lewy bodies contain aggregates of the α-synuclein protein which has been shown to accumulate on LD phospholipid surfaces, slowing lipolysis of LDs (Cole et al., 2002). Cole and colleagues also found that two mutant forms of α-synuclein, A30P and A53T, showed decreased capacity to reduce LD turnover in neurons compared to WT α-synuclein. These results suggest LD turnover in neurons is contingent on proper α-synuclein function, and that LD lipolysis may contribute to PD (Cole et al., 2002). However, these findings are complicated by the work of Outeiro and Lindquist (2003), who found that both WT and A53T α-synuclein caused an accumulation of LDs in yeast, whereas A30P synuclein did not. These findings suggest that LDs may play a cell-specific role in PD pathology and call for further study in this area.

Integrated genome wide association studies have found that key mechanisms of PD pathogenesis (oxidative stress response, lysosomal function, ER stress response, and immune response) rely heavily on genes regulating lipid and lipoprotein signaling. For example, Klemann and colleagues found that lipid and lipoprotein signaling is regulated by the same processes involved in dopaminergic neuron death, and found deficient signaling to be associated with increased risk for PD (Klemann et al., 2017). Scherzer et al. found that genes related to lipid metabolism and vesicle-mediated transport had the largest effects on increasing α-synuclein toxicity in yeast (Scherzer and Feany, 2004). This same group also classified a variety of genes related to α-synuclein expression in *Drosophila*, and again found that lipid-related genes were strongly associated with this process. The authors proposed that dysregulation in lipid processing may be an indicating factor of problems caused by A30P α-synuclein toxicity (Scherzer et al., 2003).

Additional studies have begun to investigate lipid dyshomeostasis in PD more deeply. For example, suppression of the oleic acid generating enzyme stearoyl-CoA-desaturase (SCD) was recently found to be protective against α-synuclein yeast toxicity, and SCD knockout models in roundworms was shown to prevent dopaminergic neuron degeneration

(Fanning et al., 2019). Specific genes related to lipid regulation have also been identified. ATPase cation transporting protein 13A2 (ATP13A2) functions in cation transport within the cell. Mutations to ATP13A2 are associated with PD and overexpression of ATP13A2 showed a decrease in various forms of lipids *in vitro* (Marcos et al., 2019). When looking at these findings together, it is evident that LDs and lipid homeostasis play a more significant role in PD than originally thought. This line of thought is supported by Fanning et al. (2020) who stated that α-synuclein toxicity and cell trafficking defects have been associated with aberrations in LD content and distribution. PD has classically been believed to be a "proteinopathy," but with many of the recent discoveries, lipid dyshomeostasis is rapidly becoming one of the fundamental characteristics of this disease (Fanning et al., 2020).

Alzheimer's Disease

Alzheimer's disease is the most common form of dementia worldwide (Karantzoulis and Galvin, 2011). When Alois Alzheimer wrote his seminal paper in 1907 describing the case of Auguste Deter, he noted three neuropathological hallmarks. He found "striking changes of the neurofibrils" and "minute milliary foci caused by deposition of a particular substance in the cortex." He also observed glial changes and stated, "many glia include adipose inclusions" (Alzheimer, 1907; Alzheimer et al., 1995). While the first two findings have been studied extensively by scientists interested in the contribution of tau and amyloid to disease progression, the finding of glial lipid accumulation has largely been overlooked. It wasn't until recently that this phenomenon of increased lipid accumulation in AD was revisited and examined. Hamilton et al. (2015) helped renew interest in this phenomenon in a report describing increased LD formation in the SVZ of both 3xTgAD mice and human AD samples which correlated with defects in neurogenesis. Interestingly, acute administration of intracerebral oleic acid mimicked the LD phenotype of the 3xTgAD mice, but did not alter SVZ neuron viability, suggesting that disease-associated LD accumulation is not simply a result of environmental lipid exposure. Derk et al. (2018) found a highly significant increase in both neutral lipid and diaphanous 1 (DIAPH1) expression in myeloid cells in AD brains. DIAPH1 mediates signaling for the receptor for advanced glycation end products (RAGE), an inflammatory ROS-producing pathway. This apparent correlation between neutral lipid accumulation and inflammatory signaling suggests that LDs may be key players in cerebral inflammatory responses. A recent study further validated this model, where it was discovered that astrocytes uniquely upregulate ROS management genes, seemingly to manage the import of neuron-derived lipid oxidation products (Ioannou et al., 2019). This study showed that neuronal hyperactivity alone was sufficient to initiate neuronal lipid peroxidation, neuronal lipoprotein export, and subsequent management and storage of peroxidized lipid as LDs in astrocytes. Further work done by van der Kant et al. (2019) showed that CE, which can be incorporated into LD cores through normal LD biogenesis, increase the accumulation of phosphorylated tau (p-tau) by reducing proteasome activity. The study showed that both statins and an allosteric activator of cholesterol 24-hydroxylase (efavirenz) helped lower p-tau levels in human neurons by reducing CE concentrations, thereby providing a potential mechanistic link between LDs and AD neuropathology.

A role for apolipoproteins as shuttles for oxidative waste from neurons has been described in the brain (Liu et al., 2017). Additionally, different isoforms of Apolipoprotein E (ApoE) were shown to have altered efficiency for lipid shuttling. ApoE4-laden lipoproteins appeared to be less efficacious at the delivery of lipotoxic products to glia than lipoproteins associated with ApoE3.

This is particularly interesting in light of the E4 allele of *APOE* being the strongest genetic risk factor for the development of late onset AD. Our group recently showed that astrocytes expressing E4 preferentially accumulate and utilize LDs for energetic needs (Farmer et al., 2019). Additionally, transcriptional profiling of glia derived from human iPSC lines harboring homozygous E4 or E3 alleles showed that the majority of differentially expressed genes in astrocyte-like cells involved lipid metabolism and transport (Lin et al., 2018). This group also found that a phenotype of E4 astrocytes included the accumulation of intracellular and extracellular cholesterol. They hypothesized that since cholesterol is responsible for a wide range of functions in the brain, altered cholesterol metabolism in E4 glia may be associated with pathological phenotypes in neurodegenerative disorders. Conditioned media from E4 astrocytes has been shown to induce LDs in other cell types, suggesting that E4 may also act extracellularly to induce LD formation (Tambini et al., 2016). Finally, neutral lipid staining of the choroid plexus in post-mortem AD brains proposed LDs as central hubs of an ApoE-mediated complement-cascade regulation (Yin et al., 2019). The authors found ApoE to bind to complement component 1q (C1q), a protein complex that binds antigen-antibody complexes, on LDs in the choroid plexus. Since C1q protein is involved in the activation of the classical complement pathway, this interaction effectively keeps the complement system of the immune system in check at the CNS/vasculature interface. While all ApoE isoforms showed equal binding affinity for C1q, post-mortem mice and human E4 brains were shown to accumulate LDs more abundantly. Interestingly, these lipid deposits significantly correlated with neuropathological staging of AD, pointing to *APOE* regulation of the complement cascade at the choroid plexus niche as a novel hypothesis for AD pathogenesis.

Hereditary Spastic Paraplegia

Hereditary spastic paraplegia (HSP) is a group of inherited neurological disorders that cause muscle weakness and tightness, primarily in the legs, through the degeneration of long corticospinal axons (Lo Giudice et al., 2014; de Souza et al., 2017). Studies of HSP-causing proteins suggest a link between lipid metabolism and the development of disease. For example, DDH2 domain containing protein 2 (DDHD2) is a triglyceride hydrolase in the brain that is implicated in recessive complex HSP. The systemic genetic knockout and pharmacological inhibition of DDH2 resulted in large-scale accumulation of LDs within the CNS, but not elsewhere (Inloes et al., 2018). These data indicate a

link to TAG metabolism, as the inhibition of DDHD2 affects lipid homeostasis and LD number.

One of the most common genetic mutations involved in HSP occurs in the microtubule severing protein spastin (Papadopoulos et al., 2015; Branchu et al., 2017). The M1 isoform of spastin contains a LD targeting sequence which contributes to protein targeting to LDs and LD sorting at the ER. Additionally, spastin deficiency in *Drosophila* and *C. elegans* altered LD number and TAG content. This study suggests that LD processing may contribute to the pathogenesis of HSP (Papadopoulos et al., 2015). Additionally, a study by Arrabit et al. further identifies spastin as a regulator of LD dispersion and dynamics. This group showed that mutations in the spastin M1 isoform induced ER reorganization in HeLa cells. This reorganization subsequently disrupted spastin's ability to disperse LDs throughout the cell and aberrantly modulated neutral lipids and phospholipids on membranes throughout the muscle and brain (Arribat et al., 2020). Furthermore, embryonic zebrafish cells that were treated with oleic acid (a common method to induce LD formation) indicated that a loss of spastin resulted in a higher number of smaller LDs, therefore suggesting differential generation, and/or dispersion of LDs (Arribat et al., 2020). In addition to identifying potential new HSP biomarkers, this study also proposes that HSP-causing mutations impacts lipid profiles and LD networks.

Several groups have also forged a connection between the protein spartin and LD regulation. Spartin is a multi-functional unit that associates with LD, and a lack of spartin expression contributes to a HSP form called Troyer syndrome (Hooper et al., 2010). In one study by Edwards et al. (2009) spartin surrounded LD clusters in oleic acid treated HeLa cells, thus suggesting that spartin is recruited to LDs. Additionally, in spartin knockout mice, female mice had increased LDs number and higher perilipin protein levels in adipose tissue (Renvoisé et al., 2012). Hooper and colleagues further demonstrated an interaction between spartin and LDs. Their experiments in HeLa cells showed that spartin binds to and recruits the ubiquitin ligase atrophin-1-interacting protein 4 (AIP4) to LD. This interaction subsequently promotes the ubiquitination of PLIN2. Since PLIN2 resides on LD membranes and regulates TAG turnover, spartin may play a role in LD regulation in cells and contribute to Troyer syndrome pathology (Hooper et al., 2010). Furthermore, another group reported binding interactions between spartin and protein kinase C interacting proteins (ZIPs) at the surface of LDs. Spartin-expressing HEK-293 cells exhibit co-localization of spartin with ZIP1 and ZIP3 on LDs as shown by superimposition of spartin GFP-tagged fluorescence and Oil Red O fluorescence (Urbanczyk and Enz, 2011). Interestingly, in the absence of spartin, no ZIP proteins were detected on the LD surface (Urbanczyk and Enz, 2011). These collective findings suggest that impaired LD metabolism might be one mechanism that contributes to Troyer Syndrome.

REGULATION OF LDS IN THE BRAIN

Lipid droplets in the brain may be regulated differently than more traditional LD niches. DDHD2 action in HSP implicates this protein as an important player in CNS lipid metabolism but also shows a dichotomy between peripheral LDs and central LDs. The BBB forms a tight and regulated gate for lipid import and export into the brain, and thus there are separate pools of lipids that constitute the intracellular LDs located in cells in the CNS versus the periphery. Lipid contents of the cell types that make up the BBB appear to regulate its permeability (Andreone et al., 2017; Ceafalan et al., 2019). Since enzyme mutations of key lipases are manifested differently in the brain and given the existence of lipid-regulatory mechanisms between the periphery and the CNS, it is possible that the two pools are controlled by separate mechanisms. Investigators should be cautious when applying canonical peripheral LD pathways to brain LD biology until more evidence emerges for conserved physiological pathways between the brain and the periphery.

Sex-specific regulation of lipid metabolism and LDs in the brain should also be considered. In one study, neurons of male rats were shown to be more vulnerable to starvation than neurons of female rats, with male neurons exhibiting decreased mitochondrial respiration, increased autophagosome formation, and increased cell death when compared to females (Du et al., 2009). On the other hand, the same study showed that female tolerance of starvation conditions was associated with increases in fatty acid content and LD formation in order to prolong cell survival. These findings suggest that central LD dynamics may be sex dependent. Potential sex differences should be considered in future studies in order to better understand the contribution of sexually dimorphic features to cerebral LD metabolism and its relevance to disease susceptibility and pathology.

FUTURE DIRECTIONS AND CONCLUDING REMARKS

Several important knowledge gaps remain in our understanding of the role of LD biology in the pathophysiology in neurodegenerative disease. One important future direction for the field is to better understand the full composition of lipids comprising brain LDs – in both healthy and diseased states. A clearer picture would likely be revealed by the precise distribution of lipid classes in distinct brain regions, as well as in specific neurodegenerative diseases. However, their relatively small size and the diversity of LDs by age, cell type, and disease type complicate the compilation of a lipid profile of LDs. Additionally, *in vivo* analysis of lipids in general is challenging, as they are a diverse class of macromolecules and are relatively insoluble (Cornett et al., 2007). However, improved techniques in mass spectrometry provide new ways to document the lipid composition in LDs. For example, matrix-assisted laser desorption/ionization imaging mass spectrometry (MALDI-IMS) allows for anatomically-specific direct detection of lipids within membranes through the production of lipid-derived ions (Fuchs et al., 2010). Since MALDI can detect phospholipids, sphingolipids, and glycerolipids, this technique provides the opportunity for an in depth and spatially-resolved profile of LDs (Murphy et al., 2009). The continued use of powerful imaging techniques such as MALDI will be important to providing a better understanding of lipid class distribution in cerebral LDs.

Lipid droplets are cellular fuel stores, markers of inflammation, signaling hubs, protective waste reservoirs for hyperactive neurons, products of lysosomal dysregulation, and hallmarks of age. With such a wide array of roles, it is not surprising that LD accumulation has been linked to trauma, neurodegeneration, and aberrant cerebral metabolism. LDs are promising targets for novel investigations of neurological disease diagnosis and therapeutics. Therapeutic treatments could be targeted at restoring lipid balance, decreasing droplet levels, or improving other aspects of lipid metabolic pathways. Further study on LDs and lipid metabolism will be essential in advancing our knowledge of cerebral metabolism, as well as the multifaceted etiologies of neurological disease.

AUTHOR CONTRIBUTIONS

BF initiated this work and drafted the manuscript. BF, AW, JK, and LJ wrote the manuscript. AW and JK performed the literature search for **Table 1**. All authors read and approved the manuscript in final form.

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Accumulation of Cerebrospinal Fluid Glycerophospholipids and Sphingolipids in Cognitively Healthy Participants With Alzheimer's Biomarkers Precedes Lipolysis in the Dementia Stage

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Insight into lipids' roles in Alzheimer's disease (AD) pathophysiology is limited because brain membrane lipids have not been characterized in cognitively healthy (CH) individuals. Since age is a significant risk factor of AD, we hypothesize that aging renders the amyloid precursor protein (APP) more susceptible to abnormal processing because of deteriorating membrane lipids. To reflect brain membranes, we studied their lipid components in cerebrospinal fluid (CSF) and brain-derived CSF nanoparticle membranes. Based on CSF $A\beta_{42}$/Tau levels established biomarkers of AD, we define a subset of CH participants with normal $A\beta_{42}$/Tau (CH-NAT) and another group with abnormal or pathological $A\beta_{42}$/Tau (CH-PAT). We report that glycerophospholipids are differentially metabolized in the CSF supernatant fluid and nanoparticle membrane fractions from CH-NAT, CH-PAT, and AD participants. Phosphatidylcholine molecular species from the supernatant fraction of CH-PAT were higher than in the CH-NAT and AD participants. Sphingomyelin levels in the supernatant fraction were lower in the CH-PAT and AD than in the CH-NAT group. The decrease in sphingomyelin corresponded with an increase in ceramide and dihydroceramide and an increase in the ceramide to sphingomyelin ratio in AD. In contrast to the supernatant fraction, sphingomyelin is higher in the nanoparticle fraction from the CH-PAT group, accompanied by lower ceramide and dihydroceramide and a decrease in the ratio of ceramide to sphingomyelin in CH-PAT compared with CH-NAT. On investigating the mechanism for the lipid changes in AD, we observed that phospholipase A_2 (PLA_2) activity was higher in the AD group than the CH groups. Paradoxically, acid and neutral sphingomyelinase (SMase) activities were lower in AD compared to the CH groups. Considering external influences on lipids, the clinical groups did not differ in their fasting blood lipids or dietary lipids, consistent with the CSF lipid changes originating from brain pathophysiology. The lipid accumulation in a prodromal AD biomarker positive stage identifies perturbation of

lipid metabolism and disturbances in APP/Amyloid beta (Aβ) as early events in AD pathophysiology. Our results identify increased lipid turnover in CH participants with AD biomarkers, switching to a predominantly lipolytic state in dementia. This knowledge may be useful for targeting and testing new AD treatments.

Keywords: Alzheimer's disease biomarker, amyloid, cerebrospinal fluid, dementia, glycerophospholipids, nanoparticles, sphingolipids, tau

INTRODUCTION

Specific mutations lead inevitably to familial Alzheimer's disease (FAD; Lacour et al., 2019; Wong et al., 2020), and gene polymorphisms are associated with late-onset Alzheimer's disease (AD; Morelli et al., 1996; Casadei et al., 1999; Dursun et al., 2008; Du et al., 2016; Zuin et al., 2020), the most common form of AD. Some risk factors such as age, education, apolipoprotein ε4 (*ApoE*-4), hypertension, diabetes, hyperlipidemia, obesity, smoking, and head trauma have been identified, but none is necessary or sufficient to cause AD (Devanand et al., 2013; Guimas Almeida et al., 2018; Takahashi et al., 2018). Nevertheless, early AD pathology, similar to FAD, is exemplified by extracellular β–amyloid, predominantly amyloid$_{42}$ (Aβ_{42}) deposition, with intracellular neurofibrillary tangles in the later stages that include abnormally modified tau protein (Vanmechelen et al., 2001). Amyloid beta (Aβ) peptides are enzymatically cleaved from the extracellular neuronal surface of the transmembrane amyloid precursor protein (APP; Frederikse et al., 1996; Lorenzo et al., 2000). The neurotoxic Aβ_{42} aggregates and accumulates in plaques that characterize AD pathology (Perry et al., 1988; Arai et al., 2019). Apolipoprotein ε4 reduces Aβ_{42} clearance, consistent with the increased AD risk of this inherited isoform (Castellano et al., 2011; Verghese et al., 2013). In the absence of autosomal dominant mutations, it is less clear how amyloid pathology is initiated in sporadic AD. Since Aβ_{42} derives from the transmembrane APP, clues to the mechanism that links these multiple risk factors to the Aβ_{42} cascade may, therefore, be revealed in the neuronal membrane environment at the earliest pathology stage of AD.

The most significant risk factor for AD is aging, and post-mitotic neurons are especially vulnerable in aging: oxidative damage is more significant in mitochondria of neurons from the aged brain (Chakrabarti et al., 2011), mitochondrial proteins have an age-dependent loss of expression (Shevchenko et al., 2012), and lipids, the major membrane constituent, are more susceptible over time to peroxidative damage (Hulbert, 2010). Lipids, mainly in membranes, constitute about 50% of the brain's dry mass (O'Brien and Sampson, 1965; Svennerholm et al., 1991); though their major classes have been identified, most of their molecular species remain uncharacterized. Thus, the biggest challenges to understanding the APP processing and the origins of Aβ_{42} formation are to characterize the APP's lipid environment in CH individuals with AD biomarkers.

Of eight lipid classes (Fahy et al., 2009), glycerophospholipids (GPs) and sphingolipids (SPs) constitute diverse molecules whose perturbation may be associated with neuronal injury, neuroinflammation (Russo et al., 2018; Leishman et al., 2019), and neurodegeneration (Raszewski et al., 2016; Aufschnaiter et al., 2017; Dorninger et al., 2020; Hernandez-Diaz and Soukup, 2020), all features associated with AD pathology. Some brain lipid components have been measured in AD (Patel and Forman, 2004; Lim et al., 2014; Garner, 2010; Chew et al., 2020; Kao et al., 2020). However, studies of the prodromal pathology phase have not been undertaken, and the availability of fresh brain tissue to explore lipid chemistry is thus a major barrier. An approach to accessing brain-derived lipids through cerebrospinal fluid (CSF) was enhanced with the discovery that CSF has an abundance of membranous nanoparticles (billions per mL) and includes typical synaptic and large dense-core vesicles (Harrington et al., 2009). CSF is readily obtained in research studies *in vivo*, potentially repeatedly, allowing testing of brain-derived fluid and membrane lipids in CH individuals with or without expression of CSF biomarkers of AD.

We characterized the most abundant GPs in the CSF from an elderly, cognitively healthy (CH) population and found GPs in the CSF nanoparticle membranes differed from the supernatant fluid (Fonteh et al., 2013). Moreover, the CSF Aβ_{42} biomarker, most commonly combined with total tau protein as a ratio, can identify AD pathology long before dementia by distinguishing CH people with normal versus abnormal CSF biomarkers of AD: CH participants with normal Aβ_{42}/Tau (CH-NAT) versus CH participants with abnormal or pathological Aβ_{42}/Tau (CH-PAT; Fagan et al., 2007).

Here, we examined if lipid metabolism differed in CH volunteers negative or positive for AD biomarkers, or those with clinical AD, by measuring GP and SP lipid classes and their molecular species in CSF supernatant fluids (SF) and membrane nanoparticles (NP). We tested the hypothesis that lipolysis contributes to the pathological process by measuring the activity of two enzymes that hydrolyze membrane lipids: phospholipase A$_2$ (PLA$_2$) and sphingomyelinase (SMase) activities. PLA$_2$ activity has been shown to increase in CSF in AD and associates with amyloid plaques (Stephenson et al., 1996), while SMase activity changes in CSF and brain fractions from AD patients (Jana and Pahan, 2004; Lee et al., 2014; Fonteh et al., 2015). Our data show an accumulation of GP in the supernatant fluid and SPs in the membrane nanoparticles of CH study participants with CSF AD biomarkers and lower lipid compositions in samples from those with clinical AD. We propose that our findings of enhanced lipid turnover in CH individuals with AD biomarkers, followed by lipolysis in the AD dementia stage, may be useful for targeting and testing AD treatments.

MATERIALS AND METHODS

Clinical Methods

The local Institutional Review Board of Huntington Memorial Hospital approved our protocol and consent form, and study participants gave written informed consent. Clinical details of our classification have been reported (Harrington et al., 2013). In brief, 70 participants were classified at a clinical consensus conference using UDS and NACC criteria (Harrington et al., 2013) as CH based on their having no evidence of cognitive impairment after uniform clinical and neuropsychological examinations. Of 40 participants with dementia, 29 were diagnosed after fulfilling the criteria for clinically probable AD (Harrington et al., 2013). Participants completed the National Cancer Institute Diet History Questionnaire (DHQ; Subar et al., 2001, Version 2, 2010) that estimates the annual intake of 176 nutrients and food groups. We only trusted the memories of the CH subgroups for reliable DHQ data. Answers on paper questionnaires were entered online by study staff, and the Diet*Calc 1.5 software was used to obtain dietary intake measurements. Fasting CSF and blood samples were obtained between 8:00 and 10:00 h within one month of neuropsychological testing, prepared and stored within 2 h of collection in polypropylene tube aliquots. Lumbar CSF was immediately examined for cells and total protein, and the remainder stored in 1 mL aliquots at −80°C.

CSF $A\beta_{42}$ and Tau

The concentrations of CSF $A\beta_{42}$ and Tau were measured using a sandwich enzyme-linked immunosorbent assay kit ([ELISA] Innotest β-amyloid$_{(1-42)}$ and Innotest hTAU-Ag, Innogenetics, Gent, Belgium) according to the manufacturer's protocol (Harrington et al., 2013). In brief, to determine the concentration of $A\beta_{42}$, we added 25 μL of CSF sample and standards in duplicate into the monoclonal antibody (21F12) pre-coated plate and incubated with biotinylated antibody (3D6). We determined the $A\beta_{42}$ concentration using a standard curve, between 125 and 2,000 pg/mL, with an assay detection limit of ± 50 pg/mL. In the Tau ELISA assay, we added 25 μL of CSF sample and standards in duplicate into the monoclonal antibody (AT120) pre-coated plate and incubated overnight with two biotinylated Tau-specific antibodies (HT7 and BT2). We quantified tau in CSF samples using a standard curve ranging between 75 and 1,200 pg/mL and an assay detection limit of ± 59.3 pg/mL.

Determination of Protein in CSF

Supernatant fluids and NP fractions were diluted using phosphate-buffered saline, and protein contents were determined using a fluorescence-based Quant-iT^{TM} Protein Assay detection kit (Invitrogen, Eugene, OR, united States) with 0–500 ng BSA as standards.

Nanoparticle (NP) Quantification in CSF

We determined the number and size distribution of NPs in CSF using a NanoSight NS300 instrument (Malvern Panalytical, Inc., Westborough, MA, United States; Fonteh et al., 2020). Briefly, after centrifugation at 3,000 RCF for 3 min to remove cellular debris, we diluted CSF (10×) using dd-H_2O. The diluted CSF was continuously infused into the NS300 previously calibrated with polystyrene beads (30, 100, and 400 nm). Light scattering data was recorded at 432 nm for 60 s (×5) and processed using the Nanoparticle Tracking Analysis software (Malvern Panalytical, Inc.).

CSF Fractionation and Glycerophospholipid Extraction

Supernatant fluids and NP fractions were isolated from CSF as described (Harrington et al., 2009). Briefly, starting with 4 mL of CSF (SF1), we obtained SF2 and NP2 after centrifugation at 17,000 *g*. SF2 was centrifuged again at 200,000 *g* to obtain the SF and NP fractions. The SF fraction was stored at −80°C, and the NP containing CSF nanoparticles was washed with 4 mL of phosphate-buffered saline, re-pelleted at 200,000 g, and re-suspended in 50 μL of phosphate-buffered saline.

Materials for Lipid Analyses

HPLC grade water, 2-isopropanol, and acetonitrile (ACN) were purchased from VWR (West Chester, PA, United States). Ammonium acetate and butylated hydroxyl toluene (BHT) were purchased from Sigma (St Louis, MO, United States). Phosphatidylcholine (17:0 PC and 11:0 PC), phosphatidylethanolamine (17:0 PE), phosphatidylserine (17:0 PS) and lysophosphatidylcholine (11:0 LPC), sphingomyelin (SM), ceramide (CM), and dihydroceramide (dhCM) standards were purchased from Avanti Polar Lipids (Alabaster, AL). N-arachidonoyl phosphatidylethanolamine (NAPE), D_4-platelet-activating factor (PAF), and D_4-lysophosphatidylcholine (LPC) were purchased from Cayman Chemical (Ann Arbor, MI, United States).

