CHOLESTEROL AND NEURODEGENERATIVE DISEASES: PRESSING QUESTIONS AND HOW TO ADDRESS THEM

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