Lipid Extraction

Internal standards (IS) and retention time calibrants [11:0 PC (5 ng), D_4-PAF (1 ng), 11:0 LPC (5 ng), and D_4-LPC (5 ng)] were added to 1 mL SF from the original 4 mL of CSF, and GPs were extracted using a modified Bligh and Dyer (1959) procedure. Briefly, to limit lipid oxidation, we added 2 mL methanol containing 0.2 mg/mL BHT and performed all extraction at room temperature. A lipid-rich chloroform layer was aspirated to clean borosilicate culture tubes. Similarly, 40% of NP from the original 4 mL of CSF was suspended in 1 ml water containing 1 M NaCl and extracted as described above. The GP-rich chloroform layers from SF or NP were dried under a stream of N_2 and reconstituted in HPLC solvent for LC/MS analyses.

Hydrophilic Interaction Liquid Chromatography (HILIC) of GPs and SPs

Hydrophilic interaction liquid chromatography was performed using an HP-1100 system equipped with an autosampler, a column oven maintained at 35°C, and a binary pump system using TSK-Gel Amide-80 Column (2.0 × 150 mm). GPs and SPs were isolated using a binary solvent system of 20% acetonitrile

in isopropanol (Solvent A) containing 8% solvent B (20% water in isopropanol containing 10 mM ammonium acetate) at a flow rate of 0.2 ml/min (Fonteh et al., 2013, 2015). Solvent A was maintained for 5 min followed by a linear increase to 20% solvent B in 20 min, maintained at 20% B for 25 min, and then equilibrated with solvent A for 15 minutes before subsequent injections.

Positive Ion Electrospray Ionization (ESI) Mass Spectrometry (MS)

Glycerophospholipids and SPs eluting from the HILIC column were positively ionized using an electrospray ionization (ESI) probe and detected using several MS scanning modes in a TSQ mass spectrometer (Thermo Fisher Scientific, San Jose, CA, United States; Fonteh et al., 2013, 2015). The MS was operated with a spray voltage of 4.5 kV, a heated capillary temperature of 300°C, and nitrogen (50 units) and argon (5 units) as the sheath gas and the auxiliary gas, respectively. All GPs (PE, PE1, NAPE, PC, C11:0 PC internal standard, PS, PAF-like lipids [PAF-LL], and LPC) and SPs (SM, CM, dhCM) scans were optimized for collision energies, acquisition mass range, and retention times. To better isolate lipid classes, we collected data using three different scan windows from 0–5, 5–16, and 16–40 min (Fonteh et al., 2013, 2015).

GP and SP Analyses

Peak areas for all GPs were integrated using the Qual Browser module of the Xcalibur software (Thermo Fisher, San Jose, CA, United States) normalized to the IS, C11:0 PC. For quantification, standard curves of GPs and SPs were obtained from each lipid amount plotted against each lipid's intensity ratio to the C11:0-PC internal standard. Quantities of GPs and SPs were calculated for SF (ng/ml CSF) or NP (ng/ml CSF equivalent) using the ensuing standard curves. PC, PE, PS, and SM molecular species were identified using the Qual browser's spectra function. The major peaks were identified using Lipid Maps MS tools[1].

Phospholipase A_2 (PLA_2) Activity Assay

A modified liposomal-based fluorescent assay was used to measure PLA_2 activity in CSF samples. Briefly, a PLA_2 substrate cocktail consisting of 7-hydroxycoumarinyl-arachidonate (0.3 mM), 7-hydroxycoumarinyl-linolenate (0.3 mM), hydroxycoumarinyl-6-heptanoate (0.3 mM), 10 mM dioleoylphosphatidylcholine (DOPC), and 10 mM dioleoylphosphatidylglycerol (DOPG) was prepared in ethanol. Liposomes were formed by gradually adding 77 μl substrate/lipid cocktail to 10 ml PLA_2 buffer [50 mM Tris-HCl (pH 8.9), 100 mM NaCl, 1 mM $CalCl_2$] while stirring rapidly over 1 min using a magnetic stirrer. CSF containing 10 μg total protein was added to 96 well plates, and PLA_2 activity was initiated by adding a 50 μl substrate cocktail. Fluorescence (Excitation at 360 nm and emission at 460 nm) was measured, and specific activity was determined [relative fluorescent units (RFU)/μg protein] for each sample determined (Fonteh et al., 2013).

[1]www.lipidmaps.org

Sphingomyelinase (SMase) Activity Assay

Fluorescence (Excitation at 360 nm and emission at 460 nm) was measured, and specific activity was determined (RFU/μg protein) for each sample (Fonteh et al., 2015).

Statistical Analyses

All GP and SP data are presented as the mean ± SEM with 95% CI for each cognitive or $A\beta_{42}$/Tau subgroups. One way ANOVA on ranks (Kruskal–Wallis test) and correction for multiple comparisons using statistical hypothesis testing using Dunn's method were performed to determine within-group differences of GPs and SPs classes and molecular species. Spearman correlation was used to determine the association of lipids species. One way ANOVA analyses were performed using GraphPad Prism software (La Jolla, CA, United States). Data normalization was performed using MetaboAnalyst software. Briefly, GP and SP data were converted to tab-delimited text (.txt) before import into the MetaboAnalyst Statistical Analysis platform (Mendes et al., 2018; Chong and Xia, 2020). Data normalization and scaling used globalized logarithm transformation (glog) and mean-centering to obtain a Gaussian distribution and compare lipid levels over several orders of magnitude in the SF and NP fractions. Hierarchical clustering data presented in the form of a heatmap used Euclidean for distance measure and Ward for the clustering algorithm (Mendes et al., 2018; Chong and Xia, 2020). Data were considered significant if $p < 0.05$ after adjustment for multiple comparisons.

RESULTS

CSF $A\beta_{42}$ and Tau Levels Establish the Presence of AD Biomarkers in Some CH Individuals

An expert research consortium offered a working definition of three preclinical stages of AD pathology, based on molecular and neuroimaging biomarkers (Mendes et al., 2018): stage 1, CH with abnormal amyloid in CSF or brain; stage 2 adds to evidence of neurodegeneration to stage 1; stage 3 adds subtle cognitive change (insufficient to diagnose MCI) to stage 2. The authors modeled these preclinical stages to hypothetical pathophysiology that merits further dissection to unravel the diversity of dementia risk factors and the heterogeneous pathophysiology, exemplified by cases confirmed with AD at autopsy, but with prior normal amyloid imaging or normal CSF $A\beta_{42}$. To study CH individuals with early AD pathology and compare it with dementia, we analyzed CSF $A\beta_{42}$ and tau from individuals with clinical diagnoses ranging from healthy to dementia, including a CH cohort with an increased likelihood of preclinical pathology because of advancing age (Harrington et al., 2013). Our study participants with normal cognitive function are separated into two subgroups based on CSF $A\beta_{42}$/Tau levels that have either CH-NAT or CH-PAT, **Table 1**. The CH-PAT group is cognitively healthy based on normal neuropsychometry, but with

TABLE 1 | Demographic data, mini-mental state examination scores (MMSE), and $A\beta_{42}$/tau of cognitively healthy (CH) and Alzheimer's disease (AD) participants.

Parameters	CH-NAT (*n* = 35)	CH-PAT (*n* = 33)	AD (*n* = 25)	*P*-value[#2]
Age, mean, SD (95% CI)	77 ± 7 (74-79)	78 ± 7 (75-80)	75 ± 9 (72-79)	0.6876
Female (n female/n total)[#1]	25/35	20/33	12/25	
Neuropsychology data (mean, SD, 95% CI)				
MMSE	28.9 ± 1.2	28.5 ± 1.8	15.9 ± 7.8	< 0.0001
Amyloid and tau (means SD, 95% CI)				
$A\beta_{42}$ (pg/ml)	898 ± 216 (825-971)	487 ± 213 (410-566)	458 ± 199 (373-542)	< 0.0001
tau (pg/ml)	216 ± 121 (175-257)	345 ± 187 (276-413)	505 ± 200 (421-590)	< 0.0001
$A\beta_{42}$/T-tau	4.9 ± 1.8 (4.2-5.5)	1.7 ± 0.8 (1.4-1.9)	1.0 ± 0.5 (0.8-1.2)	< 0.0001

[#1]Fisher's Exact test showed no significant difference in the female to male ratio for CH-NAT and CH-PAT (p (=0.3458), CH-NAT, and AD (p (=0.0.0657), and CH-PAT and AD (p (=0.3391). [#2]One way ANOVA (p-values) and Dunn's multiple comparison test was performed for MMSE, and $A\beta_{42}$, tau, and $A\beta_{42}$/tau ratio. MMSE scores are similar in the CH groups but significantly higher than the AD group. $A\beta_{42}$ levels are higher in the CH-NAT group than in CH-PAT and AD. Tau is lower in CH-NAT compared with CH-PAT and AD resulting in a significantly higher $A\beta_{42}$/tau ratio in CH-NAT than in AD.

significantly lower Stroop Color Word performance than the CH-NAT group (Harrington et al., 2013), suggesting early executive function deterioration. Longitudinal follow-up over 4 years shows that 40% of CH-PAT participants cognitively decline while none of the CH-NAT participants decline (Harrington et al., 2019). Our CH subgroup with normal CSF protein, CH-NAT, fits Stage 0, while the subgroup with abnormal protein (CH-PAT) includes stages 1 and 2.

GP Metabolism Is Increased in CH Individuals With AD Biomarkers

We investigated whether GP classes (PC, LPC, PAF_LL, PE, and PS; Fonteh et al., 2013) and their molecular species differed between the CH-NAT, CH-PAT, or the AD groups.

PC Changes in the SF and NP Fractions

In the SF fraction, there is a significant increase in PC in CH-PAT compared to AD (**Figure 1A**). Further examination showed five PC species, PC-SAFA, and PC-MUFA exhibit group differences (**Figure 1B**). Multiple comparisons show significantly higher levels of these PC species in CH-PAT compared with AD. Similar to the SF fraction, there is a trend for higher PC levels in the NP fraction of CH-PAT compared with CH-NAT and AD (**Figure 1C**). The examination of PC species shows a higher PC32a:1 in CH-PAT than in AD (**Figure 1D**).

Other Choline-Containing GPs in the SF and NP Fractions

There is a trend for higher LPC (**Figure 2A**) and PAF_LL (**Figure 2B**) in CH-PAT and a progressive increase in the LPC/PC ratio in CH-PAT and AD (**Figure 2C**) in the SF fraction. We observe a similar increase in LPC (**Figure 2D**), PAF-LL (**Figure 2E**), and the LPC/PC (**Figure 2F**) ratio in the NP fraction of CH-PAT.

Figures 2G,H are the clustering result illustrated as a heatmap showing changes in the PC measures in the SF and NP fractions, respectively. In general, the mean levels of GPs are higher in the CH-PAT than the CH-NAT group, which contrasts with the general decrease of GPs in AD. Of the 30 choline-containing GP measures in the SF fraction, three are highest in CH-NAT, three measures are highest in AD, while the rest of the measures are highest in CH-PAT (**Figure 2G**). In the NP fraction, two choline-containing GP measures are highest in AD, 10 measures are highest in CH-NAT, and 15 are highest in CH-PAT (**Figure 2H**).

Choline-Containing GPs Normalized to the Number of NPs

We normalized NP lipids levels to the number of particles to determine if the membrane concentration could account for group differences. When normalized to the number of NPs in CSF, we find a progressive decrease in PC (**Figure 3A**), SAFA-PC (**Figure 3B**), MUFA-PC (**Figure 3C**), PUFA-PC (**Figure 3D**), and total choline-containing GPs (**Figure 3E**). We do not observe similar changes in LPC (**Figure 3F**), LPAF (**Figure 3G**), and the LPC/PC (**Figure 3H**) ratio in the NP fractions.

Correlation of PC to LPC

In the SF fraction, there is positive correlation between PC and LPC in CH-NAT (**Figure 4A**, $r = 0.34$, $p = 0.0398$) and this level of correlation increases in CH-PAT (**Figure 4B**, $r = 0.50$, $p = 0.0029$), and in AD (**Figure 4C**, $r = 0.51$, $p = 0.0096$). In contrast to the SF fraction, PC correlates better in the NP fraction of CH-NAT (**Figure 4D**, $r = 0.47$, $p = 0.0035$) than in CH-PAT (**Figure 4E**, $r = 0.35$, $p = 0.0505$) and AD (**Figure 4F**, $r = 0.26$, $p = 0.2128$).

Phosphatidylethanolamine (PE) and Phosphatidylserine (PS) in SF and NP Fraction

We did not measure significant clinical group changes in PE (**Figure 5A**), PE1 (**Figure 5B**), NAPE (**Figure 5C**), and PS (**Figure 5D**) in the SF fractions. However, one PE species (PE38a:5) was significantly higher in CH-PAT than in CH-NAT, and a plasmalogen PE specie (PE40p:4) was significantly higher in CH-PAT than in AD (**Figure 5E**). In the NP fraction, we did not measure group differences in the levels of PE (**Figure 5F**), PE1 (**Figure 5G**), and NAPE (**Figure 5H**). However, when normalized to the number of NPs to determine if membrane concentration varied between groups, there was a significant decrease in PE in CH-PAT and AD compared with CH-NAT (**Figure 5I**), but not in

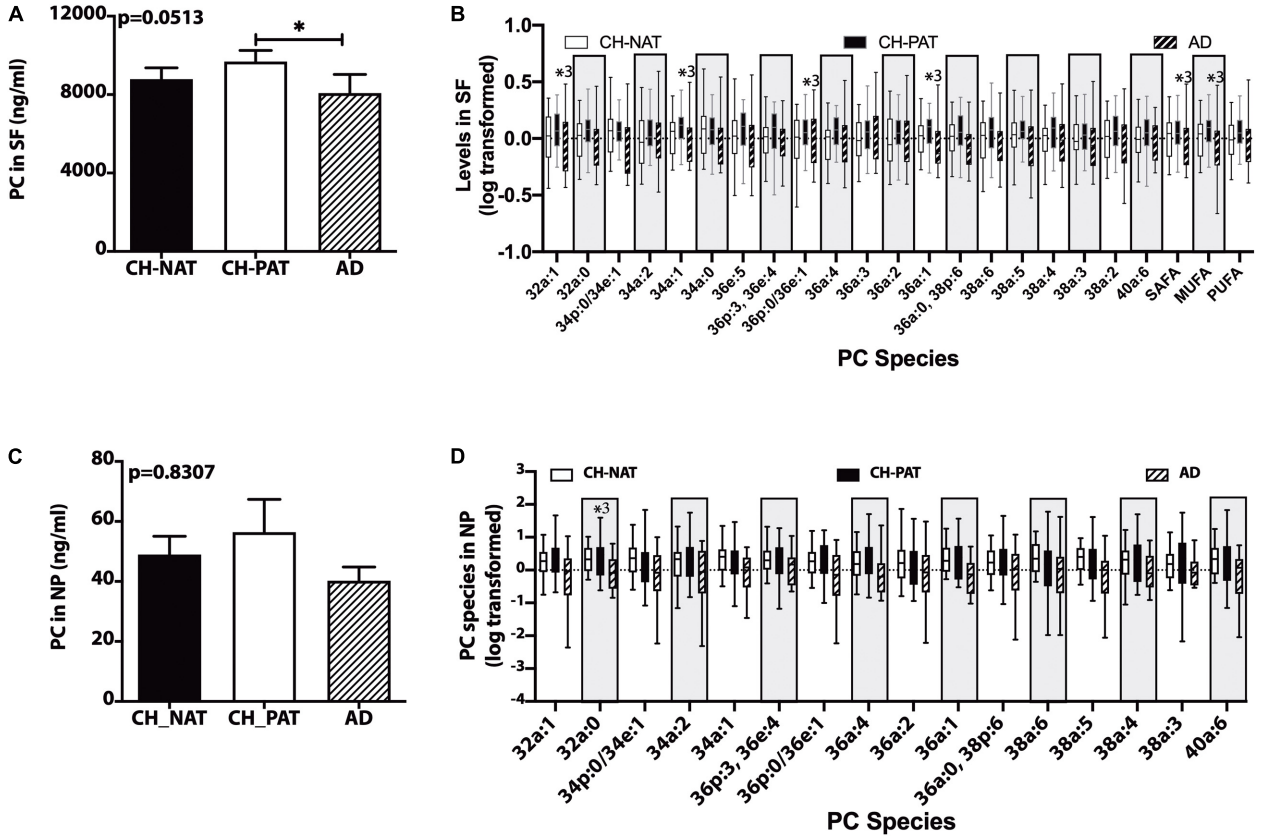

FIGURE 1 | Phosphatidylcholine (PC) and PC species in the supernatant fluids (SF) and nanoparticles (NP) fractions – Levels of PC in the SF fraction **(A)** and PC species in the SF fraction **(B)**, PC in the NP fraction **(C)**, and PC species **(D)** in the NP fractions were determined using Hydrophilic interaction liquid chromatography (HILIC) chromatography coupled with mass spectrometry. The mean ± SEM for PC and PC species were normalized and log 10 transformed to display species of different concentrations. Group comparisons were made using One way ANOVA (Kruskal–Wallis test) combined with Dunn's multiple comparisons. The *p*-values are from One way ANOVA, and significant changes ($p < 0.05$) from Dunn's are indicated with an asterisk(*).

the levels of PE1 (**Figure 5J**). Mean NAPE levels generally trended lower in CH-PAT and AD than in CH-NAT (**Figure 5K**).

GP Summary

These data show subfraction-specific changes in GP classes and molecular species that reflect altered lipid metabolism from both extracellular fluid-derived SF and brain tissue-derived NP in the CH group with AD biomarkers. The increase of GPs in this CH-PAT group is most notable for being in the opposite direction from AD, in which the GPs other than LPC decrease.

Changes in Sphingolipids (SPs) in Preclinical AD

Sphingolipids are essential in brain structure and function and are implicated in AD pathology (van Echten-Deckert and Walter, 2012; Martinez Martinez and Mielke, 2017). Therefore, we compared SP levels in our CH groups with normal or pathological $A\beta_{42}$/tau ratio and with the AD group (Harrington et al., 2019). The SP classes we quantified in CSF fractions include SM, CM, and dhCM (Fonteh et al., 2015).

SM Changes in the SF and NP Fractions

In the SF fraction, there is a trend for lower levels of SM in CH-PAT and AD compared to CH-NAT (**Figure 6A**). Examining SM species levels showed no significant differences between our three clinical groups (**Figure 6B**). In contrast to the SF fraction, SM levels trended on a higher side for CH-PAT than CH-NAT (**Figure 6C**), and seven SM species were significantly higher in CH-PAT than in CH-NAT and AD (**Figure 6D**).

Other Sphingolipids in the SF and NP Fractions

In the SF fraction, there is a trend for higher CM in AD (**Figure 7A**). While not significant, dhCM increases progressively in CH-PAT and AD compared with CH-NAT (**Figure 7B**), while total SP levels show a decreasing trend in CH-PAT and AD compared with CH-NAT (**Figure 7C**). The increase in CM concomitant with the SM decrease resulted in a significantly higher CM/SM ratio in AD than CH-NAT and CH-PAT in the SF fraction (**Figure 7D**). In the NP fraction, the mean CM level is significantly lower in AD than in CH-NAT (**Figure 7E**). In contrast to the increase of dhCM in the SF fraction (**Figure 7B**),

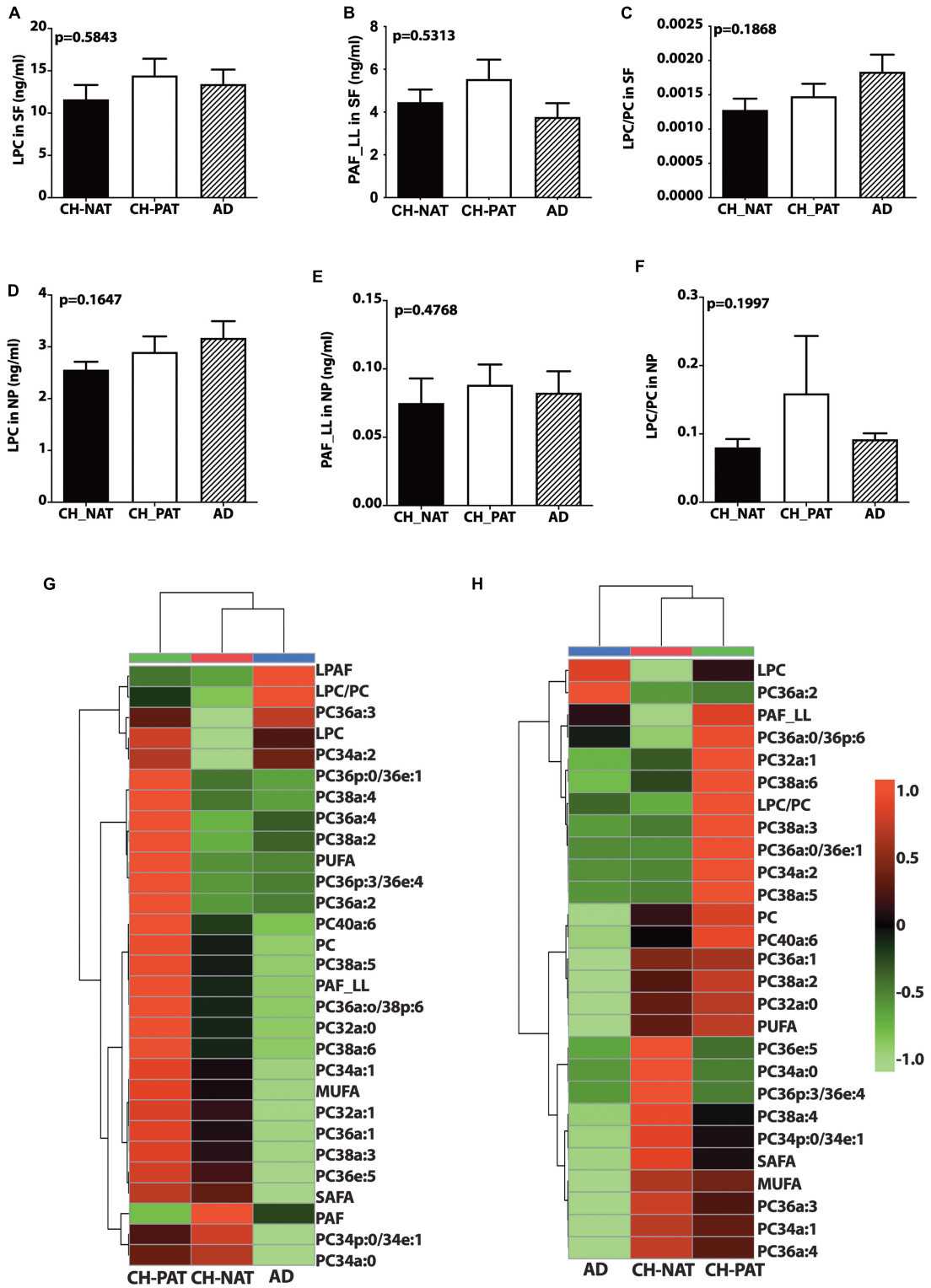

FIGURE 2 | Phosphocholine glycerophospholipids and hierarchical clustering of all phosphatidylcholine (PC) glycerophospholipids in the supernatant fluids (SF) and nanoparticles (NP) fractions – Levels of lysophosphatidylcholine (LPC; **A**), PAF_LL **(B)**, and the LPC to PC **(C)** ratios were determined in the SF fraction of CH-NAT, CH-PAT, and AD. LPC **(D)**, and PAF_LL **(E)** and the LPC/PC ratio **(F)** were similarly determined in the NP fractions. Group comparisons were made using One way ANOVA (Kruskal–Wallis test) combined with Dunn's multiple comparisons. The *p*-values are from One way ANOVA, while significant changes ($p < 0.05$) from Dunn's are indicated with an asterisk (*). The heatmap displays the hierarchical clustering of PC-glycerophospholipids in the SF fraction **(G)** and the NP fraction **(H)**. Distance measures of the heatmap use Euclidean while the clustering algorithm uses Ward on the Metabanalyst 4.0 platform.

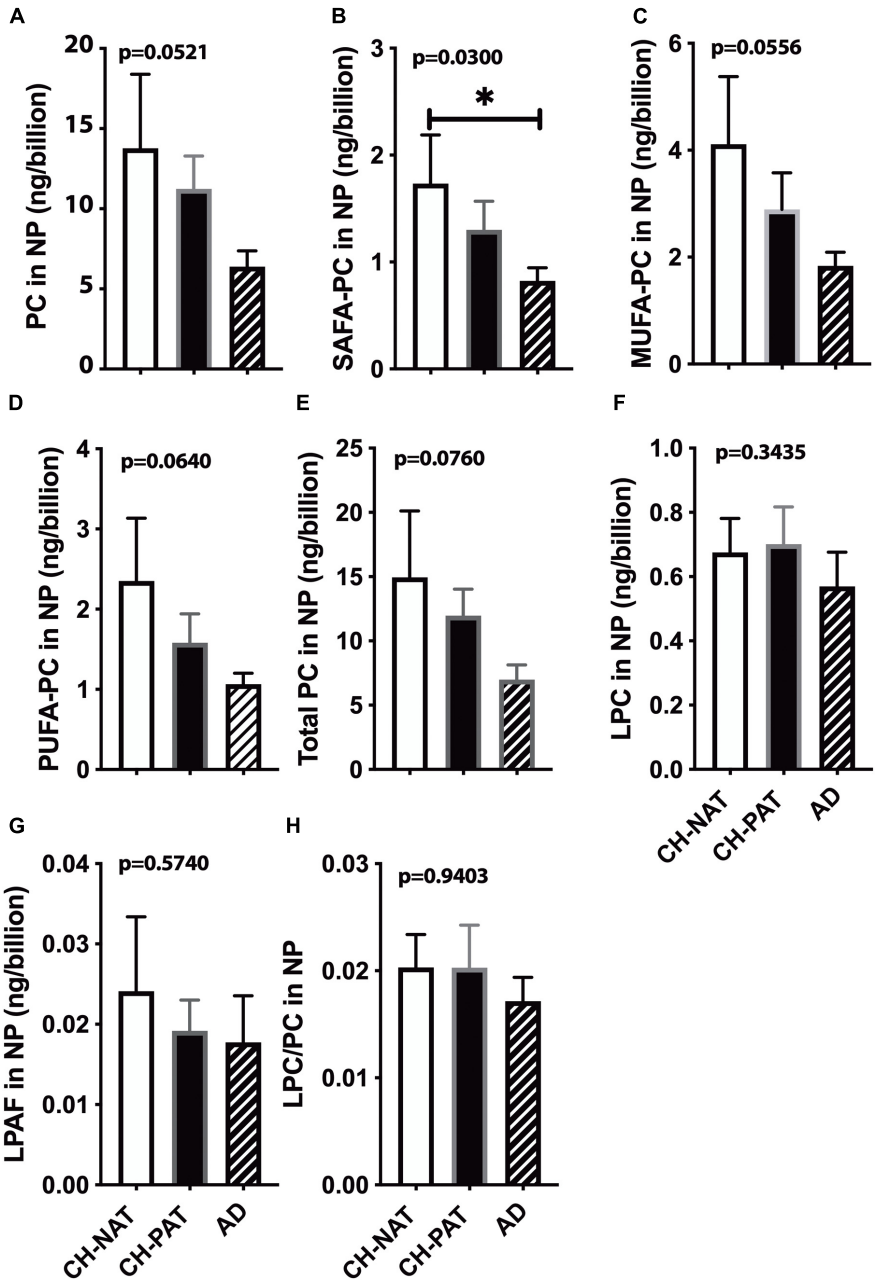

FIGURE 3 | Phosphatidylcholine (PC) levels normalized to the nanoparticles (NP) concentration in cerebrospinal fluid (CSF) – The levels of PC **(A)**, SAFA-containing PC **(B)**, MUFA-containing PC species **(C)**, PUFA-containing PC species **(D)**, LPC **(E)**, LPAF **(F)**, total PC lipids **(G)**, and the LPC to PC ratio **(H)** were normalized to the number of NPs (billions) in CSF. These data are the mean ± SEM, and group comparisons were made using One way ANOVA (Kruskal–Wallis test) combined with Dunn's multiple comparisons. The *p*-values are derived from One way ANOVA, and significant changes ($p < 0.05$) from Dunn's are indicated with an asterisk (*).

dhCM levels trend lower in the NP fraction of AD (**Figure 7F**). Total SP in the NP fraction is significantly higher in CH-PAT than in CH-NAT and AD (**Figure 7G**). The NP fraction's CM/SM ratio is significantly lower in CH-PAT than in CH-NAT (**Figure 7H**). **Figures 7I,J** are clustering results illustrated as a heatmap showing changes in 20 SP measures in the SF and NP fractions, respectively. Of the 20 SP measures in the SF fraction, seven are highest in CH-NAT, five are highest in AD, while the rest of the measures are highest in CH-PAT (**Figure 7I**). In the NP fraction, three SP measures are highest in CH-NAT, and 17 measures are highest in CH-PAT (**Figure 7J**).

SM Levels Normalized to the Number of NPs

To determine if membrane SP concentrations varied between groups, we normalized NP lipids levels to the number of

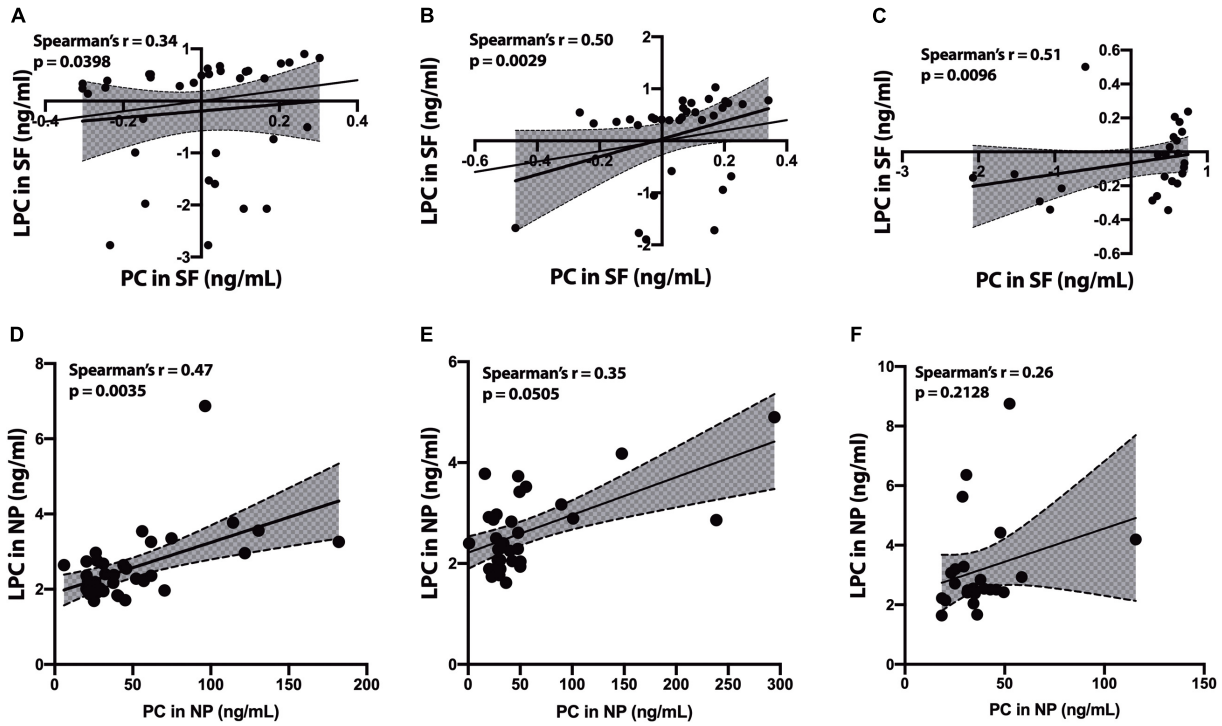

FIGURE 4 | Correlation of PC to lysophosphatidylcholine (LPC) in cerebrospinal fluid (CSF) fractions – We performed Spearman's correlation of PC to LPC in the SF fraction for CH-NAT **(A)**, CH-PAT **(B)**, and AD **(C)**. Similarly, we performed a correlation of PC to LPC in the nanoparticles (NP) fraction for CH-NAT **(D)**, CH-PAT **(E)**, and AD **(F)**. The data for the SF fractions was log 10 transformed to enable us to plot all the data on the same scale while the unnormalized data is shown for the NP correlation analyses. Spearman's rho (*r*) and the *p*-values are indicated for each correlation analysis.

particles. Normalized SM (**Figure 8A**), CM (**Figure 8B**), dhCM (**Figure 8C**) are significantly higher in CH-PAT than in AD. The SM and CM changes result in a significantly lower CM to SM ratio in CH-PAT than in AD (**Figure 8D**). Total SP per billion NPS is significantly higher in CH-PAT (**Figure 8E**).

Correlation of SM to CM

In the SF fraction, there is positive correlation between SM and CM in CH-NAT (**Figure 9A**, $r = 0.64$, $p < 0.0001$), CH-PAT (**Figure 9B**, $r = 0.64$, $p < 0.0001$), and in AD (**Figure 9C**, $r = 0.84$, $p < 0.0001$). In contrast to the SF fraction, SM does not correlates with CM in the NP fraction of CH-NAT (**Figure 9D**, $r = 0.11$, $p = 0.5247$), CH-PAT (**Figure 9E**, $r = 0.18$, $p = 0.3067$) and AD (**Figure 9F**, $r = 0.26$, $p = 0.2120$).

Summary of SP Data

No changes in SM molecular species are measured in SF for the CH subgroups and AD. In contrast, there is an increase in 17 of 20 SP measures in CH-PAT compared with CH-NAT and AD. These data show an increase in membrane-bound SM and its main molecular species in CH-PAT, indicating modification in SP metabolism in the CH group with CSF biomarkers of AD. Modifications in SP metabolism may typify increased lipid membrane particle formation and enhanced oxidative stress in CH-PAT attributed to the increase in dhCM (Idkowiak-Baldys et al., 2010; Munoz-Guardiola et al., 2020) that may precede the neuronal apoptosis in AD. Thus, measuring SP in CSF fractions can be crucial in monitoring early AD pathology or metabolic screening modifiers at the earlier stages of AD pathology even before clinical features are evident.

Increased PLA_2 Activity Contributes to GP Lipolysis in AD Dementia

Phospholipase A_2 hydrolyzes membrane lipids, and cerebrospinal PLA_2 activity increases in AD (Fonteh et al., 2013). Immunohistochemical studies associate PLA_2 also with AD plaques in human brain samples (Stephenson et al., 1996). Therefore, we measured PLA_2 activity in CSF to determine if higher lipolysis may account for the decrease in GP levels in AD fractions compared with the CH samples. Mean PLA_2 activity was similar in the CH-NAT and CH-PAT groups but significantly increased in AD (**Figure 10A**). These data suggest that PLA_2 activity does not contribute to GP metabolism in the absence of clinical signs of AD. However, the higher LPC and lower PC levels found in CSF supernatants and pellet fractions from AD participants may result at least partly from increased PLA_2 activity.

Correlation of Phospholipase A_2 Activity With Lysophopholipids

Lysophosphatidylcholine in NP did not correlate with PLA_2 activity for CH ($r = 0.24$, $p = 0.0685$) and AD ($r = -0.32$,

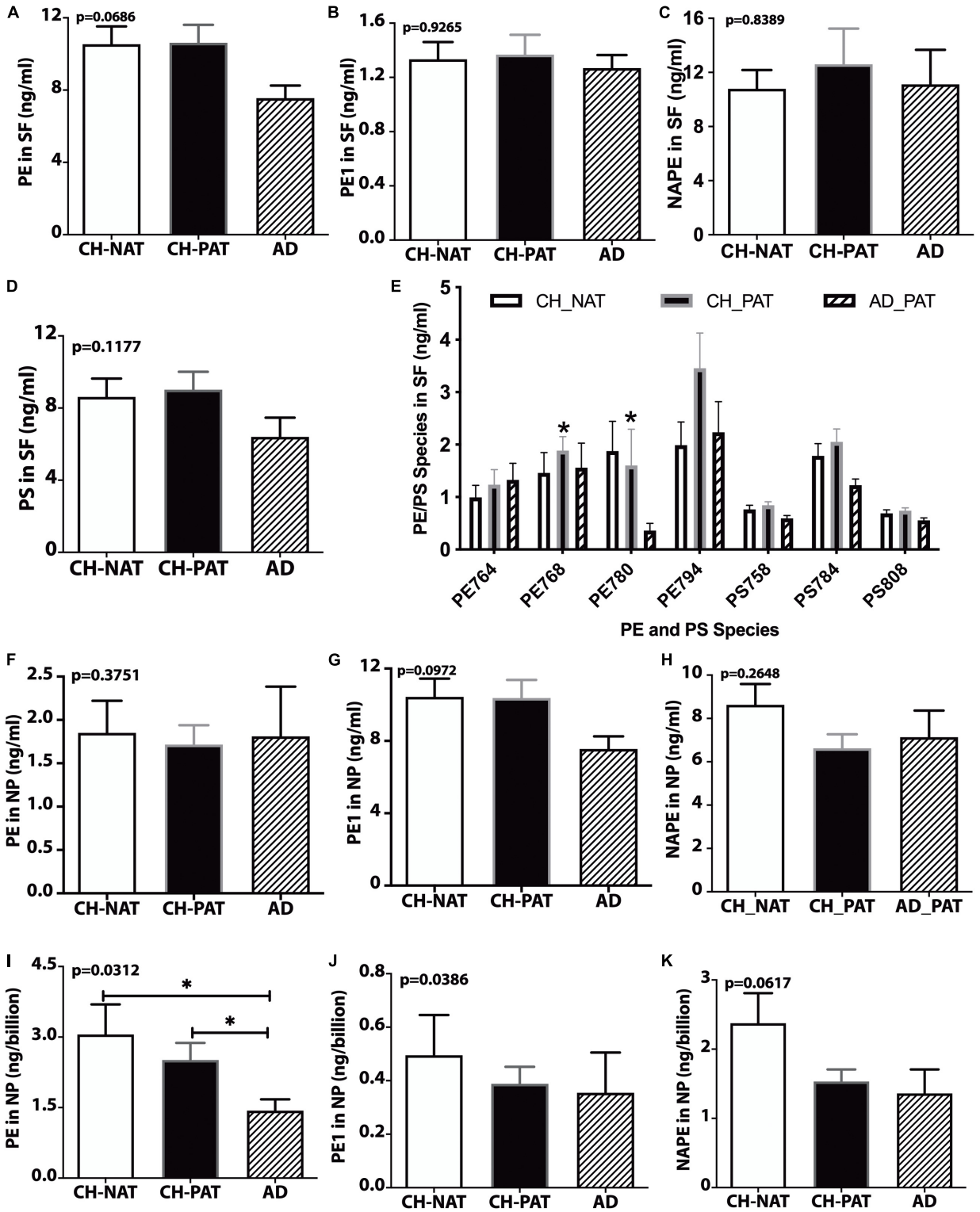

FIGURE 5 | PE, PS, and their molecular species in cerebrospinal fluid (CSF) fractions – PE **(A)**, PE1 **(B)**, NAPE **(C)**, PS **(D)**, and PE and PS **(E)** species in the SF fractions are plotted for CH-NAT, CH-PAT, and AD. PE species identified in CSF include PE38a:8 (PE764), PE38a:5 (PE768), PE40p:4 (P780), and PE40a:5 (PE794). PS species include PS34a:3 (PS758), PS36a:4 (PS784), and PS38a:6 (PS808). **(F–H)** show the levels of PE, PE1, and NAPE in the nanoparticles (NP) fractions of CSF of CH-NAT, CH-PAT, and AD. For these same glycerophospholipids, we normalized the levels of PE **(I)**, PE1 **(J)**, and NAPE **(K)** to the number of nanoparticles. These data are the mean ± SEM and significant changes in GP amounts ($p < 0.05$) are indicated with a symbol (*). Group comparisons were made using one-way ANOVA (p-values) and Dunn's multiple comparisons (asterisks).

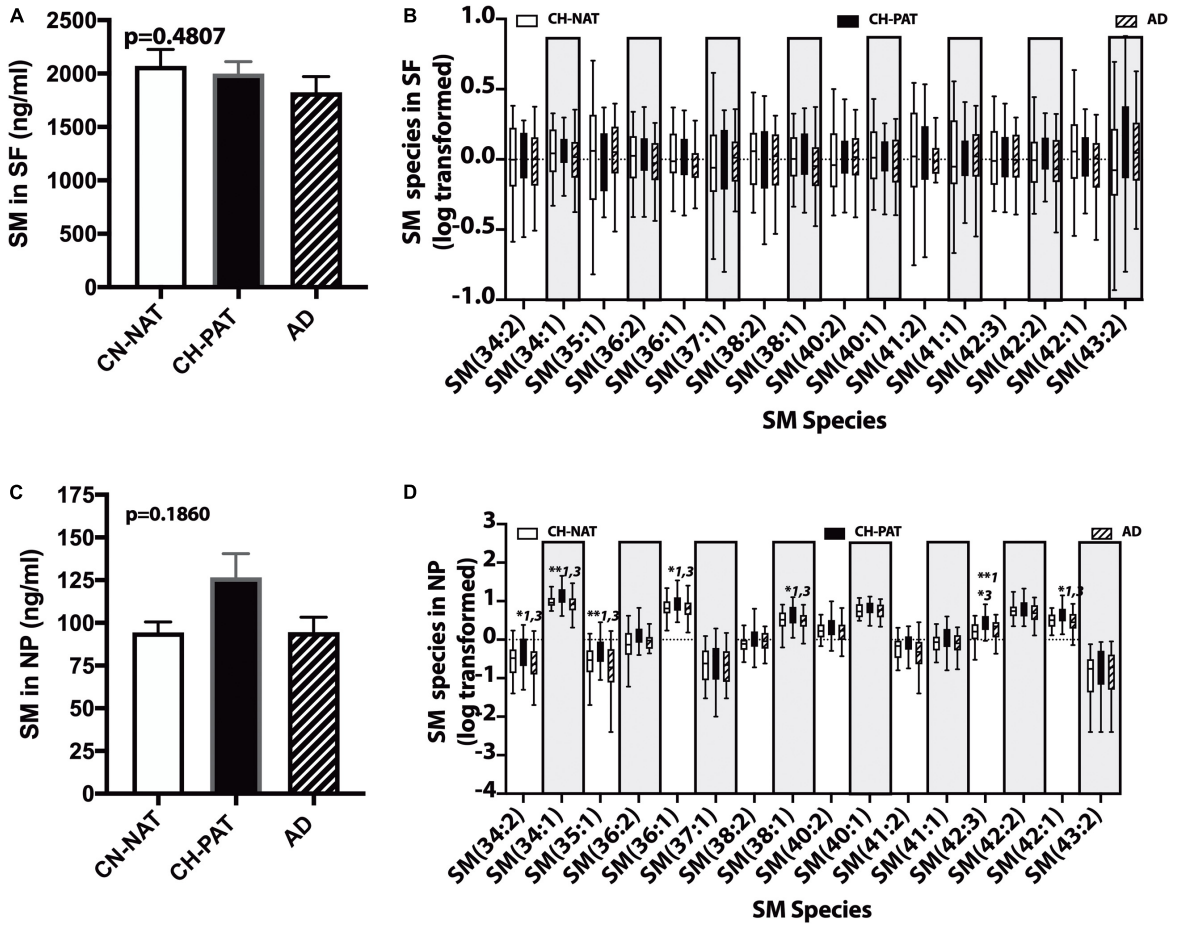

FIGURE 6 | Changes in cerebrospinal fluid (CSF) sphingomyelin in the CH-PAT group – SM **(A)** and SM species levels **(B)** in the SF fractions were determined for CH-NAT, CH-PAT, and AD samples. We also determined SM **(C)** and SM species **(D)** levels in the nanoparticles (NP) fraction. The untransformed data are plotted for SM (mean ± SEM), while the log 10 transformed data (Tukeys plot) is presented for SM species. Multiple comparison tests (Dunns) significance is indicated with an asterisk and a number for CH-NAT versus CH-PAT (*1), CH-NAT, and AD (*2), and CH-PAT and AD (*3).

$p = 0.2159$). LPC did not correlate with PLA_2 activity in the NP ($r = -0.09$, $p = 0.6495$) and SF ($r = 0.15$, $p = 0.4442$) fraction of CH-NAT participants. In contrast, LPC correlated with PLA_2 activity in the SF ($r = -0.35$, $p = 0.0495$) and NP ($r = 0.41$, $p = 0.0270$) fractions of CH-PAT. We detected LPAF and PAF in the SF but not in the NP fraction. LPAF significantly correlated with PLA_2 activity from CH ($r = 0.34$, $p = 0.0049$) but not AD ($r = -0.13$, $p = 0.6007$) participants. LPAF ($r = 0.44$, $p = 0.0090$) and PAF ($r = 0.37$, $p = 0.0379$) correlated with PLA_2 activity in CH-NAT but not CH-PAT and AD.

Sphingomyelinase Activity

Our studies have detected acid sphingomyelinase (aSMase) and neutral sphingomyelinase (nSMase) activities in CSF (Fonteh et al., 2015). Since the changes in SMase activities can influence SP metabolism and influence AD pathology (Jana and Pahan, 2010; Lee et al., 2014; Dinkins et al., 2016), we measured both aSMase and nSMase activities in CSF from CH clinical subgroups and AD. Compared with CH-NAT, aSMase activity decreased in CSF from CH-PAT (5.2%) and in AD (45.6%, $p < 0.05$, **Figure 10B**). nSMase activity was only slightly decreased in CH-PAT (−1.0%) and in AD (−12%, **Figure 10C**) compare with CH-NAT. The ratio of aSMase to nSMase was significantly higher in CH-NAT and CH-PAT than in AD (**Figure 10D**). Although we detected acid and neutral SMase in CSF and their putative product, CM, neither activity correlated with SP levels in the SF and NP fractions (data not shown).

Dietary Lipid Sources, Lipid Modulating Medications, and Lipid Transport in the Blood Do Not Explain the Lipid Changes in the Healthy, AD Biomarker Stage, or Dementia Stages

We surmise that the distinct changes in the SF and NP fractions' lipid composition between our clinical groups represent brain pathology differences. However, altered lipids in the CSF fractions might originate from their local source, from altered clearance or delivery from the systemic circulation, different

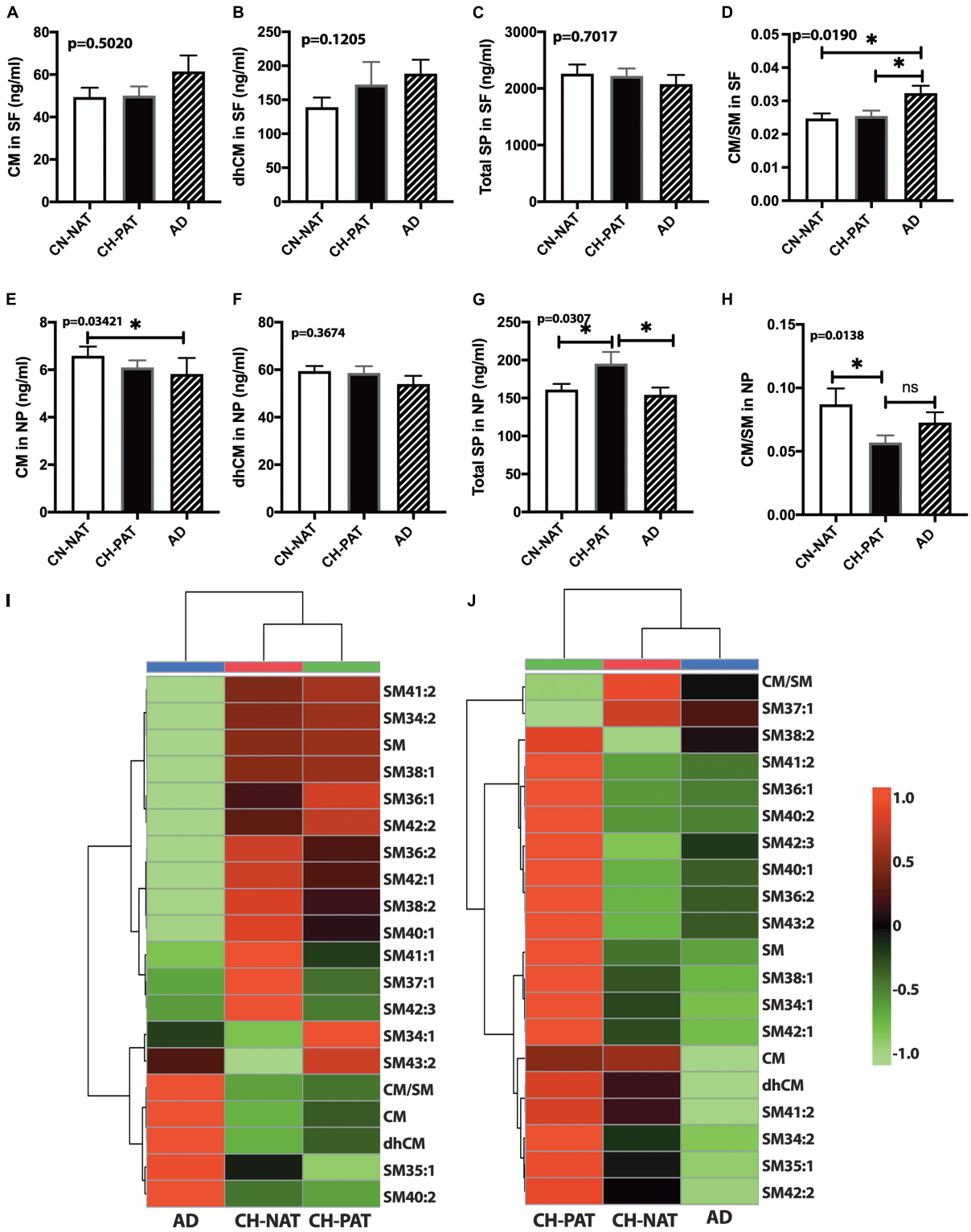

FIGURE 7 | Alteration in cerebrospinal fluid (CSF) sphingolipids (SPs) in the CH-PAT group – SP levels in the supernatant fluids (SF) and nanoparticles (NP) fractions were determined for CH-NAT, CH-PAT, and AD samples. CM **(A)**, dhCM **(B)**, total SP in the SF fraction **(C)**, and the CM/SM ratio **(D)** were determined in the SF fraction. In the NP fraction, we also quantified CM **(E)**, dhCM **(F)**, total SP **(G)**, and the ratio of CM to SM **(H)**. These data are the mean ± SEM and a significant change ($p < 0.05$) in SP amounts way ANOVA and Dunns multiple comparisons) are indicated with a symbol (*). The heatmaps show the clustering data of SP changes in the SF fraction **(I)** and the NP fraction **(J)**. The distance measures used Euclidean while the clustering algorithm used Ward on the Metabanalyst 4.0 platform.

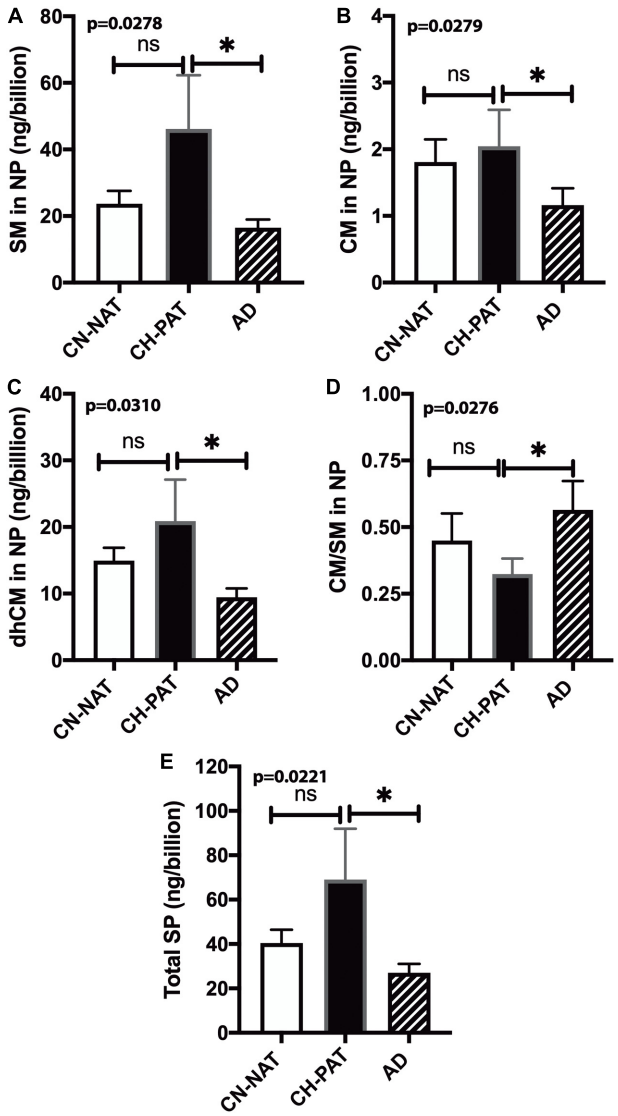

FIGURE 8 | Sphingomyelin levels normalized to the nanoparticles (NP) concentration in cerebrospinal fluid (CSF) – The levels of SM **(A)**, CM **(B)**, dhCM **(C)**, CM/SM ratio **(D)**, and total SP **(E)** in the NP fraction were normalized to the number of NPs (billions) in CSF. These data are the mean ± SEM, and group comparisons were made using One way ANOVA (*p*-values) combined with Dunn's multiple comparisons (asterisks). Significant changes ($p < 0.05$) are indicated with an asterisk (*).

diets, or lipid modulating medications. To investigate these possibilities, we measured the mean fasting blood levels of triglycerides, cholesterol, HDL, and LDL, assessed diets over the previous year, and compared the prescription, over-the-counter, and nutritional supplements taken by the participants. Neither medications nor measured blood lipids differ between the CH-NAT, CH-PAT, and AD groups (Harrington et al., 2013; Chew et al., 2020). The comprehensive dietary questionnaire was only analyzed with confidence in the CH sub-groups to avoid any bias from impaired memories in those with dementia; the dietary lipid intake did not differ between CH-NAT and CH-NAT participants (Fonteh et al., 2020). These results suggest that the changes mentioned above in GPs and SPs in biochemical and dementia stages likely result from the brain pathophysiology.

DISCUSSION

The structural and functional importance of lipids underscores their roles in several brain disorders, yet brain lipid composition and turnover in early AD pathology has not received much study. Moreover, despite substantial progress in defining genetic, clinical, cellular, and molecular components of the pathophysiology (Laske and Stellos, 2014; Kumar et al., 2015; Koch et al., 2018; Guo et al., 2020), our knowledge of how the different dementia risk factors associated with early AD pathology is also insufficient for diagnosis and effective treatments. These two shortcomings are connected because of the substantial loss of the lipid-rich brain tissue in AD starts many years before symptoms arise. We hypothesize that changes in lipid metabolism are an important component of the biomarker/non-clinical AD stage. To test this, we developed methods to study the composition of brain-derived lipids in the CSF to assess *in vivo* brain metabolism. 30–200 nm-sized particles that we described in CSF to have neurotransmitters, lipids, active enzymes, and signaling functions and are likely derived from brain cells via exocytosis and vesicle signaling (Harrington et al., 2009). We also reported that these nanoparticles are rich in the GP class of lipids, with compartmentalization between the SF and NP fractions of CSF (Fonteh et al., 2013, 2015, 2020). Here, we provide evidence to support this hypothesis by finding that GPs and SPs in both SF and NP fractions are higher in CH-PATs and lower in dementia. In the SF fraction, the most significant changes are in the PC species in the SF fraction (**Table 2**). In the NP fractions, most of the changes occur with SM species **Table 2**). Levels of two major lipid metabolizing enzymes are also altered in AD (**Table 2**).

We summarize lipid and enzyme changes in our clinical groups (**Table 3**). For CH-NAT versus CH-PAT, the most significant changes are the increases in SP species in the NP fraction. A lower ceramide to sphingomyelin ratio in the SF fraction and higher ceramide in the NP fraction characterizes CH-NAT versus AD (**Table 3**). In contrast, we measured higher phosphatidylcholine species and lower ceramide to sphingomyelin ratio in the SF fraction of CH-PAT compared with AD. In the NP fraction, total SPs and three sphingomyelin species are higher in CH-PAT than in AD (**Table 3**). Both enzyme activities (PLA_2 and SMase) are similar in the CH groups, but PLA_2 activity is higher and SMase lower in the AD group than the CH groups (**Table 3**). Thus, an overall dysregulation of $A\beta_{42}$, tau, GPs, SPs, and lipid enzymes may differentiate the AD biomarker stage from the clinically established dementia stage of AD.

Classification of Study Participants

The study participants who are CH with pathological CSF $A\beta_{42}$/tau have been recently confirmed to be at higher risk for a cognitive decline when we found 11/28 (40%, $p < 0.0001$) of this CH-PAT group declined cognitively with an AD pattern

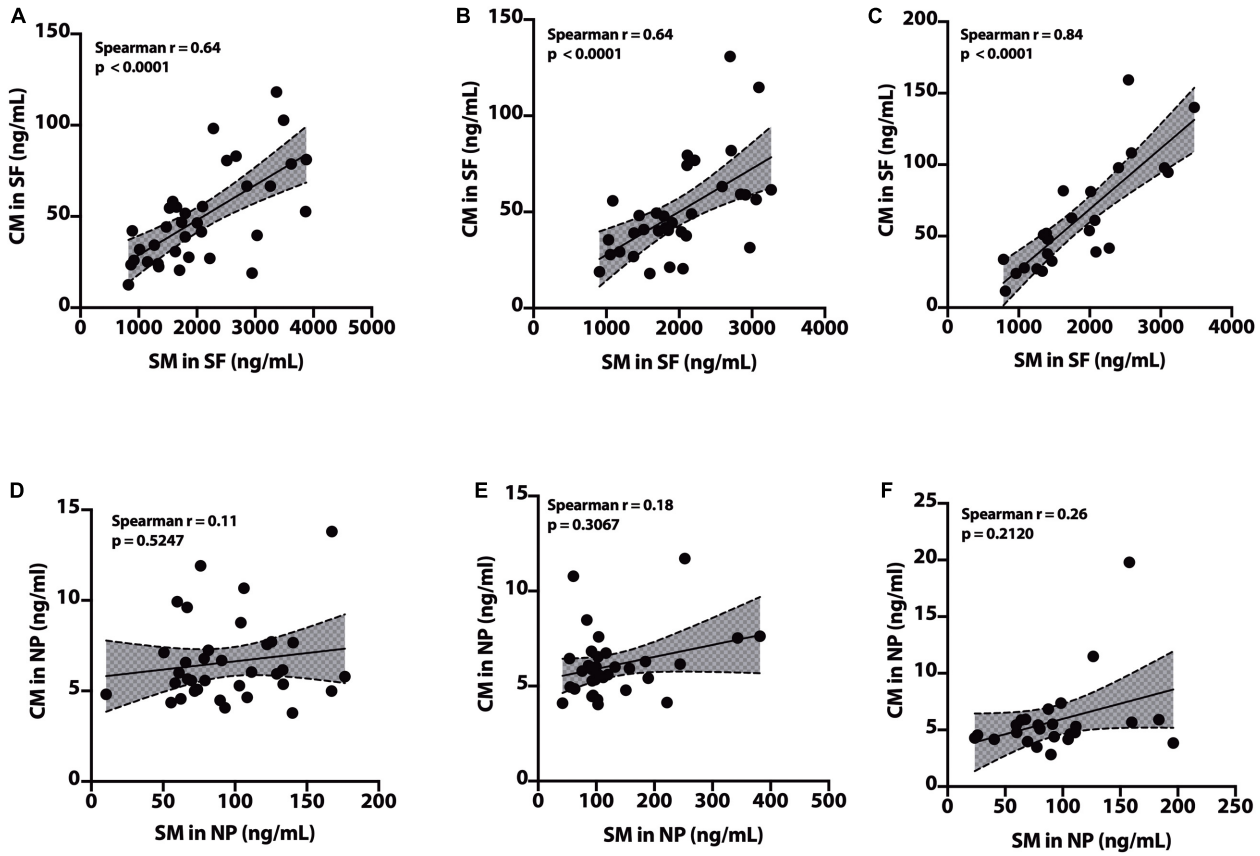

FIGURE 9 | Correlation of SM to CM in cerebrospinal fluid (CSF) fractions – We performed Spearman's correlation of SM to CM in the SF fraction for CH-NAT **(A)**, CH-PAT **(B)**, and AD **(C)**. Similarly, we performed a correlation of SM to CM in the nanoparticles (NP) fraction for CH-NAT **(D)**, CH-PAT **(E)**, and AD **(F)**. Spearman's rho (*r*) and the *p*-values are indicated for each correlation analysis.

after 4 years, while none of the CH-NAT group had measurable decline (Harrington et al., 2019). We have not studied the CSF of these subgroups after follow-up. However, this CH-PAT group is distinguished from the CH-NAT group by two measures: normal but reduced executive function at baseline (Harrington et al., 2013) and a higher risk for longitudinal decline (Harrington et al., 2019). The CH-PAT individuals are thus a subgroup that has developed CSF biomarkers of AD that precedes symptoms, and cognitive impairment occurs more rapidly in the CH-PAT than in the CH-PAT group.

GP Dysfunction in the CH-NAT Group

We expected to see lipid changes in neurodegenerative pathology. However, we were surprised to find the different lipid metabolic events at the AD biomarker stage of the CH group compared to the dementia stage. This expansion of GPs in the CSF fluid and membrane fractions reflects a higher turnover of membrane lipids; this may signify an increased propensity for forming inflammatory lipid mediators since the expansion of lipids in cells is consistent with the enhanced formation of inflammatory lipids (Luo et al., 2012; Mirmiran et al., 2014; van Dierendonck et al., 2020). A more dramatic increase in CSF GPs is found in traumatic brain injury, where survival depends on how fast CSF lipids return to normal after injury (Adibhatla and Hatcher, 2008; Pasvogel et al., 2008; Abdullah et al., 2014). A less subtle injury may be in play with CH-PAT study participants, where there is an increase in the release of brain lipids in the CSF. Over the many years of the AD biomarker stage in CH individuals, prolonged release of brain lipids will eventually deplete neuronal cells of vital molecules required for their structure and function. For example, a higher LPC to PC level changes the membrane composition and may indicate altered blood-brain barrier (BBB) transport since LPC species are effectively transported and metabolized across the BBB than unesterified fatty acids (Alberghina et al., 1994; Bernoud et al., 1999).

Other metabolic mechanisms that may lead to the lipid accumulation we report in CH-PAT include changes in uptake, biosynthesis, and lipid remodeling by CoA-dependent and CoA-independent acyltransferases (Chilton et al., 1996; Fonteh et al., 2001). Taken together, our data of the distinct changes in the CH-PAT group suggest that those at risk for AD (CH-PAT) are in a "pro-inflammatory," "pre-apoptotic" state.

SP Dysfunction in Preclinical AD

In addition to alterations in GP composition, we show an increase in SM and SM molecular species in CH-PAT compared with

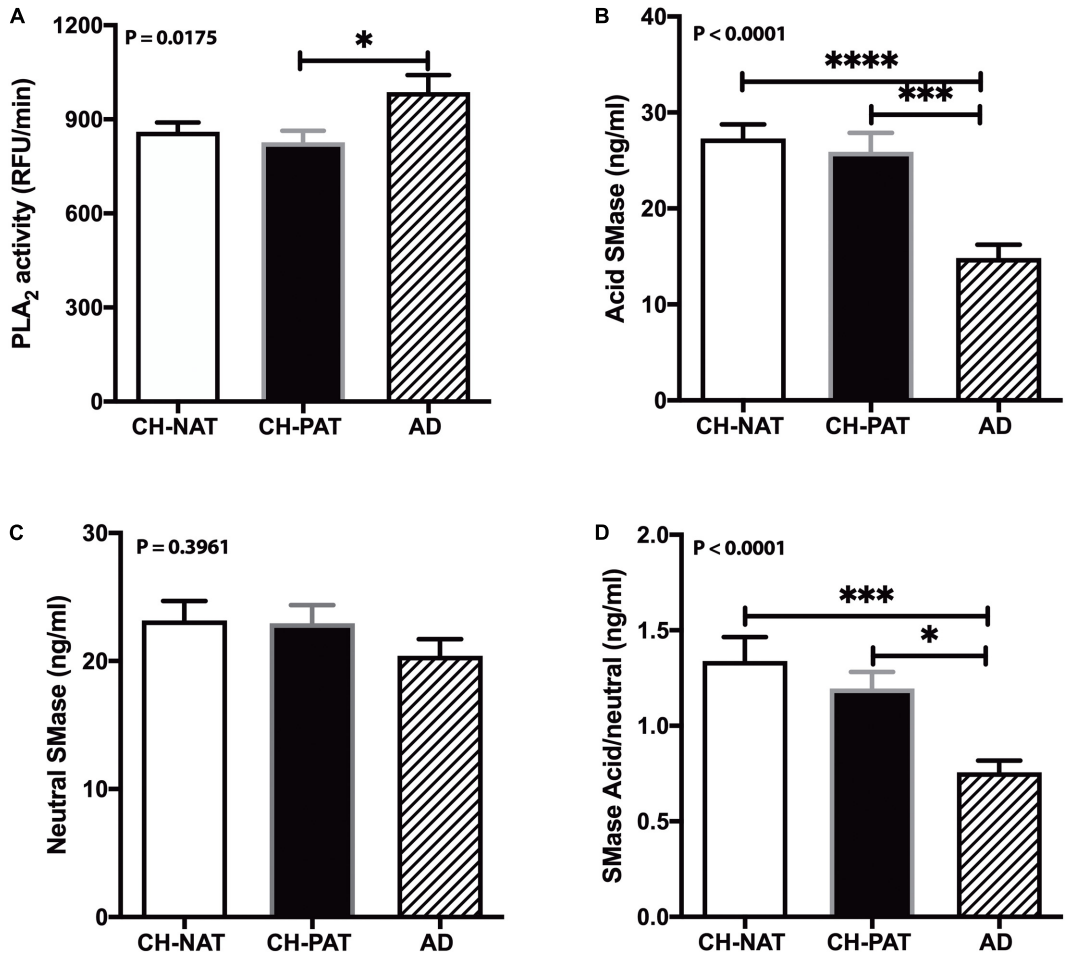

FIGURE 10 | Phospholipase A_2 (PLA_2) and SMase activity in cerebrospinal fluid (CSF) – PLA_2 activity in CSF was determined for CH-NAT, CH-PAT, and AD **(A)**. Similarly, aSMase **(B)** and nSMase **(C)** activities were measured in CSF using a coupled fluorescent assay. The ratio of the acid SMase to nSMase activity is shown in **(D)**. These data are the mean ± SEM, and group comparisons were made using One way ANOVA (*p*-values) combined with Dunn's multiple comparisons (asterisks). * indicate $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, **** $p < 0.0001$.

CH-NAT. Whereas the increase in PC is mainly in SF, SM's increase is higher in the NP fraction. These data show that SP metabolism changes can distinguish CH from AD. A subgroup of CH study participants with abnormal CSF proteins similar to AD can be distinguished from AD by their NP-specific increase in SM species.

Changes in Phospholipases in AD

Several metabolic events may account for the perturbation of CSF lipids in CH-PAT and AD (Chew et al., 2020). GP breakdown by enzymes can alter membrane properties and initiate the formation of inflammatory lipids. For example, PLA_2 has inflammatory properties and has been shown by immunohistochemical studies to co-localize with amyloid plaques in the brain of AD (Stephenson et al., 1996). We demonstrate that CSF PLA_2 activity is increased in AD, indicating a role for PLA_2 in the pathology. It is noteworthy that this increase in AD PLA_2 activity is not apparent in the CH-PAT stage, nor do the products of PLA_2 activity (LPC) appreciably change in the CH-PAT stage, which is characterized by the accumulation of lipids including PUFAs in the CSF fractions (Fonteh et al., 2020) and, by inference, in brain cells. The PUFAs are susceptible to oxidation with age that can generate pro-inflammatory lipid products in addition to altering the physical properties of neuronal membranes (Chew et al., 2020). Plasmalogen-specific PLA_2 that has been reported to play a role in AD (Farooqui and Horrocks, 1998) may account for the depletion of plasmalogen-PE (Senanayake and Goodenowe, 2019; Kling et al., 2020). Examining the major pools of plasmalogen species in CSF, we notice enhanced PE depletion. If PLA_2 activity is responsible for decreasing plasmalogen (Farooqui and Horrocks, 1998), then there should be an increase in the expression or activation of a plasmalogen-specific PLA_2 in CSF or within brain cells. Class-specific depletion of lipids coupled with the increase in PLA_2 product (LPC) in both SF and NP argues for the presence of different enzyme isoforms in CSF. Whereas the decrease in GPs dovetails with the increase in PLA_2 activity in CSF, there is a paradoxical increase in both PC and LPC in the SF fractions of all

TABLE 2 | Lipids and enzyme activities that change in cerebrospinal fluid (CSF) fractions from participants with normal $A\beta_{42}$/tau compared with pathological $A\beta_{42}$/tau and Alzheimer's disease (AD) participants.

Lipids in SF fraction			
Lipids	**CH-NAT (*n* = 35)**	**CH-PAT (*n* = 33)**	**AD (*n* = 25)**
PC[#3]	8787 ± 3433 (7607.7-9966.2)[a]	9688.8 ± 3254.4 (8534.9-10842.8)	8076.4 ± 4746.4 (6117.1-10036.6)
PC-SAFA[#3]	931.8 ± 452.6 (778.7-1085)	945.5 ± 345.1 (823.1-1068)	760.9 ± 452.0 (574.3-947.4)
PC-MUFA[#3]	2600.4 ± 1067.7 (2239.1-2961.7)	2915.6 ± 1007 (2558.5-3272.6)	2279.6 ± 1372.3 (1713.2-2846.1)
PC32a:0	382.2 ± 148.8 (331.0-433.3)	439.6 ± 173.0 (378.3-500.9)	340.8 ± 201.0 (257.8-423.8)
PC34a:1[#3]	2247.6 ± 887.1 (1943-2552)	2503.6 ± 873.1 (2194.1-2813.2)	1954.4 ± 1207.2 (1447.1-2443.7)
PC36a:1[#3]	274.6 ± 104.9 (238.6-310.6)	300.3 ± 100.7 (264.6-336.0)	239.9 ± 135.8 (183.9-296.0)
PC36a:0[#3]	53.4 ± 21.2 (46.1-60.7)	59.7 ± 25.3 (50.8-68.7)	48.8 ± 36.7 (33.6-63.9)
PC34p:0/34e:1	95.7 ± 49.5 (78.7-112.7)	88.3 ± 35.4 (75.7-100.8)	71.3 ± 48.0 (51.5-91.1)
CM/SM[#2,#3]	0.025 ± 0.001 (0.02-0.03)	0.026 ± 0.01 (0.02-0.03)	0.032 ± 0.011 (0.028-0.037)
Lipids in the NP fraction			
	CH-NAT (*n* = 35)[b]	**CH-PAT (*n* = 34)**	**AD (*n* = 25)**
PC32a:0	1.77 ± 1.07 (1.32-2.21)	3.0 ± 3.47 (1.75-4.35)	2.73 ± 2.69 (1.83-3.63)
CM[#2]	6.59 ± 2.29 (5.81-7.38)	6.10 ± 1.72 (5.49-6.71)	5.83 ± 3.35 (4.44-7.21)
Total SP[#1,#3]	161.1 ± 44.0 (132.2-184.9)	195.2 ± 87.8 (164.1-226.1)	154.3 ± 48.0 (134.5-174.2)
SM(34:2)[#3]	0.43 ± 0.35 (0.31-0.66)	0.71 ± 0.65 (0.45-0.93)	0.37 ± 0.43 (0.20-0.55)
SM(34:1)[#3]	10.83 ± 5.77 (8.85-12.81)	15.58 ± 10.31 (11.99-19.18)	9.63 ± 6.63 (6.89-12.36)
SM(35:1)[#1,#3]	0.44 ± 0.40 (0.30-0.57)	0.81 ± 0.67 (0.57-1.05)	0.39 ± 0.47 (0.19-0.59)
SM(36:2)[#1]	1.02 ± 0.82 (0.74-1.30)	1.68 ± 1.48 (1.16-2.19)	1.16 ± 1.10 (0.70-1.61)
SM(42:3)[#1]	1.77 ± 0.96 (1.44-2.10)	2.97 ± 2.04 (2.26-3.68)	2.16 ± 1.21 (1.66-2.66)
SM(42:1)	3.89 ± 2.30 (2.89-4.48)	4.88 ± 2.63 (3.96-5.80)	3.43 ± 2,04 (2.59-4.27)
CM/SM[#1]	0.09 ± 0.01 (0.06-0.11)	0.06 ± 0.03 (0.05-0.07)	0.07 ± 0.04 (0.06-0.09)
Enzyme activities			
	CH-NAT (*n* = 36)[b]	**CH-PAT (*n* = 33)**	**AD (*n* = 23)**
PLA_2 [#3]	860.5 ± 173.1 (800.1-920.9)	827.1 ± 202.2 (752.9-901.2)	986.7 ± 230.9 (871.9-1102)
aSMase[#2,#3]	27.2 ± 8.7 (24.4-30.2)	25.9 ± 11.4 (21.9-29.9)	14.8 ± 6.7 (11.9-17.7)
aSMase/nSMase[#2,#3]	1.3 ± 0.8 (1.0-1.4)	1.2 ± 0.5 (1.02-1.37)	0.8 ± 0.3 (0.6-0.9)

[a] *Lipid levels and enzyme activities are presented as the mean ± SD and 95% confidence intervals of the mean. We compared lipid levels or enzyme activities in CSF fractions from CH-NAT, CH-PAT, and AD using One way ANOVA (Kruskal–Wallis test) with multiple comparisons (Dunn's multiple comparisons). The above lipids and enzyme activities showed significant ($p < 0.05$) group differences.* [#1] *Indicates CH-NAT is different from CH-PAT,* [#2] *indicates CH-NAT is different from AD, and* [#3] *indicates CH-PAT is significantly different from AD using multiple comparison tests.* [b] *We did not detect lipids in samples with low recovery of nanoparticles (NPs), resulting in a smaller sample size for some lipid species in the NP fractions. For PLA_2, n = 34, 31, and 18 for CH-NAT, CH-PAT, and AD, respectively.*

three clinical states and mainly in the CH-NAT state for the NP fractions. These data suggest that lipolysis alone does not account for lipid changes in AD. The transport of lipids to a healthy brain, enhanced oxidation in a damaged brain, and increased lipolysis may contribute to lipid abnormalities in preclinical AD (Chew et al., 2020). Thus, the characterization of the different PLA_2 isoforms in different clinical states, determination of oxidative stress, and the modes of lipid transport into the brain will be fertile areas of future studies to identify mechanisms responsible for plasmalogen depletion in AD.

The Potential Role of Enzymes in SP Metabolism in the CH-PAT Stage

Examination of SP metabolic pathways suggests that any differences may occur at the synthesis level or via catabolic enzymes such as SMase. Some studies have shown increases in SPMase in AD, and inhibitors of SMase are proposed to have anti-inflammatory and beneficial effects in AD (Chen et al., 2012; Sala et al., 2020). SMase activity was analyzed to determine if the heightened activity was causing elevated CM levels. The assay of neutral SM showed no significant differences in activity among CH and AD. Although SMase activity is the most studied for CM formation, CM may also be derived from secondary pathways such as the breakdown of dhCM by DSE1, the formation of CM by CM synthase, or the breakdown of glucosylceramide by ceramidase. Also, both neutral and acidic SMase is implicated in AD while we have not delineated these activities in our CSF fractions. Acid SMase has recently been shown to influence vesicle formation in glial cells, and several SP metabolic enzymes are associated with Aβ toxicity. We do not expect to detect any of these enzyme changes in CSF if they occur in brain tissues. However, there is evidence in our studies that several of these enzymes are involved in CM formation.

The increase in dhCM can be imparted by enzyme changes also. Several studies show that enhanced oxidative stress can inhibit DSE resulting in the buildup of dhCM in cells (Idkowiak-Baldys et al., 2010; Munoz-Guardiola et al., 2020).

TABLE 3 | Summary of lipids and enzyme activities changes in cerebrospinal fluid (CSF) fractions.

Changes in the Supernatant Fluid Fraction		
CH-NAT/CH-PAT	**CH-NAT/AD**	**CH-PAT/AD**
	The ceramide to sphingomyelin ratio is significantly is lower in CH-NAT than in AD.	PC, PC-SAFA, PC-MUFA, PC34a:0, PC36a:0, and the PC36a:1 levels are higher in CH-PAT than in AD. The ceramide to sphingomyelin ratio is significantly is lower in CH-PAT than in AD.
Changes in the nanoparticle fraction		
Total sphingomyelin is lower in CH-NAT than in CH-PAT. SM(35:1), SM(36:2), and the SM(42:3) levels are lower in CH-NAT than in CH-PAT. The ceramide to sphingomyelin ratio is higher in CH-NAT than in CH-PAT.	Ceramide levels are higher in CH-NAT than in AD.	Total sphingomyelin is higher in CH-PAT than in AD. SM(34:2), SM(34:1), and SM(35:1) levels are higher in CH-PAT than in AD.
Changes in enzyme activities		
	Acid sphingomyelinase activity and the acid to neutral sphingomyelinase ratio are lower in AD than in CH-NAT.	Phospholipase A_2 activity is higher in AD than in CH-PAT. Acid sphingomyelinase activity and the acid to neutral sphingomyelinase ratio are lower in AD than in CH-PAT.

Such a process will account for the increase in dhCM in CH-PAT and AD, providing evidence of oxidative stress in preclinical AD. Finally, while the buildup of SM in NP may be due to changes in vesicle formation or "blebbing" during apoptosis of post-mitotic neurons, a decrease in SMase activity and/or an increase in SM synthase may also account for the increase in SM. A precedent for this is provided by the increase in cellular SM in brain diseases due to a defect in SMase activity. Of importance is the fact that such an increase in SM is associated with neuronal degeneration. Could the increase in SM in NP be due to defects in enzyme activities and represent the first signs of neuronal degeneration? We can only glean the answer to this question by characterizing the various enzyme activities in SP metabolism. Additional proteomic studies or genetic knockout studies will tease out the AD defect that accounts for the increase in CM, or in the CH-PAT stage for the increase in SM (NP) or dhCM (SF).

Implications of GP Changes in AD

What are the implications of altered phospholipid metabolism for the treatment of preclinical pathology and, eventually, AD? Approaches that may reduce abnormal GP metabolism in the CH-PAT stage may include dietary supplementation with omega fatty acids or antioxidants. Though PLA_2 is not activated in CH-PAT, PLA_2 activity is increased in AD; therefore, preventing its increase may slow the progression of symptomatic AD. PLA_2 inhibitors (Farooqui et al., 2006) may help slow the progression of AD in conjunction with modulators of the other known pathologies from $A\beta_{42}$ and oxidative stress. Increased oxidative stress in the presence of higher PUFA concentration in CH-PAT presents a unique environment for the formation of oxidized lipid products with inflammatory or neurotoxic properties. Abrogation of these early biochemical events has the potential for preventing neuronal cell death and thus preventing disease transition from the CH-PAT to the AD stage.

Regulating SP Metabolism in Preclinical AD –Changes in SP Metabolism in CH-PAT and AD

The major SPs we detect in CSF fractions are sphingomyelin (SM), ceramide (CM), and dihydroceramide (dhCM; Fonteh et al., 2015). SM is formed by SM synthase, dhCM is synthesized by dhCM synthase. CM can be formed from SM by one of three different SMase (neutral SMase, acid SMase, and alkSMase), *de novo* synthesis, or using sphingosine and a fatty reacylation. In the NP fraction from CH-PAT, there is an increase in SM and dhCM. The increase in SM may be accounted for by an increase in SM synthase and/or a decrease in SMase activities. Alternatively, defects in autophagogocytosis can result in the abnormal breakdown of membrane lipids with a subsequent increase of SM in NP.

Similarly, the increase in dhCM levels in NP from CH-PAT may be accounted for by increasing dhCM synthase and/or decreasing dhCM desaturase. In contrast to CH-PAT, there is an increase in CM in AD. An increase in CM may be due to an increase in the SMase activity or the other enzyme pathways that form CM via *de novo* synthesis or the Salvage pathway. The net effect of these enzymatic changes is the alteration in SP metabolism resulting in neuronal dysfunction. At the center of our data is the enhanced breakdown of SM in SF for AD shown by a significant increase in the CM/SM ratio. This increase accompanies an increase in SM and total SP in the preclinical samples (CH-PAT). These data suggest that compounds that alter SM breakdown can be useful in AD. An example is minocycline, an anti-inflammatory compound, an inhibitor of SMase activity reported to be a useful agent for controlling the progression of AD (Chen et al., 2012) . If an increase in apoptosis of neurons accounts for the increase in SM in NP, then agents that prevent this process can also be monitored. Known small molecule enhancers of autophagocytosis affect lipid signaling and have the potential beneficial effects on AD. Thus, our study identified a subset of CH subjects with abnormal amyloid/tau and skewed SM metabolism that may benefit from this type of intervention.

Study Limitations

Our study has significant implications for GP and SP regulation in aging. However, it is limited by the cross-sectional design and by the fact that there is no a *priori* study that we could use to design our study better. The clinical and CSF biomarker classification of our groups is based on neuropsychology and measures of CSF $A\beta_{42}$/tau, and we are still establishing an association of our classification with the conventional NIA/AA (ATN) trajectories (Tan et al., 2020). We did not control for

dietary intake of lipids and do not know how medications and other confounders (genetics, age, gender, race) may affect brain lipids. We attempt to mask any interference by these confounders by matching them in our clinical groups (Harrington et al., 2013; Fonteh et al., 2020). The preparation of the SF and NP fractions involves complex fractionation using differential centrifugation. The analyses of complex lipids involve manipulations that may introduce errors in our analyses. None the less, we have attempted to normalize our data using internal standards for the most accurate quantification of CSF lipids.

CONCLUSION

There is a pressing need for sensitive biomarkers for early diagnosis and monitoring treatments of AD. The CH-PAT and AD have similar $A\beta_{42}$/tau pathology; however, GP and SP alterations in the CSF can distinguish these groups. GPs and SPs differences also distinguish the CH-PAT from CH-NAT biomarker CH groups. Therefore, the measured phospholipid composition in CSF fractions not only indicates a mechanistic disturbance in the CH-PAT stage but can be combined with $A\beta_{42}$/Tau as a biomarker of early AD pathology. The increase in SM species in NP opens another exciting area of research to determine whether the CH-PAT cohort with increased SM in NP undergoes a faster transition to AD and test whether agents that enhance phagocytosis (Roig-Molina et al., 2020) may prevent modifications in SP metabolism. Finally, the increase in dhCM in CH with abnormal proteins points to oxidative stress in the CH-PAT group. It may be useful in monitoring the efficacy of antioxidants in preventing the onset of AD. Overall, our data reveal striking changes in GP and SP metabolism in a CH group distinguished with AD CSF biomarkers that can motivate the development of early detection methods and therapies to lessen lipid imbalances in the complex pathophysiology of AD.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Huntington Memorial Hospital, Pasadena. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AF and MH contributed to the conceptualization and study design, writing of original draft and manuscript preparation, project administration, resources, and funding acquisition. AF, AC, SE, and MH contributed to the methodology. AF contributed to validation and supervision and the formal data analyses. AC, MH, SE, and AF contributed to the data curation. XA, MH, and AF contributed to the expert manuscript review and editing. All authors contributed to the article and approved the submitted version.

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REVIEW
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Lipid Transport and Metabolism at the Blood-Brain Interface: Implications in Health and Disease

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Many prospective studies have shown that a diet enriched in omega-3 polyunsaturated fatty acids (n-3 PUFAs) can improve cognitive function during normal aging and prevent the development of neurocognitive diseases. However, researchers have not elucidated how n-3 PUFAs are transferred from the blood to the brain or how they relate to cognitive scores. Transport into and out of the central nervous system depends on two main sets of barriers: the blood-brain barrier (BBB) between peripheral blood and brain tissue and the blood-cerebrospinal fluid (CSF) barrier (BCSFB) between the blood and the CSF. In this review, the current knowledge of how lipids cross these barriers to reach the CNS is presented and discussed. Implications of these processes in health and disease, particularly during aging and neurodegenerative diseases, are also addressed. An assessment provided here is that the current knowledge of how lipids cross these barriers in humans is limited, which hence potentially restrains our capacity to intervene in and prevent neurodegenerative diseases.

Keywords: n-3 PUFA, blood-brain-barrier, blood-cerebrospinal fluid barrier, cholesterol, lipid transport

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INTRODUCTION

The brain is rich in lipids since it contains 24% phospholipids (PLs) and 22% cholesterol by dry matter (Svennerholm et al., 1997). However, the brain has a poor capacity to synthesize lipids which must be supplied from peripheral blood circulation and cross the barriers protecting the entrance of toxic molecules within the central nervous system (CNS) (Igarashi et al., 2007; Lacombe et al., 2018). The liver is the major site of lipids production where the long chain polyunsaturated fatty acid (PUFA) are synthetized. Alpha-linolenic acid (ALA) and linoleic (LA) are designated essential fatty acids because they cannot be synthesized *de novo* and must be obtained from the diet (Bourre, 2006). In the liver, the precursors ALA and LA acids are converted into docosahexaenoic acid (DHA) and arachidonic acid (ARA), respectively (Scott and Bazan, 1989), but this process is not efficient with less than 1% of ALA and LA converted (Plourde and Cunnane, 2007). In addition to long chain PUFA synthesized in the liver, shorter chains precursors such as ALA are provided by

the plasma. These PUFAs are additional lipid sources for the brain. Therefore, long-chain PUFAs must be supplied by the peripheral blood circulation and cross the barriers protecting against the entrance of toxic molecules into the central nervous system (CNS) (Igarashi et al., 2007; Lacombe et al., 2018). A certain level of ALA and LA are thought to be converted into DHA and ARA within the central nervous system (Spector, 2001; Qi et al., 2002). However, the efficiency of this process is unknown and is thought to be insufficient to fulfill the brain requirement for long-chain PUFAs.

The brain has three main blood-brain interfaces: the blood-brain barrier (BBB), the blood-cerebrospinal fluid barrier (BCSFB), and the blood-arachnoid barrier (BAB) (Strazielle and Ghersi-Egea, 2013). Although its role in CNS homeostasis is now well recognized (Holman et al., 2010; Dias et al., 2019), the BAB is avascular and has a smaller exchange area than the BBB and BCSFB (Gomez-Zepeda et al., 2020). Moreover, to our knowledge, the contribution of the BAB to lipid transport and metabolism is unclear and not very well documented. Therefore, this review will focus on the BBB, which is located at the endothelium of the brain microvessels, and the BCSFB, which is located at the epithelium of the choroid plexuses (CPs). The exchange of substances between the blood and the brain is regulated by various mechanisms, including the low permeability of the barrier to most substances and the selective transport of nutrients, ions, peptides, drugs, and hormones using transporters (Zlokovic, 2011; Banks, 2016; Galea and Perry, 2018). The BBB is not only composed of brain microvascular endothelial cells sealed together by tight junctions but can also be extended to the neurovascular unit, which is formed by the basement membrane and neighboring pericytes, astrocytes, neurons, and microglia (Iadecola, 2017). The CP is a tissue found in each brain ventricle and consists of a network of capillaries and epithelial cells. CP capillaries are fenestrated and highly permeable, while cerebral capillaries are highly impermeable. CP epithelial cells (CPECs) surrounding fenestrated capillaries filter water and other substances from the blood and transport them through the epithelial layer into brain ventricles (Lun et al., 2015b). This clear fluid produced by the CP is the cerebrospinal fluid (CSF), which fills the ventricles, cisterns and sulci of the brain as well as the central canal of the spinal cord. In humans, the CP of a young adult produces approximately 500 mL of CSF per day, but only 150 mL is present inside the brain and spinal cord as the CSF is constantly reabsorbed (Damkier et al., 2013). The CP is an evolutionarily conserved structure that is present from lower vertebrates to humans.

Tight junctions link CPECs together to form the CP epithelium, which represents the BCSFB. Although the BBB and BCSFB share the same overall structure, there are significant differences in their permeability. BBB junctions are extremely tight, as they protect the immediate neuronal environment, whereas BCSFB junctions are more permeable and allow the slow leakage of plasma proteins into the CSF. Although there are strong differences in permeability between the BCSFB and BBB, they are both vulnerable to pathophysiological modifications. For instance, defective cholesterol and fatty acid homeostasis in the brain is associated with age-related diseases, especially neurodegenerative diseases (Dietschy and Turley, 2001). Therefore, a defect in these processes will likely modify brain lipid homeostasis. Hence, because lipids are a major component of brain membranes, it is important to understand how they are transported from the blood to the CNS and how modifications in the BBB and BCSFB fatty acid profiles might change their permeability to lipids.

LIPID TRANSPORT ACROSS THE BLOOD-BRAIN BARRIER (BBB)

In mammals, brain neurons are highly enriched in cholesterol and in the long-chain PUFAs DHA and ARA. The next section will describe in more details how PUFAs and cholesterol cross the BBB, and a schematic representation of these passages is shown in **Figure 1**.

Transport of PUFAs Across the BBB

Passive Diffusion

For a long time, the passive diffusion of albumin-associated free fatty acids was thought to be the main form of lipid transport across the BBB (Dhopeshwarkar and Mead, 1973). Transfer via passive diffusion through the membranes of endothelial cells requires the dissociation of the non-esterified fatty acid from albumin and takes place in three steps that do not involve binding to proteins or receptors, namely, adsorption, transmembrane movement, and desorption (Kamp and Hamilton, 2006; Hamilton and Brunaldi, 2007; Lacombe et al., 2018). According to Strosznajder et al., 1996, the permeability of the BBB for an FA depends on three main factors: (1) the relative affinity of the FA for albumin circulating in the bloodstream, (2) the rate of dissociation between the FA and albumin, and (3) the metabolic flow and FA utilization by endothelial cells and nerve cells. Using FAs labeled with a radioactive isotope, these authors demonstrated that ARA diffuses more rapidly through the BBB than palmitic acid (16:0), which itself diffuses more rapidly than DHA (Alberghina et al., 1993, 1994; Strosznajder et al., 1996). The authors also suggested that the slower transport of DHA across the BBB than palmitic acid and ARA potentially limits the passage of DHA from the brain to the blood, hence contributing to the retention of DHA within the CNS. Hence, this mechanism might also participate in the selective retention of DHA within the membrane composition of nervous system cells. Interestingly, these studies also suggested that FA transport through the BBB is not affected during aging (Alberghina et al., 1993, 1994; Strosznajder et al., 1996). Other studies using *in situ* brain perfusion in which radioactive DHA was infused directly into one of the brain carotids reported that less than 10% of radioactive DHA remained in the endothelial cells of the brain vasculature, indicating that DHA crossed the BBB (Ouellet et al., 2009). The binding of DHA to albumin reduced the passage of DHA through the BBB. Since the brain transport coefficient was not saturable, it was suggested that DHA crosses the BBB by simple diffusion (Ouellet et al., 2009). These authors also showed that providing a long-term high DHA diet reduced the brain transport coefficient by 20%

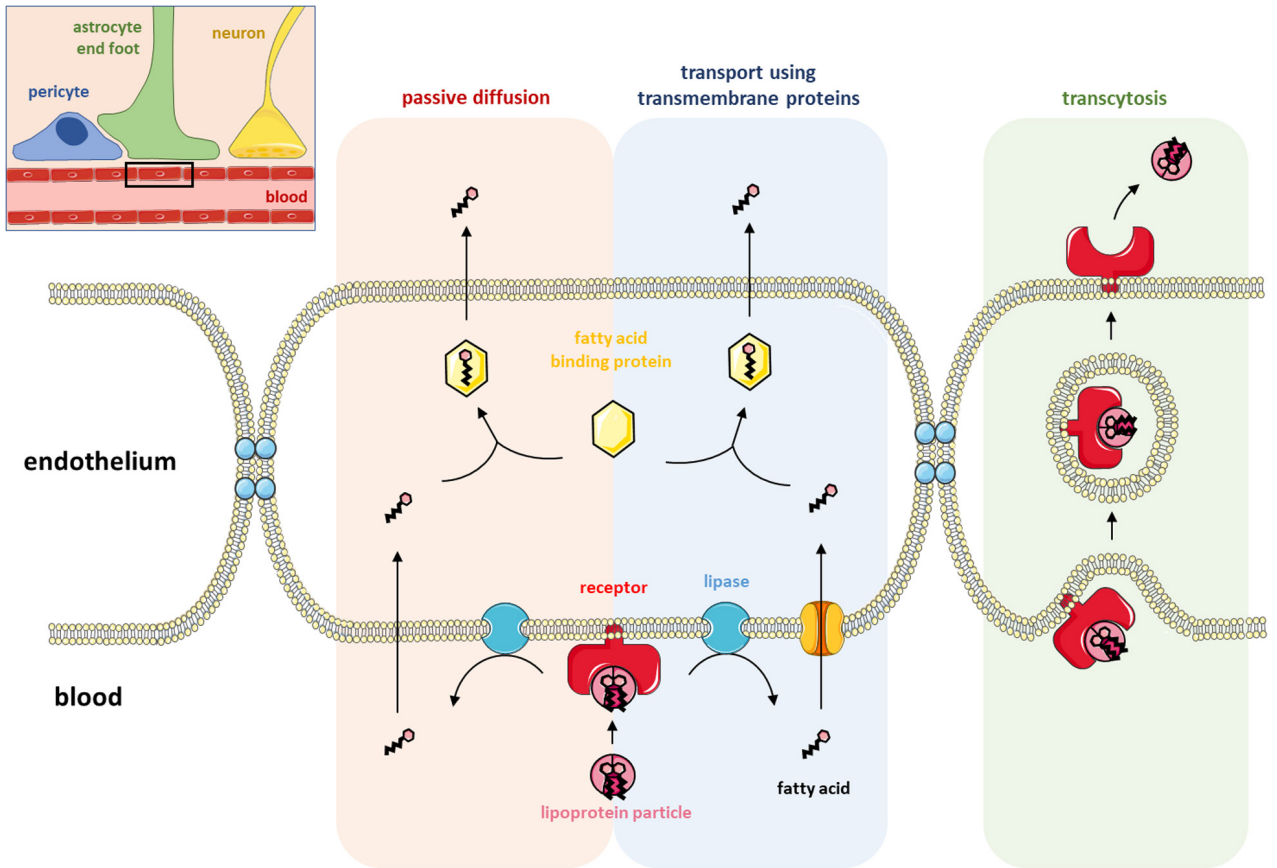

FIGURE 1 | Lipid transport at the blood-brain barrier. The top right corner shows a schematic representation of the blood-brain barrier (BBB) with the blood capillary, a pericyte, an astrocyte end-foot and a neuron. The rest of the figure (a magnified view of the black rectangle) illustrates the three ways lipids can cross the BBB: passive diffusion, transport using transmembrane proteins and transcytosis. Fatty acids (FAs) are locally produced in the endothelium after the hydrolysis of lipoproteins by membrane-associated lipase enzymes. FAs can either passively diffuse across the endothelial membrane or be transferred inside the endothelium by the activity of a transmembrane protein. Once in the endothelium of the BBB, FAs shuttle through the cytosol by binding to FA-binding proteins before being transported into the brain. Lipoproteins can also be directly transported into the brain via receptor-mediated transcytosis involving either caveolin- or clathrin-coated vesicles.

(Ouellet et al., 2009). Interestingly, the brain transport coefficient of eicosapentaenoic acid (EPA) was similar to that of DHA, even though brain membranes do not contain EPA.

Transcytosis

The second mode of transport of lipids across the BBB is the transcytosis pathway. Two mechanisms of transcytosis involving LDL receptors have been described: clathrin-dependent and caveolae-mediated endocytosis. Clathrin-dependent endocytosis is based on the existence of vesicles coated with clathrin. The assembly of this protein into a structure called a clathrin coat allows the formation of endocytosis pits (Smith et al., 1998). These structures, associated with adapter proteins, can recognize specific sequences of transmembrane proteins and internalize them. Clathrin-dependent endocytosis directs LDL to lysosomes that degrade them to release cholesterol and FAs, while receptors are recycled to the plasma membrane (Kirchhausen et al., 2005; Robinet et al., 2006). Caveolae are membrane vesicles whose formation depends directly on the presence of cholesterol within the membranes (the lipid composition of caveolae is similar to that of lipid rafts). These vesicles contain a transmembrane protein necessary for their formation, caveolin, which has the property of binding cholesterol (Rothberg et al., 1992; Yang et al., 2020). LDL receptors have been observed in these structures at the level of BBB endothelial cells (Dehouck et al., 1997). They can transfer LDLs across the plasma membrane, preventing their degradation. Coculture of endothelial cells with astrocytes showed that the expression of LDL receptors on the surface of endothelial cells was stimulated by the release of a soluble astrocytic factor (Dehouck et al., 1994, 1997).

Transport Across the BBB Requiring a Transport Protein

FAs can also cross the BBB when they are esterified to a glycerol backbone such as lysophosphatidylcholine (lysoPC), which has been shown to be the most efficient form of passage through the BBB (Brossard et al., 1997; Bernoud et al., 1998; Lacombe et al., 2018). In addition to the abovementioned mechanisms,

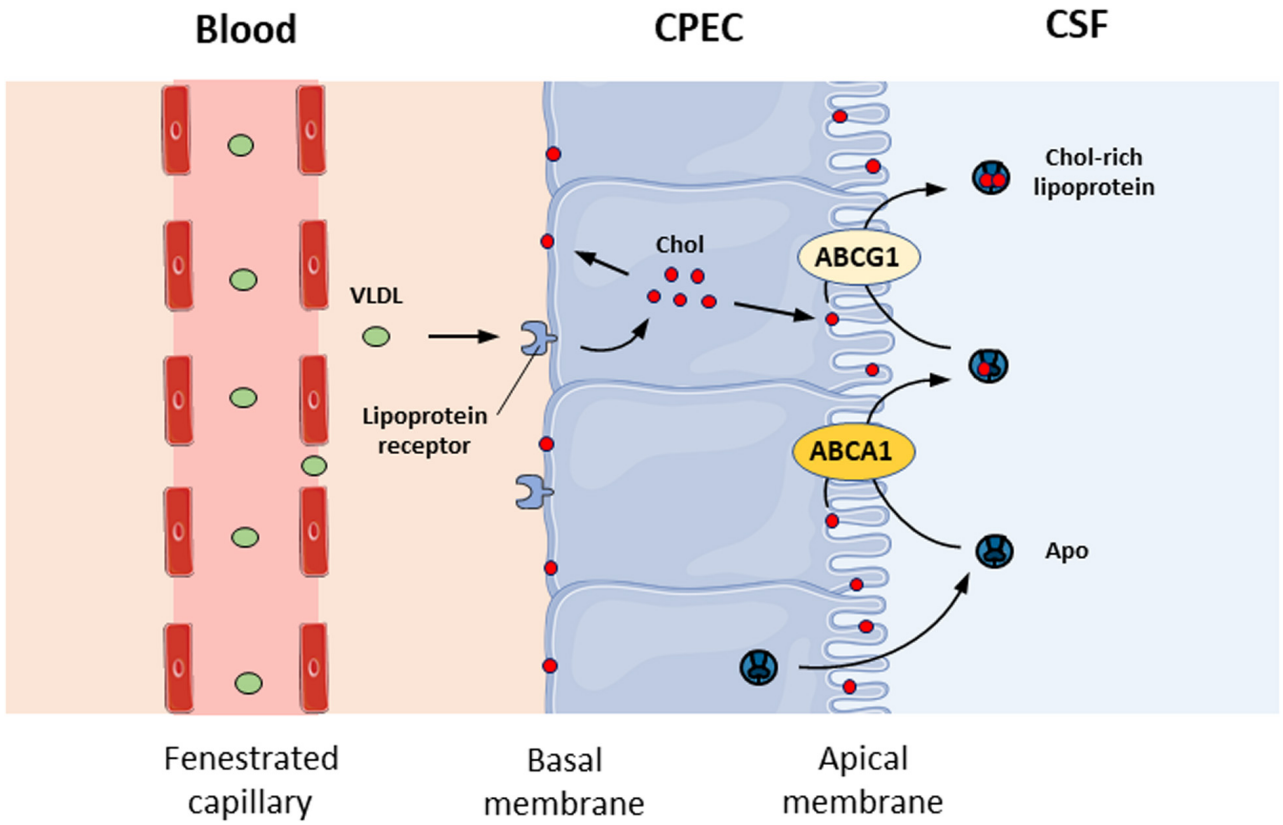

FIGURE 2 | Cholesterol transport at the blood-CSF barrier. Schematic representation of cholesterol transport from the circulating blood to the CSF across the blood-CSF barrier. CPEC, choroid plexus epithelial cell; CSF, cerebrospinal fluid; Chol, cholesterol; VLDL, very low-density lipoprotein; Apo, apolipoprotein.

Edmond (2001) proposed a model for the specific transport of PUFAs. In the model, lipoprotein receptors are located at the luminal membrane of endothelial cells and are thus not in direct contact with the brain parenchyma. Edmond hypothesized that lipoprotein receptors of the luminal membrane allow the internalization of lipoproteins into endothelial cells. The endothelial cells then process the lipids of the lipoproteins, releasing the PUFAs, which are then selectively transferred to their specific transporters at the abluminal membrane of the endothelial cells. There are a number of candidate transporters at the abluminal side of the membrane that include but are not limited to monocarboxylic acid transporters (MCTs) and/or FA transport proteins (FATPs). Because of the structural variety of PUFAs, there are different FATPs allowing selective transfer across the abluminal membrane (Edmond, 2001). More recent work demonstrated that FATP-1 and FATP-4 are the predominant FA transport proteins expressed at the BBB interface, while FA translocase/CD36 also appears to play a prominent role in transporting FAs across human brain endothelial cells (Mitchell et al., 2011). In 2014, the identification of a member of the major facilitator superfamily, Mfsd2a, as the major transporter for DHA uptake into the brain shifted our view of how PUFAs are transported across the BBB (Nguyen et al., 2014). Mfsd2a is expressed in the endothelium of the brain and transports DHA in the form of lysoPC or PC [see Lacombe et al. (2018) for extensive review]. Mfsd2a-knockout mice had markedly reduced levels of DHA in brain membranes, and this hallmark was accompanied by neuronal cell loss in the hippocampus and cerebellum as well as cognitive deficits, severe anxiety, and microcephaly, demonstrating the importance of that specific transporter in brain function (Nguyen et al., 2014; Zhou et al., 2019; Razmara et al., 2020; Wong and Silver, 2020). Mfsd2a deletion had major consequences on the development and proper functioning of the BBB (Ben-Zvi et al., 2014; Andreone et al., 2017). Interestingly, Mfsd2a also seems to be involved in the regulation of brain endothelial cell lipid composition, particularly in maintaining DHA levels, which may, in return, be unfavorable for caveolae formation and then contribute to the Mfsd2a-mediated regulation of transcytosis and barrier permeability (Andreone et al., 2017).

Transport of Cholesterol Across the BBB

Unlike PUFAs, cholesterol in the CNS is almost entirely synthesized within the brain since the BBB prevents any direct transfer of sterols from the blood to the brain, especially when they are contained in lipoprotein particles (Dietschy and Turley, 2001; Björkhem et al., 2004). However, to compensate for the steady-state synthesis of cholesterol within the brain, there is a specific brain clearance mechanism. Cerebral microvessels have a certain level of ATP binding cassette protein A1 (ABCA1),

a protein known to efflux cholesterol from the intracellular compartment to systemic and brain apolipoproteins. Do et al. (2011) reported that free cholesterol can be effluxed from the brain by crossing the BBB (Do et al., 2011). Therefore, the BBB might allow a certain level of free cholesterol to cross into the CNS, but one of the major limitations to this transfer is how cholesterol is transported within the blood, since the BBB seems to limit its uptake and more strongly favors its efflux from the CNS. Thus, the majority of cholesterol is directly synthesized in the brain parenchyma, mainly by glial cells and, to a lesser extent, by neurons (Mahley, 2016). Interestingly, dysfunction of or damage to the BBB led to altered cholesterol metabolism in the brain. Using a pericyte-deficient mouse model, Saeed et al. (2014) demonstrated that BBB disruption led to increased flux of cholesterol from the blood into the mouse brain and to a loss of 24(S)-hydroxycholesterol (the oxysterol regulating brain cholesterol synthesis) from the brain into the circulation, resulting in increased brain cholesterol synthesis.

In summary, a large proportion of lipids reach the CNS by crossing the BBB by using different strategies, including passive diffusion and specific and non-specific transporters. There is, however, another barrier where lipids can enter the CNS and reach the CSF, namely, the BCSFB, which is described in the following sections.

LIPID TRANSPORT ACROSS THE BLOOD-CSF BARRIER (BCSFB)

Transport of Fatty Acids Across the BCSFB

So far, most of the studies performed on the transfer of PUFAs from the blood to the brain were performed at the BBB level. However, it is an open question whether PUFAs can enter the brain by crossing through the BCSFB. A way to address this question is to directly inject radioactive FAs into animals and test the *in vivo* metabolic activity of the CP. One study involved stereotaxic injection of radioactive palmitic acid in the left ventricle of anesthetized rabbits (Marinetti et al., 1971). One to eight hours after injecting the tracer, the animals were sacrificed, and the left, right, third, and fourth CPs were removed to determine how much radioactivity was recovered in the TG and PL classes in each CP. They reported that in the TG class, the level of the tracer peaked at 2 h post injection in the left ventricle and at approximately 5 h post injection in the fourth ventricle (Marinetti et al., 1971). With respect to PLs, the tracer peaked 4 h post injection, and the relative radioactivity of the tracer was higher in left ventricle PLs than in TGs. This group also evaluated the *in vitro* metabolic potential of each CP. After dissection, each CP was incubated in Krebs-Ringer buffer with radioactive palmitic acid for 1 h, and afterward, the lipids were extracted (Marinetti et al., 1971). Contrary to the *in vivo* study described above, the tracer was more highly incorporated into TGs than PLs in the *in vitro* study (Marinetti et al., 1971). The incorporation of the tracer was different among the four CPs, suggesting that the metabolism of palmitic acid is not the same in each CP and that each CP may have its own metabolism. The authors also suggested that CPs may use lipids as a source of energy for their metabolic functions, which is unlike white and gray matter. This is supported by the much greater enzymatic esterification of palmitic acid into TG in the CP than in white and gray matter (Marinetti et al., 1971). These data are in line with another study suggesting that TG accumulated in the CP when the PNPLA2 gene encoding adipose TG lipase (ATGL), which catalyzes the rate-limiting reaction of lipolysis, was knocked down in mice (Etschmaier et al., 2011). Another group tested whether the CP has delta-6 desaturase activity, as delta-6 desaturase is the key enzyme regulating chain elongation and desaturation of LA and ALA. They incubated CP with radioactive LA and evaluated the presence of radioactivity in the different lipid classes of the CP (Bourre et al., 1997). Approximately 50% of the radioactivity was recovered in the CP PCs, 20% in cholesteryl esters and 10% in phosphatidylethanolamine. An additional 2–5% of the radioactivity was detected in other PLs and in non-esterified FAs (Bourre et al., 1997). Taken together, these results suggest that CPs are involved in CNS PUFA metabolism and that there is a need to better understand their roles in brain PUFA homeostasis.

Cholesterol Transport Through the BCSFB

Cholesterol is essential for the structure and function of the CNS. It is the major constituent of myelin and plays a key role in synaptogenesis and neurotransmitter release (Mauch et al., 2001). The transfer of cholesterol to apolipoproteins and lipoproteins within the CSF plays an important role in brain homeostasis. Several genes involved in cholesterol metabolism are regulated by liver X receptors (LXRs) (Schultz et al., 2000). LXRs act as cholesterol sensors and modify the expression of genes that regulate the transport, catabolism, and elimination of cholesterol, such as cholesterol ester transfer protein, lipoprotein lipase, and apolipoprotein E (APOE) (Edwards et al., 2002). The mRNA expression of LXRα and LXRβ has been detected in rat CP (Fujiyoshi et al., 2007), suggesting that they could be important for the BCSFB. This is supported by the fact that CPs are barely detectable in LXR$\alpha^{-/-}\beta^{-/-}$ mice because the size of lateral ventricles is greatly decreased, with little empty space left (Wang et al., 2002). The absence of LXRs in mice disrupts CNS lipid homeostasis since the accumulation of lipid droplets has also been observed within CPECs and around all four CPs of LXR$\alpha^{-/-}\beta^{-/-}$ mice (Wang et al., 2002), suggesting a defect in cholesterol transport through the BCSFB. At a mechanistic level, several receptors, such as lipoprotein receptor-related protein 1 (LRP1), LRP2, and apolipoprotein E receptor 2 (apoER2), are expressed at the basal membrane of the CP epithelium (Kounnas et al., 1994; Kim et al., 1996) and involved in the uptake of very low-density lipoprotein (VLDL) from the circulating blood (**Figure 2**). The mechanisms by which cholesterol in the cytoplasm is integrated into the apical membrane of the CP are not known. CPECs have the capability to transfer cholesterol in the apical membrane to apolipoproteins and lipoproteins in the CSF with the help of ABCA1 and ABCG1, two membrane-bound transporters of the ABC transporter family that are

expressed in the CP (Fujiyoshi et al., 2007). ABCA1 mediates the transfer of cholesterol but also PLs from cell membranes to either lipid-free or lipid-poor apolipoproteins such as apoA-I and apoE (Wang et al., 2000), while ABCG1 mediates cholesterol transfer to partially lipidated lipoproteins formed by the action of ABCA1 (Wang et al., 2004; **Figure 1**). Cholesterol release from CPECs occurs in an apical- and apoE isoform-dependent manner (Fujiyoshi et al., 2007).

The multiple functions of the CP include the release of biologically active molecules into the CSF by CPECs; these molecules are then distributed globally throughout the CNS (Lun et al., 2015a). This library of proteins consists of six major categories based on their biological functions, i.e., carrier proteins, matrix- or matrix-associated proteins, proteases, neurotrophic factors, and anti-inflammatory proteins (Thouvenot et al., 2006). It is possible that CPECs might coordinate cholesterol transport concomitantly with the release of apoE into the CSF. Interestingly, in ABCA1$^{-/-}$ mice, the levels of apoE in the brain were reduced by 80% (Hirsch-Reinshagen et al., 2004), while apoA-I levels were dramatically increased in both the brain and CSF (Karasinska et al., 2009). These observations suggest that ABCA1 at the apical side of the CPECs also regulates the release of Apo proteins by the CP. Together, these studies show that the BCSFB plays an important role in regulating the transport of cholesterol from the peripheral blood circulation into the CSF. However, it is important to note that we currently do not know whether other mechanisms, such as the passive diffusion of FAs and the transcytosis pathway described for the BBB, take place in the BCSFB.

HOW TO MODULATE FATTY ACID MEMBRANE COMPOSITION

At the BBB Level

BBB endothelial cells possess the metabolic capacity to synthesize ARA and DHA from their respective precursors (Moore, 2001). However, the synthesis of DHA from ALA or EPA, 20:5 is very limited. When ^{14}C-labeled 22:5 n-3, 24:5 n-3, and 24:6 n-3 were used in endothelial cells, these intermediates were metabolized into DHA. The level of DHA synthesis from 22:5 n-3 was less than 1%, whereas the synthesis rate was higher when using 24:5 n-3 and 24:6 n-3 as precursors (Moore, 2001). According to Moore, the limiting step in the synthesis of DHA in endothelial cells involves the elongation of 22:5 n-3 into 24:5 n-3 and the desaturation of 24:5 n-6 into 24:6 n-3, the latter of which is catalyzed by Δ6 desaturase (Moore, 2001). In addition, several *in vitro* studies suggested that when 22:5 n-3 was supplied, the newly synthesized DHA was preferentially released into the culture medium, whereas the newly synthesized ARA was preferentially incorporated into the endothelial cell membranes (Benistant et al., 1995; Delton-Vandenbroucke et al., 1997). Numerous authors have thus suggested that BBB endothelial cells participate in the enrichment of DHA in the brain by supplying DHA directly to neurons (Delton-Vandenbroucke et al., 1997; Moore, 2001). In a study using coculture of endothelial cells and astrocytes, it was shown that these two cell types cooperated to provide more DHA to neurons (Moore, 2001). Endothelial cells in coculture actively converted over 50% of ALA into EPA, while astrocytes converted this precursor and, more specifically, EPA into DHA. This group also showed that more than 80% of synthesized n-3 PUFAs are not incorporated into the membrane PLs but are secreted into the culture medium. This longer-chain PUFA synthetic route would also be valid for the conversion of 18:2 n-6 to ARA. Therefore, it seems that there is metabolic cooperation between endothelial cells of the BBB and astrocytes to synthesize DHA and ARA and transport them into neurons (Moore, 2001). These results corroborate an *in vivo* study evaluating the FA composition of BBB endothelial cell membranes. Indeed, data obtained from animals fed a standard diet indicated that the brain microvessels of rats have a particularly high content of ARA compared to DHA (Selivonchick and Roots, 1977). In another study with rats fed a standard diet providing both LA and ALA, freshly isolated brain endothelial cells had higher levels of ARA and lower levels of DHA than the cortex, as evaluated in three PL classes [phosphatidylcholine (PC), ethanolamine phosphoglycerolipid (EPG), and phosphatidylserine (PS)] (Pifferi et al., 2005). In the microvessel PC fraction, saturated FAs accounted for 56% of the total fatty acids, and the ARA content was 60% lower than that in the cortex; DHA represented only 1.6% of the total fatty acids in the endothelial cell PC fraction, whereas it accounted for 7.6% of the cortex PC fraction. The capillary EPG fraction was ∼5 times richer in ARA than in DHA, whereas the ratio of these FAs was the opposite in the cortical EPGs (9.7% ARA and 22.5% DHA). ARA accounted for ∼4.5% of the total FAs in the PS fractions of microvessels and the cortex, but the PS DHA content was 84% lower in the microvessels than in the cortex. Thus, we concluded that the ARA:DHA ratios in the three PL classes were opposite in the microvessels and the cortex, confirming the specific enrichment of the brain parenchyma in n-3 fatty acids (Pifferi et al., 2005).

At the BCSFB Level

To our knowledge, the first study reporting lipids in the CP was published by Helmy and Hack (1963). They evaluated the histochemistry and lipids of human CPs from individuals between 35 and 78 years of age. They performed thin-layer chromatography to separate the lipid classes but did not evaluate the FA profile; perhaps gas chromatography was not available at that time. Therefore, this study provided qualitative rather than quantitative data. They identified two phosphoinositides that seemed to be present in a greater relative proportion in the CP than in the CSF (Helmy and Hack, 1963). They also reported that unlike the CSF, the CP does not contain plasmalogens. Two decades later, Homayoun et al. (1988) reported the lipid and FA profile of CP. They performed FA profiling of the CP in rats fed for three generations with a diet with very low levels of ALA or with adequate levels of ALA. Regardless of the diet provided to the animals, Homayoun et al. (1988) reported that the CP was composed of 20% palmitic acid (16:0), 20% stearic acid (18:0), 10% oleic acid (18:1 n-9), and 25% ARA. When a diet low in n-3 PUFAs (low ALA, no DHA) was provided, CP had 25%

higher behenic acid (22:0) and 60% lower DHA levels than rats fed adequate n-3 PUFAs (Homayoun et al., 1988). The CP of mice provided an ALA-deficient diet had 18% higher saturated FAs, 13% higher monounsaturated fatty acids (MUFAs), and 31% lower PUFAs than that of mice provided an adequate diet (Homayoun et al., 1988). There are similar modifications in the FA profile of brain capillaries during aging (Tayarani et al., 1989). Between 2 months and 24 months, the relative percentage of MUFAs in the brain capillaries of rats doubled, while that of PUFAs was reduced by half, suggesting that aging partially reproduces some of the outcomes of an n-3 PUFA-deficient diet (Tayarani et al., 1989). Although not statistically significant, one interesting change observed in the paper of Homayoun et al. (1988) was that the relative percentage of ARA was 35% lower in mice given the n-3 PUFA deficient diet than in mice given the adequate n-3 PUFA diet. This result is interesting since the relative percentage of ARA usually remains stable following dietary modification. In the paper of Tayarani et al. (1989), ARA in brain capillaries was reduced by more than half in 24-month-old rats compared to 2-month-old rats. Overall, the study of Homayoun et al. (1988) showed that providing a diet with very low levels of ALA shifted the FA profile to higher saturated and MUFA and lower PUFA; this is unusual since in brain membranes, the relative percentage of PUFA remains stable with lower % in n-3 PUFA shifting to a higher relative percentage of omega-6. They then investigated how long it would take to return the CP FA profile to "normal" by shifting the diet of animals fed the deficient diet to the "normal" n-3 PUFA diet. They concluded that 46 days of dietary treatment were required to reverse the n-3 PUFA-deficient profile of CP (Homayoun et al., 1988). It is therefore anticipated that these FAs are mostly incorporated into the PL bilayer of endothelial cells. It was a surprise to us that few studies have investigated the PUFA composition of the CP given the important functions of the CP-CSF system in brain homeostasis.

IMPLICATIONS FOR BRAIN LIPID METABOLISM AND EFFECTS ON HEALTH AND DISEASE

Both the BBB and BCSFB undergo gradual modification during aging in which morphological and functional changes occur. Regarding the composition of brain lipids, between 20 and 100 years old, the brain will lose 42% in PL and 47% in cholesterol (Svennerholm et al., 1997). The effects of aging on the BBB have already been thoroughly documented [for review, see Mooradian (1988), Keaney and Campbell (2015), Erdö et al. (2017)]. These effects include (1) histological changes such as loss of capillary endothelial cells and decrease of their diameter, changes in endothelial cell morphology and decreased number of their mitochondria and (2) functional changes such as decreased BBB glucose and choline transport. Many studies have shown that diet and nutrition can improve cognitive functions during normal aging and can also prevent the development of Alzheimer's disease (Ngandu et al., 2015; Rosenberg et al., 2018). However, for decades, researchers have not elucidated the DHA blood-to-brain link and its relation to cognitive scores. A recent meta-analysis of 11 cohort studies reported that higher DHA levels in blood were associated with better cognitive function in 22,887 individuals (van der Lee et al., 2018). However, whether this link exists because there is more DHA in the participants' brain membranes remains unknown, largely due to the inaccessibility of brain samples and the quality of the samples collected at death to perform fatty acid profiling. We are aware of one study in humans that evaluated the link between plasma and postmortem brain DHA levels in non-cognitively impaired aged adults vs. cognitively impaired participants (Cunnane et al., 2012). The only significant correlation they obtained was between % DHA in plasma total lipids and % DHA in phosphatidylethanolamine of the angular gyrus, and this correlation was only observed in the non-cognitively impaired group. However, the brain requires a constant supply of DHA from the blood to replace metabolized DHA since astrocytic and neuronal synthesis of DHA from ALA is insufficient. Quantitatively, plasma non-esterified DHA (NE-DHA) is the main pool supplying the brain, whereas lyso-PC DHA is the most efficient, per dose, at targeting the brain (Chouinard-Watkins et al., 2018). Using *in situ* cerebral perfusion, brain DHA transport was studied in mice with targeted replacement knock-in of the human apolipoprotein E epsilon 4 allele (*hAPOE4*) since this is the main genetic risk factor for late onset Alzheimer's disease. The brain uptake rate in *hAPOE4* mice perfused with NE-^{14}C-DHA in the carotid was 24% lower than that in *hAPOE2* mice, in line with the reduced cortical DHA levels measured in *hAPOE4* mice during aging (Vandal et al., 2014). Conversely, in ∼35-year-old humans, a positron emission tomography study with [1-^{11}C]-DHA reported that the mean global gray matter incorporation of DHA in the brain of *APOE4* carriers was 16% *higher* than that in non-carriers (Yassine et al., 2017), and this difference was particularly pronounced in the entorhinal region, an area affected early in AD pathogenesis, whereas the rate in the hippocampus was independent of the *APOE* genotype. This result means that when NE-DHA is available in the blood in a compartment that the brain can take up, the brains of young *E4* carriers can take up DHA, but the lyso-PC DHA pool might also be important in this population. In the 3x-TG transgenic mouse model of Alzheimer's disease, the transport coefficient of DHA using *in situ* cerebral perfusion was 25% lower than that in non-transgenic littermates (Calon, 2011). Obviously, a reduction in the uptake of DHA through the BBB could lead to a deficit in DHA in the Alzheimer's brain over the long term. Interestingly, this mouse model is vulnerable to a DHA-deficient diet since brain DHA levels were significantly lower than those in non-transgenic littermates after chronic exposure to the same deficient DHA diet as the transgenic model (Calon, 2011). There are evidence suggesting that mice with *hAPOE4* and transgenic models of Alzheimer's disease might have issues in transferring plasma DHA to the brain membranes, and these issues might be accentuated during aging. Indeed, two studies reported a marked decrease in ARA in cerebral microvessels in aged rats compared with young rats (Tayarani et al., 1989; Bourre, 1991). Another group reported, however, that the FA composition of cerebral microvessels was unchanged between young and old rats, but they reported an increase in conjugated dienes, which could indicate increased free radical damage in old mice compared

to young mice (Mooradian and Smith, 1992). A decrease in PUFAs within brain microvessel membranes will likely modify membrane fluidity. One of the potential consequences arising from such membrane FA modifications is the induction of membrane rigidity, which will impair the ability of the membrane to move to expose the active sites of receptors used to transport molecules across the BBB. Another lipid important to the brain membrane is cholesterol.

For the BCSFB, CPECs undergo physiological changes during aging, such as decreases in size, volume and microvilli length (Serot et al., 2003). Epithelial cells lose approximately 20% of their height, and their morphological shape flattens along with the shortening of their microvilli (Serot et al., 2003). Furthermore, the stroma becomes thicker, as do the walls of the blood vessels (Balusu et al., 2016). In addition, deterioration of the tight junctions, which act as a physical barrier in the BCSFB, has been observed during aging, and such barrier atrophy leads to a increased molecular trafficking and efflux into the CSF and the brain parenchyma (Balusu et al., 2016). These morphological changes in the CP also modify CSF secretion during aging; however, the release of apoE, one of the most highly expressed and secreted lipid carriers, does not exhibit any changes during aging (Silva-Vargas et al., 2016).

To our knowledge, there is no published data on the lipid transport and metabolism at the BCSFB during diseases. In the later stages of life, the CP-CSF axis shows a decline in all aspects of its functions, including protein synthesis and CSF secretion and turnover, which may themselves increase the risk of developing late-life diseases, such as AD. Both BBB and BCSFB modifications occurring during aging worsen in those with neurodegenerative diseases like AD (Damkier et al., 2013; Erdö et al., 2017). For instance, in patients with Alzheimer's disease (AD), there is an additional loss to normal aging of 20% in PL in the frontal cortex (Svennerholm et al., 1997). In addition of the accumulation of beta-amyloid plaques and neurofibrillary tangles, one neurophysiopathological feature that has been underinvestigated by the research community is the presence of lipid droplets within the CNS. These hallmarks are also seen in non-AD patients, with a plateau at the age of 70 years (Marques et al., 2013), and correlate with observations made in aged mice (Sturrock, 1988). Although many efforts have been made to analyze and dissect the composition of CSF, it is important to emphasize that similar to plasma, CSF composition reflects the balance between the uptake and release of its metabolites between the CNS and peripheral circulation. Less effort has been made to investigate CP, the tissue that secretes CSF. Understanding the mechanisms that underlie the dysfunction of the CP-CSF system during aging will greatly advance the field. The brain utilizes a large amount of energy and requires tight regulation of nutrient intake and controlled management of the toxic molecules generated by its high catabolism. The regulation of brain input/output is closely tied to CSF turnover. CSF homeostasis is also important for maintaining proper brain structure and function. Given the importance of CSF turnover and composition, we believe that in-depth study of the human CP is now required.

GENERAL CONCLUSION, FUTURE DIRECTIONS

In this review, we outlined some of the specific factors involved in the transport of long-chain PUFAs from the blood into the CNS. We found that there are a limited number of studies in this field, especially those pertaining to the BCSFB We believe that advancing the field requires the use of an advanced lipidomic approach, especially when studying the CP, which is a very small tissue. For instance, determining the lipidomic profiles of microvessels and CPECs would improve our knowledge of the physiological modifications of these two cell types that are central to the BCSFB. Such profiling could be performed in different species, such as rats, mice, primates, and humans, to appreciate the similarities and the differences among species. Modifications among species could provide important information as to whether lipid changes are involved in brain homeostasis mechanistic pathways or in the prevention of neurocognitive diseases. Our group has already shown that the metabolism of peripheral plasma n-3 PUFAs is modified during aging, which hence modifies the amount of n-3 PUFAs available for uptake by the aging brain. There are therefore many gaps in this field that could help prevent neurocognitive diseases that occur during aging, and new technologies, such as liquid chromatography coupled with mass spectrometry, are now well-equipped to make progress in this important and understudied field.

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FP, BL, and MP wrote the manuscript, commented the manuscript, and contributed to improving the manuscript by providing comments. MP was responsible of the final version of the manuscript and its correspondence with the editorial board and the subsenquent comments this review may generate. All authors read the final version of the manuscript and agreed with its content.

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REVIEW
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Understanding the Exchange of Systemic HDL Particles Into the Brain and Vascular Cells Has Diagnostic and Therapeutic Implications for Neurodegenerative Diseases

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High-density lipoproteins (HDLs) are complex, heterogenous lipoprotein particles, consisting of a large family of apolipoproteins, formed in subspecies of distinct shapes, sizes, and functions and are synthesized in both the brain and the periphery. HDL apolipoproteins are important determinants of Alzheimer's disease (AD) pathology and vascular dementia, having both central and peripheral effects on brain amyloid-beta (Aβ) accumulation and vascular functions, however, the extent to which HDL particles (HLD-P) can exchange their protein and lipid components between the central nervous system (CNS) and the systemic circulation remains unclear. In this review, we delineate how HDL's structure and composition enable exchange between the brain, cerebrospinal fluid (CSF) compartment, and vascular cells that ultimately affect brain amyloid metabolism and atherosclerosis. Accordingly, we then elucidate how modifications of HDL-P have diagnostic and therapeutic potential for brain vascular and neurodegenerative diseases.

Keywords: HDL, Alzheimer's disease, APOE, ApoA-I, vascuar, imaging

INTRODUCTION

High-density lipoprotein (HDL) particles (HDL-P) are found in the peripheral circulation as well as in the central nervous system (CNS), where they protect against disease states through a variety of physiological functions. HDL-P gain access to both parenchymal and vascular cells based on their lipid and protein composition. This allows for various degrees of exchange between blood, lymph, cerebrospinal fluid (CSF), and interstitial brain fluid compartments that is largely determined by their apolipoprotein (apo) content. Notable dissimilarities in structure and apo content distinguish periphery HDL-P from CNS HDL-P. This is likely a consequence of limited crossover between these two compartments (Mahley, 2016), however, these mechanisms have not been fully elucidated. It is pertinent to explore this gap in the literature as the manipulation of HDL-P has become

a research focal point in recent years for their potential use as therapeutic and imaging agents, especially in the CNS.

The blood–brain barrier (BBB) and the blood–CSF barrier (BCSFB) compartmentalize CNS lipoprotein/apolipoprotein synthesis and metabolism separately from the periphery. HDL-P are secreted by the liver and small intestine (Timmins et al., 2005; Brunham et al., 2006), whereas CNS-derived HDL-P are generated by glial cells (Fagan et al., 1999; Vitali et al., 2014). The BBB, formed by multiple cell types, including the tightly knit endothelial cells within brain microvessels, is the greatest barrier to HDL exchange. The BCSFB, in contrast, is comprised of choroid plexus (CP) epithelial cells (McPherson et al., 2007a) that are more permeable than the BBB. This is because the junctions formed by the CP (McPherson et al., 2007a) epithelium allow for some transport of plasma proteins into the CSF (Johanson et al., 2011). In addition to these structural distinctions, the expression of transporters at the BBB and BCSFB are not the same, and the differences between their endocytic and transcytotic pathways are not clear (Strazielle and Ghersi-Egea, 2016).

While the structural, chemical, and functional properties of plasma HDL-P have been extensively studied, the properties of CSF HDL-P remain elusive due to their low abundance and high complexity (Montine et al., 1998; Yamauchi et al., 1999; Demeester et al., 2000; Koch et al., 2001; Yassine et al., 2016). Methods using gradient gel electrophoresis (Remaley et al., 2001), ion-mobility analysis (IMA), and nuclear magnetic resonance (NMR) spectroscopy have characterized three major groups of HDL-P in plasma by particle size: small (7–8.5 nm), medium (8.5–10.5 nm), and large (10.5–15 nm) (Nichols et al., 1986; Otvos et al., 1992; Jeyarajah et al., 2006; Caulfield et al., 2008). Proteomic analysis of plasma HDL isolated by density ultracentrifugation and size exclusion chromatography has identified an extensive list of over 90 proteins associated with HDL (Gordon et al., 2010; Holzer et al., 2016). In both plasma and CSF, small HDL-P are comprised of apoA-I, apoA-II, apoA-IV, apoC-I/II/III, apoD, transferrin, and other proteins, whereas apoE and apoJ are found on both smaller and larger HDL-P. Also, in both compartments, HDL-P can contain single or multiple apolipoproteins (apos) (Davidson et al., 2009) that affect their structure and function. What mainly distinguishes plasma HDL-P from CSF HDL-P is that they are enriched with apoA-I (von Zychlinski et al., 2014), while CNS HDL-P are primarily comprised of apoE (Koch et al., 2001). ApoA-I is not synthesized in the CNS, and the CSF apoA-I concentration is only 0.3% that of plasma. Furthermore, CNS-derived apoE has not been shown to cross into the periphery (Koch et al., 2017). Nevertheless, there is evidence that apoA-I is protective against CNS disease (Kawano et al., 1995; Mangaraj et al., 2016), and CNS-derived apoE has an important role in mediating amyloid-beta (Aβ) clearance (Kanekiyo et al., 2014). More broadly, enhancement of endothelial transcytosis via HDL-surface modifications has been explored as a potential drug delivery strategy to the brain (Balazs et al., 2004). Therefore, the ability to support HDL-apoA-I and/or HDL-apoE transport across the BBB may provide significant therapeutic breakthroughs in neurodegenerative diseases. This will require a deep understanding of how the BBB and BCSFB mediate the exchange of HDL-P and their components.

Not only do apos play an important role in HDL transport and component exchange, they are also involved in acute-phase response, proteolysis, immunity, Aβ clearance, and vasoprotective roles (Chait et al., 2005; Getz, 2005; Vaisar et al., 2007). Specifically, HDL-P and their components play important, protective roles against both Alzheimer's disease (AD) and vascular dementia (VD) risk through mechanisms related to atherosclerosis, cerebral amyloid angiopathy (CAA), and inflammation (Gearing et al., 1995). However, it is not clear whether these neuroprotective properties are mediated by CNS-derived apos, through peripheral apos entering the CNS from the periphery, or via both (Remaley et al., 2001). Understanding the neuroprotective properties of apos and how they are exchanged between the periphery and CNS is crucial to understanding how HDL-P can be modified to facilitate brain delivery. In this review, we explore the known structural and functional properties of HDL-P that enable access to the brain and vascular cells, as well as their neuroprotective and vasoprotective properties. We also present evidence to support the exchange of small, lipid-poor HDL-P between the CSF and plasma compartments across the BCSFB and acknowledge that the evidence for subsequent BBB exchange is weak, and therefore requires more careful, elaborate investigations. To help bridge this gap, we present evidence that HDL-P can be modified to facilitate transport across the BBB and for imaging atherosclerosis in vessel walls.

Mechanisms of Lipoprotein Exchange Between the Periphery and the CNS

Transport via the BBB

The BBB is formed by multiple cell types including endothelial cells, pericytes, smooth muscle cells that shields the brain from the periphery. The highly selective nature of the BBB is primarily orchestrated by receptors, which help regulate blood–CNS exchange and maintain CNS homeostasis. This results in a minimal exchange between systemic and CNS-derived HDL-P; however, small HDL and/or their components are suspected to traverse the BBB (Ladu et al., 2000; Koch et al., 2001; Wang and Eckel, 2014). **Figure 1** and **Table 1** show the relevant ligands and receptors of interest thought to play a role in the exchange of HDL across the BBB. ApoA-I have been shown in a more folded comformational state around small and lipid-poor HDL, giving them a discoidal form in lieu of spherically larger shape typlical of lipid-rich HDL-P. These discoildal forms were able to cross the BBB *in vitro* (Dal Magro et al., 2019), and although Martin-Nizard et al. (1989) have observed that radiolabeled HDL_3 (small HDL, d = 1.125–1.210 g/mL) can bind the luminal membrane of cultured bovine brain capillary endothelial cells with high affinity, it remains unclear which receptors were responsible for this interaction. Furthermore, whether fully intact HDL-P do cross the BBB *in vivo* remains a point of contention. HDL are suspected to transverse the BBB via transcytosis—a process by which the HDL are internalized at the luminal surface by the endothelium, then trafficked to the basal membrane (Mehta and Malik, 2006). Some of the receptors of interest that express affinity for some relevant HDL-associated apos belong to the low-density lipoprotein (LDL)

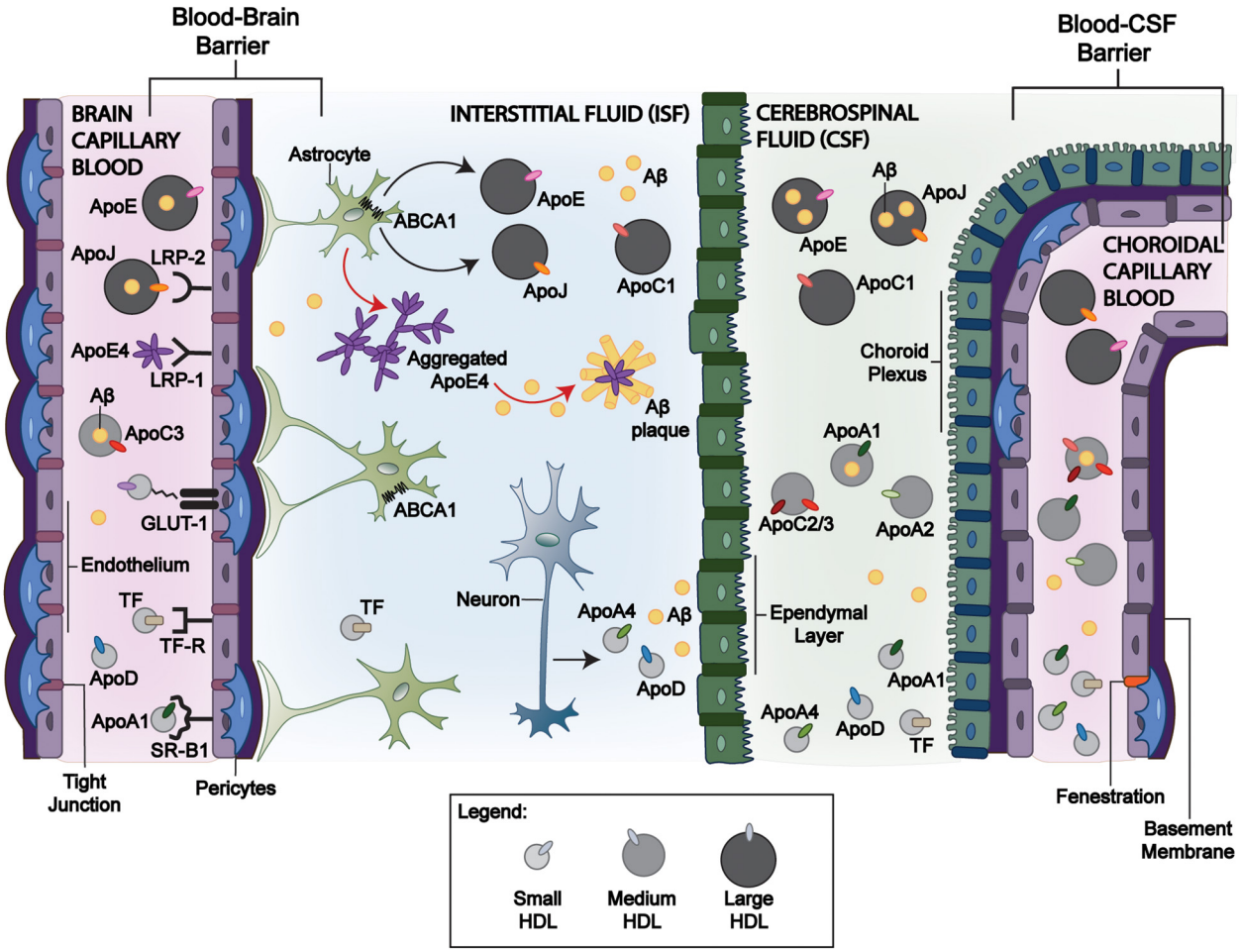

FIGURE 1 | Mechanisms of HDL-P transport across blood-brain interfaces and its relevance to Aβ clearance. Circulating large apoE, apoJ, and apoC-III containing HDL-P facilitate Aβ efflux across the BBB and BCSFB through various receptor-mediated interactions and can promote Aβ efflux from the brain despite a limited BBB exchange. Of the apoE isoforms, apoE4 has the greatest affinity to LRP-1 that may allow greater BBB transport. While larger HDL-P have limited brain delivery, smaller HDL-P may exchange among the blood, CSF, and interstitial fluid compartments. ApoJ has been demonstrated to interact with LRP-2 at the BBB and BCSFB for Aβ clearance, but lipidated apoJ and apoE3 have limited brain delivery from the periphery. While peripheral apoA-I and apoA-II cross the BCSFB and are present in CSF, the size/shape that promote this exchange remains unclear. Astrocyte-derived apoJ and apoE undergo lipidation by ABCA1. In comparison to apoE2 and apoE3, apoE4 has inefficient interactions with ABCA1 (red arrow). It remains lipid-poor and is more prone to aggregation, promoting Aβ plaque formation. In contrast, the greater lipidation of apoE2 and apoE3 compared to apoE4 by ABCA1 limits the formation of Aβ plaques. LRP-1: Low-density lipoprotein receptor-related protein 1; LRP-2: Low-density lipoprotein receptor-related protein 2; ABCA1: ATP Binding Cassette Subfamily A Member 1. Blood-Brain Barrier: BBB, and Blood-CSF Barrier: BCSFB.

receptor superfamily. These LDL receptors, as well as scavenger receptor BI (SR-BI), are suspected to be potential mediators of HDL-transcytosis at the BBB.

The LDL receptor family includes low-density lipoprotein receptor (LDLR), very low-density lipoprotein receptor (VLDLR), LDL receptor-related protein (LRP)-1, and LRP-2 (also known as megalin or gp330) (Herz and Bock, 2002). While most apos contain a receptor-binding domain, they exhibit different preferences for various LDL receptors. The binding affinity of apos to BBB-expressed receptors, such as the LDL receptor family, highlights important interactions that may facilitate the crossing of HDL through the BBB via receptor-mediated endocytosis and transcytosis.

LRP is expressed in brain capillary endothelium, glial cells, and neurons (Shibata et al., 2000; Herz and Bock, 2002). Its ligands of relevance for receptor-mediated endocytosis include apoE and apoJ (Bell et al., 2007; Lillis et al., 2008). LRP-1 is also expressed in smooth muscle cells (Ruzali et al., 2012), and endothelial LRP-1 transports amyloid peptides across the BBB, contributing to its clearance from the brain (Storck et al., 2016). LDLRs are of special interest as transmembrane receptors, because they are expressed on both the luminal and abluminal sides of the BBB and their affinity to apoE differs by its isoforms (Dal Magro et al., 2018): apoE4 > apoE3 >> apoE2 (Johnson et al., 2014). There are, however, limitations to apoE-HDL-mediated uptake. For example, CSF-derived apoE

TABLE 1 | Summary of HDL-associated proteins' source, function, and association.

Protein	Source	Cross BBB?	Cross BCSFB?	Receptor interaction	Size (Meaney et al., 2001) in Plasma	CSF/Plasma Ratio
Transferrin	Liver, choroid plexus, other tissues and organs	Yes	Yes	TF-R	Small HDL (Holzer et al., 2016; Kuklenyik et al., 2018)	1:150 (Memisogullari and Bakan, 2004; Mizuno et al., 2005)
ApoA-I	Liver, intestine	Limited BBB permeability	Yes (Stukas et al., 2014a)	Potentially involved in SR-BI mediated endocytosis of HDL at BBB [50]	Small and large HDL (Holzer et al., 2016; Kuklenyik et al., 2018)	1:700 (Koch et al., 2017)
ApoA-II	Liver, intestine	Unknown	Yes (Montine et al., 1998)	–	Small and large HDL (Holzer et al., 2016; Kuklenyik et al., 2018)	1:636*
ApoC-I	Liver, neurons, astrocytes	Some evidence (Cudaback et al., 2012)	Yes	Inhibits LRP-1, LDLR	Small and large HDL (Kuklenyik et al., 2018)	1:1,000 (Hu et al., 2020)
ApoC-II	Liver	–	Yes	Inhibits LRP-1, LDLR	Small and large HDL (Kuklenyik et al., 2018)	1:3,000 (Hu et al., 2020)
ApoC-III	Liver	Some evidence (Zhou et al., 2019)	Yes (Koch et al., 2017)	Inhibits LDLR	Small and large HDL (Kuklenyik et al., 2018), Gordts and Esko, 2018; Foley et al., 2013)	1:2,000 (Hu et al., 2020)
ApoD	Astrocytes, oligodendrocytes, various organs	Unknown; Hypothesized to act locally rather than in circulation (Provost et al., 1990)	Unknown	–	Small and large HDL (Holzer et al., 2016)	1:100 (Camato et al., 1989; Terrisse et al., 1998)
ApoE	Liver, astrocytes, macrophages	No (Elliott et al., 2010)	No (Elliott et al., 2010)	Binds LRP-1	Small and large HDL (Holzer et al., 2016; Kuklenyik et al., 2018)	1:18 (Koch et al., 2017)
ApoJ	Liver, astrocytes, neurons	Yes (Zlokovic et al., 1996; Bell et al., 2007; Merino-Zamorano et al., 2016)	Yes (Zlokovic et al., 1996)	Transport of soluble Aβ-apoJ complex and free apoJ across BBB and BCSFB via Megalin (LRP-2) (Zlokovic et al., 1996)	Small and large HDL (Stukas et al., 2014b)	1:950 (Koch et al., 2017)

**refers to unpublished data.*

is not detectable in plasma (Linton et al., 1991), and how apoE isoforms differ in brain uptake is not completely understood. Liu et al. (2012) administered adenovirus encoding human apoE3 intravenously to C57BL/6J mice, and the detection of human apoE3 in the CSF was used as a surrogate measure of central availability. In mice receiving the administered apoE3 adenovirus, human apoE3 was expressed at high levels in the liver, leading to high levels of human apoE3 in mouse plasma. In CSF, however, human apoE3 levels were undetectable.

The findings from the above study may not be applicable to apoE4. In contrast to apoE4, human apoE3 has a lower affinity to murine LDL receptors that may limit its brain uptake at the BBB (Altenburg et al., 2008). Notably, Dal Magro et al. (2018) utilized the affinity of apoE4 for surfactant-stabilized nanoparticles (NPs), particularly polysorbate-80, to create an artificial protein corona that enabled the apo-decorated NP to translocate into the brain parenchyma. This process improved brain uptake three-fold compared to uncoated particles, but only at an optimal low concentration (5 μg) when the NPs did not have to compete with excess lipid-free apoE4 (Dal Magro et al., 2018). While a simple process of incubation of the NP suspension with apoE4 might prove to be a successful strategy for clinical translation, further studies need to be done to characterize the stability of the artificial protein corona, especially in circulating blood, for true translational success. In addition, a possible toxic gain-of-function effect from injecting apoE4 on several brain functions may limit its use for neurodenegnerative diseases. However, these results corroborated the hypothesis of the involvement of LDL receptors, particularly LRP-1, in receptor-mediated uptake across the BBB for apoE4-coated NPs.

ApoJ is also an LRP binding ligand and is more specific to LRP-2 (Bell et al., 2007; Lillis et al., 2008). However *in vivo*, like apoE3, HDL-apoJ appears to have limited brain delivery. Fernández-de-Retana, et al. prepared 24 and 48 nm recombinant HDL (rHDL)-apoJ NPs by assembling dipalmitoylphosphatidylcholine (DMPC) with human recombinant apoJ (rapoJ) (Fernandez-de-Retana et al., 2017). These fluorescently labeled NPs were shown to accumulate in the cranial region, especially in old transgenic mice presenting a high cerebral Aβ load, but parenchymal brain uptake was not demonstrated.

The other receptor of interest for the transcytosis of HDL is SR-BI, which is present within brain caveolae capillary endothelial cells and allows for the bi-directional movement of cholesteryl esters mediated by apoA-I (de Beer et al., 2001; Fung et al., 2017). Furthermore, apoA-I has demonstrated cerebral vascular protection and reduced AD risk (Zhou et al., 2019). Balazs et al. (2004) found evidence that part of cerebral apoA-I originates from plasma HDL and that brain capillary endothelial cells enriched with caveolae contain SR-BI receptors, which facilitated the selective uptake of HDL at the BBB. Furthermore, they demonstrated that SR-BI co-localizes with caveolin-1 (CAV-1) on brain capillary endothelial cells. In a more recent study by Fung et al. (2017), fluorescently labeled HDL was observed via high-resolution fluorescence microscopy to be internalized by SR-BI enriched within cultured human cerebral cortex microvascular endothelial cells independent of its scaffolding protein, PDZK1. Using total internal reflection fluorescence (TIRF) microscopy, HDL was further observed to be internalized by SR-BI in a manner that was independent of proximal CAV-1 and Clathrin signaling pathways. The transcytosis of HDL was, however, determined to be dependent on an unknown dynamin and cholesterol pathway. Furthermore, it was observed that HDL uptake was inhibited by the addition of 400 μg of rapoA-I (Fung et al., 2017). The antagonistic effects of rapoA-I on HDL transcytosis were determined to be reduced by 50% in the absence of SR-BI in the same study. Rohrer et al. (2009) found that adenosine triphosphate (ATP)-binding cassette transporter, ABCG1, located on bovine aortic enthodothelium, uptook HDL via transcytosis. This suggests that ABCG1 and SR-BI receptors mediate the transcytosis of HDL. It is important to acknowledge that these findings were obtained from *in vitro* studies and there has been relatively limited information on *in vivo* transfer, as discussed in sections "Transport via the BCSFB" and "Apolipoprotein E."

Transport via the BCSFB

The BCSFB further guards against entry into the CNS, however, studies regarding lipoprotein exchange are limited compared to the BBB. The CP, which forms the BCSFB, secretes CSF as a medium for waste removal and nutrient uptake, thus acting as an independent circulatory system in this region of the CNS. While receptors at the BCSFB are not well-defined, there is evidence that LRP-2 mediates apo transport across the BCSFB (Zlokovic et al., 1996). As shown in **Figure 1** and **Table 1**, some plasma-derived proteins are known to traverse the BCSFB. For example, while apoA-I and apoA-II mRNAs are not expressed in brain cells, they are present in the CSF and have been associated with CNS lipoproteins (Demeester et al., 2000). Stukas et al. (2014a) identified that the majority of intravenously injected lipid-free apoA-I was found in the CP *in vivo*. Like apoA-I, peripheral apoA-II is suspected to cross the BCSFB through the CP. In AD and control brain tissue, apoA-II immunoreactivity was observed in the cytoplasm of CP epithelium and within blood vessels consistent with a pattern for transport across the BCSFB (Montine et al., 1998). However, it is unclear if apoA-I-containing particles aid the transport of other plasma proteins into CSF or if CNS-expressed receptors/transporters directly facilitate the transcytosis of circulating proteins (Montine et al., 1998; Stukas et al., 2014a; Koch et al., 2017). What is clear is the lipidation and subsequent shape of HDL-P are in constant flux at the BCSFB.

Exchangeable apos existing on small, lipid-poor HDL-P or circulating in their lipid-free form are suspected to become lipidated in the CSF compartment following entry from the periphery. Though not as efficient as apoA-I, exchangeable apos, such as apoA-II, apoA-IV, apoC-I, apoC-II, apoC-III, and apoE, have been identified as suitable activators of ATP-binding cassette subfamily A member 1 (ABCA1)—a transport protein responable for mediating the efflux of cholesterol and phospholipids to lipid-poor/free apos (Remaley et al., 2001; Pearson et al., 2004). Fujiyoshi et al. (2007) detected ABCA1 and ABCG1 mRNAs and proteins in isolated rat CP. Additionally, they found that both ABCA1 and ABCG1 on CP epithelium are involved in

the transfer of cholesterol and lipids to lipid-poor apos and lipoproteins in CSF (Cavelier et al., 2006; Fujiyoshi et al., 2007). This suggests that the lipidation of delipidated and/or lipid-poor apos within the CP occurs in lieu of HDL transport across the BCSFB from the periphery. This hypothesis is supported by associations among plasma and CSF apos' concentrations (Koch et al., 2017; Hu et al., 2020). The process in which lipid-poor HDL-P or lipid-free apos (made of apoA-I, apoA-II, apoA-IV, apoCs, and apoE) originating in the periphery become lipidated by CNS-expressed transporters following transport across the BCSFB likely affects brain Aβ accumulation and is discussed in section "Effect of HDL Proteins on CNS Aβ Accumulation and Related Pathology."

Surface Modifications to sHDL to Enhance Brain and Cellular Access

There are several HDL modifications shown to enhance their BBB transport. Transferrin is an iron-binding protein that is well known for its antioxidant capacity and ability to traverse both the BBB and BCSFB (**Table 1**). In plasma, transferrin has been shown to associate with apoA-I containing HDL-P isolated by selected affinity immunosorption (Kunitake et al., 1992) and denser HDL-P isolated by ultracentrifugation (McPherson et al., 2007b). With its receptors expressed on brain capillary endothelial cells (Visser et al., 2004; Johnsen and Moos, 2016), NPs modified with transferrin (Tf) are being extensively studied for drug delivery as potential treatments for brain cancers and several neurodegenerative diseases (Wiley et al., 2013; Johnsen et al., 2019; Ullman et al., 2020). Tf receptor ligands have also been incorporated into NPs in order to further facilitate transmission across the BBB. Clark and Davis (2015) demonstrated that 80 nm gold NPs that were bound to Tf by an acid-cleavable linker were better able to facilitate receptor-mediated transcytosis (RMT) and avoid BBB endothelium retention by shedding surface Tf upon acidification during transcytosis. The targeted NPs showed greater permeability across BBB models *in vitro* and entered mouse brain parenchyma in greater amounts when compared to NPs with non-cleavable Tf. Additionally, Cui et al. (2018) constructed a dual-modified HDL containing T7, a transferrin receptor ligand, and dA7R, a peptide used for its glioma-homing property, that displayed higher glioma localization than that of single ligand-modified HDL. Both these findings demonstrated that incorporating Tf-like ligands into the modification of natural HDLs could prove to be a more successful methodology for the delivery of therapeutic agents across the BBB in lieu of apo-specific receptors.

dos Santos Rodrigues et al. (2019) proposed the enhanced brain targeting and gene delivery of dual-modified (Penetratrin-Transferrin) liposomes encapsulating plasmid *APOE2* as a new gene-targeting therapeutic approach for the treatment of AD. The liposomes were surface modified with Tf, similar to previously mentioned studies, but also incorporated the conjugation of DSPE-PEG-liposomes to Penetratin (Vaisar et al., 2007)—a cell-penetrating peptide implicated with a critical enhancement of the translocation of associated cargo, such as Pen-associated liposomes—across cellular membranes, such as the BBB. A singular intravenous injection of the dual-modified liposomes loaded with plasmid *APOE2* increased apoE expression in the brain of these mice models and demonstrated successful translocation across *in vitro* triple co-culture BBB models. This study provides Tf-Pen modified liposomes as an effective method for brain delivery of plasmid *APOE2*, which has shown neuroprotective properties and a greater binding affinity to Aβ.

Indeed, one of the largest concerns in the clinical translation and the general success of synthetic HDLs is the low permeability and poor targeting property of HDLs across the BBB. Therefore, the study by dos Santos Rodrigues et al. (2019) highlights the efficacy of how the dual presence of the Tf ligand, in mediating transport across the BBB through RME, and the Pen peptide, in enhancing liposome internalization into cells, ultimately overcomes receptor saturation and promotes transfection in successfully transported HDL. Additionally, the surface modification also increased the stability of the liposome. The use of DSPE-PEG phospholipids minimized protein interaction and recognition by macrophage, while also reducing NP clearance through prolonged circulation. The plasmid DNA complexed to chitosan improved transfection by sterically hindering nucleases from degrading the nucleic acids and was also optimized to ensure nucleic acids released at target sites. Lastly, the low hemolytic activity of the liposomes at low phospholipid concentrations indicated blood compatibility safe for intravenous injection. This study demonstrated an effective method of apoE2 brain delivery that has potential for AD treatment and clinical translation.

An additional alternative method for enhanced HDL penetration across the BBB is *via* specific glycosylation. Zhou et al. (2020) developed a glycosylated siRNA NP delivery system (Gal-NP@siRNA) with "triple interaction" stabilization that specifically silenced BACE1 preemptively to decrease Aβ levels in a transgenic AD mouse model. To facilitate the transportation of the nanomedicine across the BBB, the glycosylated nano-delivery system hacked the recycling of the glycemia-controlled glucose transporter 1 (Glut1) receptor, which resulted in the movement of Glut1 from the luminal to the abluminal side of the BBB after treatment with Gal-NP@siRNA due to glucose replenishment. The "triple interaction", more specifically, the electrostatic and hydrogen bonding interaction of the guanidinium-phosphate bridge and the fluorine-mediated hydrophobic interaction between the siRNA and the galactose-modified polymer mixture, improved the biophysiological protection of the siRNA and the stability of the NPs in blood circulation. Gal-NP@siBACE1 successfully decreased BACE1 expression for at least 3 days, consequently reducing Aβ plaque levels and suppressing phosphorylated tau protein levels. This further resulted in regeneration of impaired myelin, suggesting a clearance of by-products due to biocompatibility, and contributed to the restoration of cognitive function in transgenic AD mice models. The long-term effects of these injections on Aβ levels are not clear, however. Due to the stability, ease of formulation, and successful BBB penetration (among other factors), the Gal-NP@siBACE1 model demonstrated promising potential for clinical translation, and

the study provides support for the use of RNA interference therapy for AD.

The Effect of HDL Proteins on CNS Aβ Accumulation and Related Pathology

There is a large body of research demonstrating that various apos play a large role in attenuating toxic Aβ pathology in the brain. The main apos of interest are apoE, apoJ, apoA-I, and apoD. The HDL components tested for brain delivery and impact on Aβ accumulation are summarized in **Tables 1** and **2**, and illustrated in **Figure 1**.

Apolipoprotein E

Apolipoprotein E (ApoE) is highly expressed in the CNS primarily by astrocytes (Elshourbagy et al., 1985; Mahley, 1988), but to a lesser extent in microglia (Butovsky et al., 2014), pericytes (Blanchard et al., 2020), and stressed neurons (Mahley and Huang, 2012). CNS-derived apoE is known to transfer phospholipids and cholesterol via interaction with ABCA1 and ABCG1, and promote axonal growth via interactions with the LDLRs (Fagan et al., 1998; Wahrle et al., 2004; Kim et al., 2007). ApoE lipidation via its interaction with ABCA1 has important implications toward Aβ clearance (Wahrle et al., 2004, 2005, 2008; Hirsch-Reinshagen et al., 2005; Koldamova et al., 2005; Fitz et al., 2012). Lipid-poor apoE aggregates (Hatters et al., 2006) are central to the formation of Aβ plaques, and are exacerbated by the *APOE4* genotype (Liao et al., 2018). The lipidation of apoE by ABCA1 agonists attenuates this aggregation (Rawat et al., 2019). We have shown that preserving ABCA1 function using the ABCA1 agonist, CS-6253, enhanced the ability of astrocytes to lipidate apoE4 and degrade Aβ peptides. This suggests that the transport of apos into the brain, which can activate and stabilize ABCA1, offers a therapeutic approach to limit apoE aggregation and Aβ plaque formation as illustrated in **Figure 1**.

ApoE-rHDL has been presented as a novel nanomedicine for the treatment of AD. Song et al. (2014) utilized 21–27 nm apoE3-rHDL nanostructures in an attempt to lower brain Aβ accumulation in an aging mouse model. The injected particles likely interact with ABCA1 and ABCG1 *in vivo* that modifies the shape of rHDL after injection. ApoE-rHDL injections had limited (0.4% ID/g) access to the CNS but demonstrated lower Aβ accumulation in these aging mouse models (Song et al., 2014). These effects were amplified by adding Mangostin, a model drug that accelerates Aβ degradation, to apoE containing HDL-P, and demonstrated enhanced degradation of Aβ and improved memory deficits (Song et al., 2016). In a more recent study, Song et al. (2018) examined the effects of rHDL's shape on its brain delivery, Aβ degradation, and anti-AD efficacy by comparing 27 nm spherical and discoidal apoE3-NPs nanocarriers. Spherical NPs, relative to discoidal particles, exerted the best effect due to superior brain distribution after intravenous administration, powerfully reduced Aβ deposition, decreased microglia activation, attenuated neurological damage, and rescued memory deficits in the same aging model. Notably, the NP size (27 nm) is larger than that of HDL-P (7–15 nm). It is not clear why these larger spherical particles had greater brain penetration, considering in previous studies brain delivery of apoE3-rHDL was largely unsuccessful in mouse models.

Apolipoprotein J

Apolipoprotein J (ApoJ), also known as clusterin, is an Aβ chaperone, as previously noted. It is typically associated with HDL in plasma and is a major component of CSF, wherein it is found on very dense, lipid-poor HDL-like and large HDL-P secreted by neurons and astrocytes (de Silva et al., 1990a,b; Suzuki et al., 2002). Cole et al. demonstrated that plasma-isolated apoJ lipidated with DMPC, as well as plasma-derived HDL, mediated Aβ degradation in rat microglia *in vitro* (Cole et al., 1999). These injections reduced Aβ accumulation in a similar manner to the rHDL-apoE3, possibly by facilitating Aβ efflux from the brain at the BBB. Whereas apoE, particularly apoE4, has a preference for binding LRP-1, ApoJ primarily interacts with the megalin/LRP-2 (**Figure 1**), where it facilitates Aβ clearance across the BBB and BCSFB (Zlokovic et al., 1996; Bell et al., 2007; Verghese et al., 2013). Indeed, Zlokovic et al. (1996) found that apoJ demonstrated a higher permeability–surface area product (Pearson et al., 2004) than apoE, and Bell et al. (2007) found Aβ42 complexed with apoJ was cleared 83% faster in murine models than Aβ42 alone.

Apolipoprotein A-I

Clinical studies have shown that lower plasma HDL cholesterol (HDL-C) and apoA-I concentrations are associated with increased severity of AD (Merched et al., 2000; Zuin et al., 2021). An amyloid PET brain imaging study demonstrated an association between low levels of serum HDL-C and greater cerebral amyloidosis (Reed et al., 2014). Similarly, greater serum HDL-C has been shown to be associated with greater cognitive function (Bates et al., 2017). These associations suggest a protective role for apoA-I on cognition and brain amyloidosis. While there is some evidence that plasma-derived/liver apoA-I expression may not alter parenchymal Aβ deposition, several studies reported that apoA-I attenuates cerebral Aβ angiopathy, reduces neuroinflammation, and preserves cognitive function. Specifically, Lewis et al. (2010) reported transgenic (TG) mice that overexpressed amyloid-β precursor protein (APP) and presenilin 1 (PS1), but without apoA-I expression, exhibited learning and memory deficits, higher levels of cerebral Aβ angiopathy, and Aβ-induced inflammation compared to APP/PS1/apoA-I-overexpressing mice. However, no significant differences in brain Aβ depositions between these two groups were detected (Lewis et al., 2010). Lefterov et al. demonstrated that both lipidated and non-lipidated apoA-I attenuated Aβ42 aggregation and toxicity in primary brain cells, and further showed that while apoA-I deficiency did not affect APP processing and soluble/insoluble brain parenchyma Aβ levels, 12-month-old APP/PS1 mice lacking apoA-I had higher insoluble Aβ levels in cerebral blood vessels and memory deficiencies (Lefterov et al., 2010). While these results were reaffirmed by a recent study by Robert et al. (2020), the underlying mechanisms remained unclear.

TABLE 2 | HDL-proteins for brain delivery and impact on Aβ accumulation.

Protein	Lipid	Cross BBB?	Size (nm)	Mechanism of delivery	Model*: in vitro*, *in vivo*	AD effect	References
ApoE	DMPC	Yes	21–27	RMT	microglial cells, primary astrocytes, liver cells AD animal model (SAMP8, SAMR1)	High Aβ binding affinity, accelerated Aβ degradation via lysosomal transport, rescued memory deficit	Song et al., 2014
ApoE	DMPC	Yes	27	RMT	Mouse brain endothelial cell (bEnd.3) line, microglia (BV2) cell line AD mouse model (SAMP8)	Enhanced Aβ binding affinity decreased amyloid deposition, rescued memory deficit	Song et al., 2016
ApoE	DMPC	Yes	26-27	LDLR-mediated transcytosis	Mouse brain endothelial cell (bEnd.3) line, microglia (BV2) cell line AD animal model (SAMP8, SAMR1)	Enhanced Aβ binding affinity, reduced Aβ deposition, attenuated neurological damage, rescued memory deficits	Song et al., 2018
ApoE	cetyl palmitate	Yes	211	LDLR (LRP-1) mediated uptake	human cerebral microvascular endothelial (hCMEC/D3) cells Male BALB/c mice	none (not primarily discussed)	Dal Magro et al., 2018
rApoJ	DMPC	Yes	24, 48	not discussed	Mouse J774A.1 macrophage-like cells Transgenic mouse model with high cerebral Aβ load (APP23)	Improved *in vitro* cholesterol efflux abilities, prevented Aβ fibrillization	Fernandez-de-Retana et al., 2017
ApoA-I	none	Yes	N/A	specific cellular mediated transcytosis	Human choroid plexus epithelial cells brain microvascular endothelial cells C57Bl/6 mouse model	None (not primarily discussed)	Stukas et al., 2014a
ApoA-I	none	Yes	N/A	clathrin independent cholesterol-mediated endocytosis	hCMEC/D3 endothelial cell monolayers Wild-type male rat models	None (not primarily discussed)	Zhou et al., 2019
4F ApoA-I	none	Yes	N/A	RMT	BBB endothelial cell monolayers (hCMEC/D3) B6SJLF1/J wild type AD mice models	Reductions in brain Aβ burden	Swaminathan et al., 2020

RMT, receptor-mediated transcytosis; RME, receptor-mediated endocytosis; LDLR, low-density lipoprotein receptor.

Though Contu et al. (2019) attempted to elucidate these mechanisms in apoA-I deficient AD mice models, their study obtained contrary results using the TG2576 model. This model also uses an APP mutant which expresses high Aβ levels so that by 11–13 months, these mice exhibit pathological vascular amyloid and parenchymal Aβ plaques. Furthermore, APP/PS1 mice have a different age of Aβ-pathology onset (6–9 months) than the TG2576 mice (9–12 months). Contu et al. (2019) had reported that following direct injection of Aβ into the hippocampal region, apoA-I deficient mice had higher perivascular Aβ drainage and less parenchymal and vascular Aβ pathology than the controls. They further observed that levels of apo-associated transporters, like ABCA1, LRP-2, and LRP-1, had not increased, nor had apoD and apoE levels. Clusterin/apo-J plasma and cortex levels were, however, higher in apoA-I deficient mice. These studies highlight not only the nuances in using different AD mouse models to study apo-related effects on Aβ pathology, but also how dyslipidemia affects certain apos' neuroprotective effects.

Apolipoprotein D

In contrast to other apos that are synthesized primarily in the liver and intestine, apolipoprotein D (ApoD) synthesis extends to the CNS and other peripheral organs such as the adrenal glands, kidneys, and pancreas (Drayna et al., 1986). ApoD is associated with lipoprotein subclasses in human CSF (Borghini et al., 1995; Koch et al., 2001). As a member of the lipocalin family, apoD shares little structural homology with other apos. As a result, apoD is unable to support the synthesis of nascent HDL (Flower et al., 2000; Rassart et al., 2000; Eichinger et al., 2007). However, the hydrophobic surface properties of apoD can explain its association with HDL-P and its ability to interact with lipid membranes (Eichinger et al., 2007). Known for its potent antioxidant properties, apoD is suspected to play a role in antioxidation in the brain. In an apoD knockout mouse model, increased sensitivity to oxidative stress was observed along with compromised nervous system function and decreased life expectancy, whereas overexpression of apoD in this mouse model resulted in increased resistance against oxidative stress (Ganfornina et al., 2008).

Along with apoE and apoJ, apoD is expressed at high levels in the prefrontal cortex, and apoD expression increases 5-to-10-fold during normal aging (Kim et al., 2009; Elliott et al., 2010). In AD patients, apoD expression is increased in the hippocampus, entorhinal cortex, pyramidal cells, and CSF when compared to controls (Terrisse et al., 1998; Kalman et al., 2000; Rassart et al., 2000; Belloir et al., 2001). Regarding Aβ pathology, dual-immunolabeling of temporal cortex tissue in control and AD individuals revealed that 63% of Aβ plaques co-localized with apoD, and increased immunoreactivity was observed in glial cells and cerebral vasculature (Desai et al., 2005). While the role of apoD in AD pathogenesis is unclear, it is possible that apoD partakes in Aβ-related pathology and/or the oxidative stress response in neurodegeneration.

Apo-Peptides

More recently, Swaminathan et al. (2020) looked at a therapeutic alternative to apoA-I-HDL-P due to their low permeability across the BBB in the form of 4F, an 18 amino acid apoA-I mimetic peptide, by examining the permeability-surface area product at the BBB and its effects on ^{125}I-Aβ trafficking from brain-to-blood and blood-to-brain. They demonstrated a ∼1,000-fold higher permeability for ^{125}I-4F compared to those determined for ^{125}I-apoA-I. Treatment with 4F also increased the abluminal-to-luminal flux and decreased the luminal-to-abluminal flux of ^{125}I-Aβ42 across BBB endothelial cell monolayers *in vitro*, as well as decreased the endothelial accumulation of fluorescein-labeled Aβ42. These results provided a mechanistic interpretation for the reductions in brain Aβ burden reported in AD mice after oral 4F administration, which represents a novel strategy for treating AD and CAA (Swaminathan et al., 2020). Our studies indicate that the ABCA1 agonist CS-6253 (modeled after the C-terminus of apoE) reduces brain apoE aggregation (Rawat et al., 2019), and attenuates AD pathology (Boehm-Cagan et al., 2016), although the brain delivery of CS-6253 is not clear. It is plausible that ABCA1 agonist peptides activate the peripheral sink of Aβ by promoting the formation of Aβ-binding lipoprotein particles into circulation.

Alternative Mechanisms for How Apo-HDLs Affect CNS Aβ Accumulation

Concordantly, increased apo-HDL penetration may not solely explain its neuroprotective benefits. The Peripheral-Sink Hypothesis proposes that Aβ-binding ligands in the periphery can promote CNS Aβ efflux by sequestering Aβ into the peripheral circulatory system. Indeed, increasing peripheral Aβ antibodies has been shown to increase Aβ efflux (Lemere et al., 2003; Deane et al., 2009) through LRP-1 (Kang et al., 2000; Shibata et al., 2000).

Aβ sequestration into the periphery may begin with apoJ as a chaperone from the ISF, as shown in **Figure 1**. Bell et al. (2007) demonstrated that apoJ cleared [^{125}I]-labeled Aβ40 and Aβ42 across the BBB via LRP-2. They furthermore showed that Aβ42–apoJ, compared to Aβ42 alone, crossed the BBB at an increased rate of 83%. It is possible that Aβ then crosses into the CSF following an influx of plasma-derived apoA-I. Human apoA-I overexpression in AβPP/PS1 transgenic mice was demonstrated to increase plasma HDL levels and preserve cognitive function via Aβ sequestration (Paula-Lima et al., 2009; Lewis et al., 2010). Robert, et al. demonstrated that apoE and apoA-I on HDL promoted Aβ transport across bioengineered human cerebral blood vessels, although in one of these studies recombinant apoE was injected into the "brain side" of the engineered vessel (Robert et al., 2017, 2020). Aβ preferentially binds to HDL apoA-I, but secondarily to VLDL apoE and apoC-III (Bell et al., 2012). Once in the CSF, Aβ may then cross into the periphery with an increase in plasma apoE and apoC-III concentrations. Shih et al. (2014) revealed apoC-III is an Aβ binding protein in the periphery. Interestingly, apoE4 carriers were shown to have lower peripheral apoE and apoC-III levels (Olivieri et al., 2007). These studies are indicative of higher plasma apoA-I, apoE, and apoC-III may

preserve cognitive function in AD models (Lewis et al., 2010; Shih et al., 2014; Wang et al., 2019), and further demonstrate that factors driving the exchange of HDL and its components between the periphery and CNS require further elucidation.

sHDL to Image Vascular Atherosclerosis and Its Relevance to the Brain

HDL-apos have additional important roles in imaging vascular atherosclerosis that is mechanistically linked to both AD and vascular dementia, and other CNS diseases (Chui et al., 2012). In AD mouse models, genetic apoA-I deficiency showed exacerbated memory deficits and increased CAA (Lefterov et al., 2010). Since atherosclerosis and vascular Aβ accumulation are mechanistically linked (Gupta and Iadecola, 2015), we discuss here properties that enable HDL-P to access blood vessels that could be pursued for vascular imaging of brain atherosclerosis. The mechanisms of HDL access to the vascular component are illustrated in **Figure 2**.

sHDL MRI Contrast Agents

Because of the association of vascular pathology with AD-linked biomarkers (Gupta and Iadecola, 2015) and dementia, the ability to image vasculature could be valuable for monitoring the progression of neurodegenerative diseases. HDL may be modified to include a variety of materials that generate contrast for medical imaging. These materials include radioactive or paramagnetic elements, fluorophores, and nanocrystals. One application for using HDL as an imaging agent was directed at macrophages to image atherosclerotic plaques using MRI. The first HDL contrast agent for MRI was developed by Frias et al. (2004, 2006). They reconstituted HDL with gadolinium-chelated lipids and a fluorescent dye with apoA-I and a cholesteryl ester core to make spherical particles, and later made similar Gd-HDL-P with discoidal morphology (Frias et al., 2004, 2006). The agent was administered to apoE KO mice, and MR imaging showed a significant increase in accumulation in abdominal aortas of the mice, which was confirmed to be due to the accumulation of the particles in macrophages and atherosclerotic plaques by *ex vivo* fluorescence imaging. They then improved the imaging capabilities of the particles by introducing novel gadolinium-chelating lipids, which allow for two water molecules to bind to the gadolinium instead of one, increasing the longitudinal relaxivity by a factor of four (Briley-Saebo et al., 2009). Signals can also be increased by incorporating multiple gadolinium-lipids (Gd-lipids) into each particle (Ramos-Cabrer et al., 2016). Gadolinium has also been incorporated into HDL via conjugation to apoA-I (Sriram et al., 2011; Lagerstedt et al., 2013), cholesterol (Rui et al., 2012), and to long-chain hydrophobic molecules that intercalate into the lipid coat (Carney et al., 2015). While gadolinium is the most popular element for HDL MRI contrast agents, a europium contrast agent has also been developed for use in paramagnetic chemical exchange saturation transfer (PARACEST), a highly sensitive type of MRI (Wang et al., 2015). Notably, the application of these imaging methods to either AD mice models or to human brains is still lacking and has great potential to help delineate the interaction of atherosclerosis with AD and vascular dementia pathology.

HDL MRI contrast agents have also been made with Apo-mimetic peptides. Synthetic peptides can confer advantages over native apoA-I in that they can be easily chemically synthesized and modified, and they do not require plasma-derived products and thus are safer for use in patients without extensive purification. Alpha-helical peptides from truncated apoA-I labeled with gadolinium have shown improved macrophage and plaque targeting *in vivo* compared to full-length apoA-I (Sigalov, 2014), and increasing the Gd-loading per particle increased the atherosclerotic wall/muscle normalized enhancement ratio by 160% (Shen et al., 2015). Cormode et al. developed the Gd-HDLs, prepared particles using 18A and 37pA, which are 18 and 37-residue amphipathic helical peptides mimicking apoA-I function (Cormode et al., 2008a, 2009). Both types of particles had high cholesterol efflux and were selectively taken up in macrophage cells over smooth muscle cells. The signal decreased by competition with unlabeled HDL, showing that the uptake is receptor dependent. Further studies by the same group used P2A2, a peptide derived from the LDL receptor domain of apoE, for Gd- labeled HDL-P (Chen et al., 2008). In macrophages, the uptake signal measured by MRI and fluorescence was higher than Gd-HDL made with full-length apoA-I, and the MRI signal was also higher than the signal from Gd-HDL in the aortas of apoE KO mice.

HDL as a Mechanism to Reroute Contrast Agents

HDL is directed to its natural targets by apoA-I binding. Because HDL can be reconstituted or conjugated with other peptides and proteins, it can be redirected. MRI contrast agents based on HDL have been delivered to targets other than the natural targets that are overexpressed in cardiovascular disease or cancer. HDL was conjugated with collagen-specific EP3533 peptides (EP3533-HDL) to monitor atherosclerotic plaque regression by MRI in a Reversa mouse model. Collagen, which is a marker of plaque stability, can be used as a target to distinguish between collagen and other components of the extracellular matrix. The NPs were labeled with gadolinium and administered to the atherosclerotic mice, whose regression was induced with a genetic switch. At 28 days after induction of plaque regression, there was a significant increase in MR signal from EP3533-HDL which corresponded to the increase of collagen in the plaques. *Ex vivo* confocal microscopy of aortic sections showed HDL colocalized with macrophages and not collagen, while EP3533-HDL colocalized with collagen and not macrophages (Chen et al., 2013).

SR-BI Uptake Mechanism for Direct Cytosolic Delivery

HDL and HDL-like NPs have been labeled with phospholipids containing fluorescent dyes such as rhodamine (Cormode et al., 2008b, 2009) and nitrobenzoxadiazole (Frias et al., 2004, 2006),

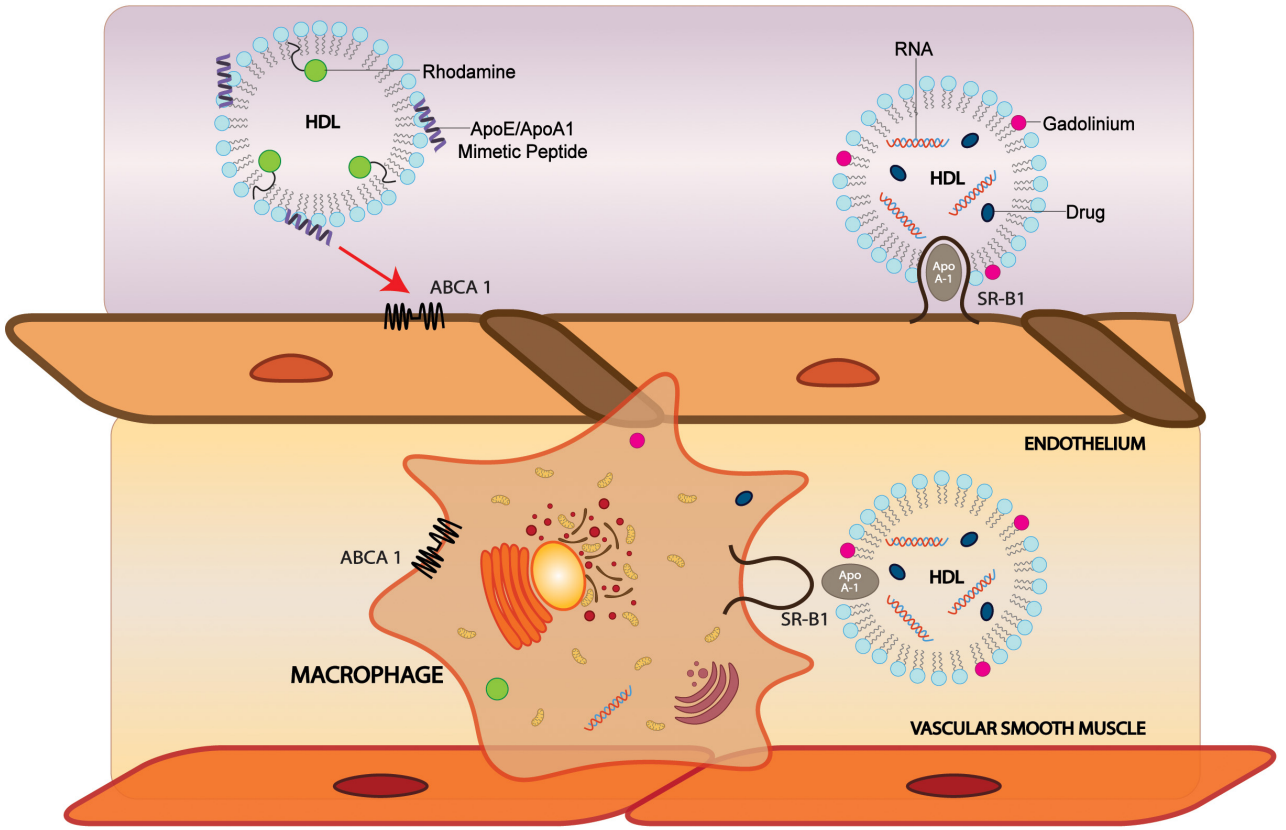

FIGURE 2 | Modification of sHDL particles can facilitate brain, vascular cell, and macrophage delivery. HDL-like NPs labeled with phospholipid-containing dyes such as rhodamine and probes conjugated with CS-6253, an ABCA1 agonist, can target ABCA1 on endothelial cells and macrophages to deliver cargo. Incorporation of MRI contrast agents such as gadolinium into HDL can increase signal. In SR-BI positive cells, sHDLs comprised of apoA-I can bind SR-BI and are taken up by the cell where cargo is delivered. ABCA1, ATP Binding Cassette Subfamily A Member 1; SR-BI, Scavenger receptor, class B type 1.

lipophilic dyes or fluorescent nanocrystals loaded into the core of the NPs (Cormode et al., 2008b; Chen et al., 2014), and with probes conjugated to the lipoprotein (Kim et al., 2014) or peptide components (Zhang et al., 2009). Because fluorescence imaging has the spatial resolution to distinguish cellular localization, it is a useful modality for elucidating the mechanism of NP interaction with cells. By labeling different NP components (core, lipid layer, protein/peptide), the localization of the components can be imaged by fluorescence.

To investigate the mechanism of drug delivery of HDL NPs, Zhang et al. (2009) developed multi-labeled nanocarriers comprised of a DiR-BOA core and an apoA-I mimetic peptide with a phospholipid coat, with fluorescein labeling on either the peptide or phospholipids. In SR-BI-positive cells, the cargo dye signal was observed in the cytosol and did not colocalize with LysoTracker, whereas the peptide and phospholipid signals were retained on the cell surface. Since SR-BI facilitates the uptake of lipids from hydrophobic cores of lipoproteins, and lipid-soluble molecules, it is not surprising that dyes and hydrophobic drugs carried to the cell by HDL can be transported into the cytosol by SR-BI. These findings suggest that via SR-BI, HDL nanocarriers are viable direct-cytosolic delivery systems for hydrophobic drugs that are prone to lysosomal degradation.

PET Imaging of Atherosclerosis With sHDL

Coupling PET tracers to HDL allows the sensitive tracers to access vascular tissues so they can be imaged with high specificity. Pérez-Medina and coworkers developed macrophage-targeting rHDLs radiolabeled with ^{89}Zr on ApoA-I (^{89}Zr-A1-HDL) or phospholipids (^{89}Zr-PL-HDL) for imaging atherosclerosis in murine, rabbit, and porcine models (Pérez-Medina et al., 2016). Biodistribution studies showed uptake in atherosclerotic tissues as well as kidneys, liver, spleen, and bone marrow. PET/CT of rabbit aortas with atherosclerotic lesions showed higher uptake of ^{89}Zr-PL-HDL than the control (0.31 ± 0.10 vs 0.16 ± 0.03 g/mL, $p < 0.05$). In pigs, atherosclerotic femoral arteries had a high accumulation of ^{89}Zr-PL-HDL at 48 h post-injection. The ability of these particles to preferentially target macrophages and plaques makes them promising imaging agents for multiple diseases.

CER-001, a pre-β-HDL mimetic containing human recombinant apoA-I and phospholipids, has also been used

for imaging atherosclerosis. Zheng et al. (2016) labeled the apoA-I component of CER-001 with ^{89}Zr and performed serial PET/CT imaging in human patients. Patients with atherosclerotic carotid artery disease ($n = 8$) were give unlabeled CER-001 (3 mg/kg) with ^{89}Zr-CER-001 (10 mg) in a 1 h infusion. PET/CT images showed carotid artery uptake of ^{89}Zr-CER-001, expressed as target-to-background ratio (TBR_{max}), was significantly increased at 24 h after infusion compared to initial scans 10 min after infusion (1.14 vs. 0.98; $p < 0.001$) and remained increased at 48 h (1.12, $p = 0.007$). TBR_{max} in plaque was 1.18, which is significantly higher than non-plaque areas (1.05, $p < 0.001$).

^{18}F-Fluorodeoxyglucose (^{18}F-FDG) accumulates in inflammatory cells associated with atherosclerotic plaques, but is non-specific and thus not ideal for imaging of atherosclerosis. Yong-Sang and coworkers synthesized ^{68}Ga-labeled HDL-P labeled on the phospholipid and compared them to ^{18}F-FDG as PET probes for imaging atherosclerotic plaques (Yong-Sang et al., 2019). The Saku group developed a PET probe to image atherosclerosis based on a 24-amino acid apoA-I mimetic peptide known as Fukuoka University apoA-I Mimetic Peptide (FAMP), which promotes macrophage reverse cholesterol transport (RCT) in a cholesterol-fed mouse model (Kawachi et al., 2013). FAMP was modified with DOTA and labeled with ^{68}Ga, then injected into the myocardial infarction animal model, Watanabe heritable hyperlipidemic rabbits (WHHL-MI). Atherosclerotic plaques and aortic atherosclerotic plaques in WHHL-MI rabbits showed high uptake of ^{68}Ga-DOTA-FAMP compared to wild-type rabbits (Kawachi, 2015).

CONCLUSION AND FUTURE PERSPECTIVES

HDL-apos are important determinants of AD pathology and VD having both central and peripheral effects on brain Aβ accumulation and vascular functions. Although the extent to which HDL-P can exchange their protein and lipid components between the CNS and the systemic circulation is still not clear, HDL-P offer untapped therapeutic potential for vascular and neurodegenerative diseases through the following mechanisms that warrant additional examination:

1. Small HDL-P appear to gain access into the brain compartment *in vitro*, but further studies are required to identify their transport *in vivo* and the small HDL components that render these BBB and BCSF transport properties, including apo and lipid composition, size, and shape.
2. Lipid-poor HDL-P entering the brain or CSF are lipidated in the brain via interactions with ABCA1/ABCG1. This process may allow for the exchange of brain lipids with peripheral lipoproteins and has important implications for Aβ production and its clearance from the brain.
3. Modifying HDL-P (e.g., the addition of Tf peptide) can enhance its brain delivery via the Tf receptors at the BBB, but applications for modifying HDL to enhance its brain delivery in neurodegenerative diseases are still lacking.
4. Even without access into the brain, some lipoproteins in the circulation can sink Aβ from the brain, a process that involves lipoprotein-Aβ binding in the circulation. This point is important for developing brain lipoprotein therapeutics without the prerequisite of crossing into the brain as a drug development milestone.
5. Since atherosclerosis is involved in the pathogenesis of brain amyloidosis and VD, imaging atherosclerosis in the brain via HDL NPs can delineate mechanisms of dementia in both parenchymal and vascular amyloidopathies, and guide drug treatments that have dual effects, ameliorating both atherosclerosis and vascular amyloid deposition.

AUTHOR CONTRIBUTIONS

HY and KC designed the review. JV, CM, AM, VK, and VS wrote the manuscript. All authors reviewed the manuscript.

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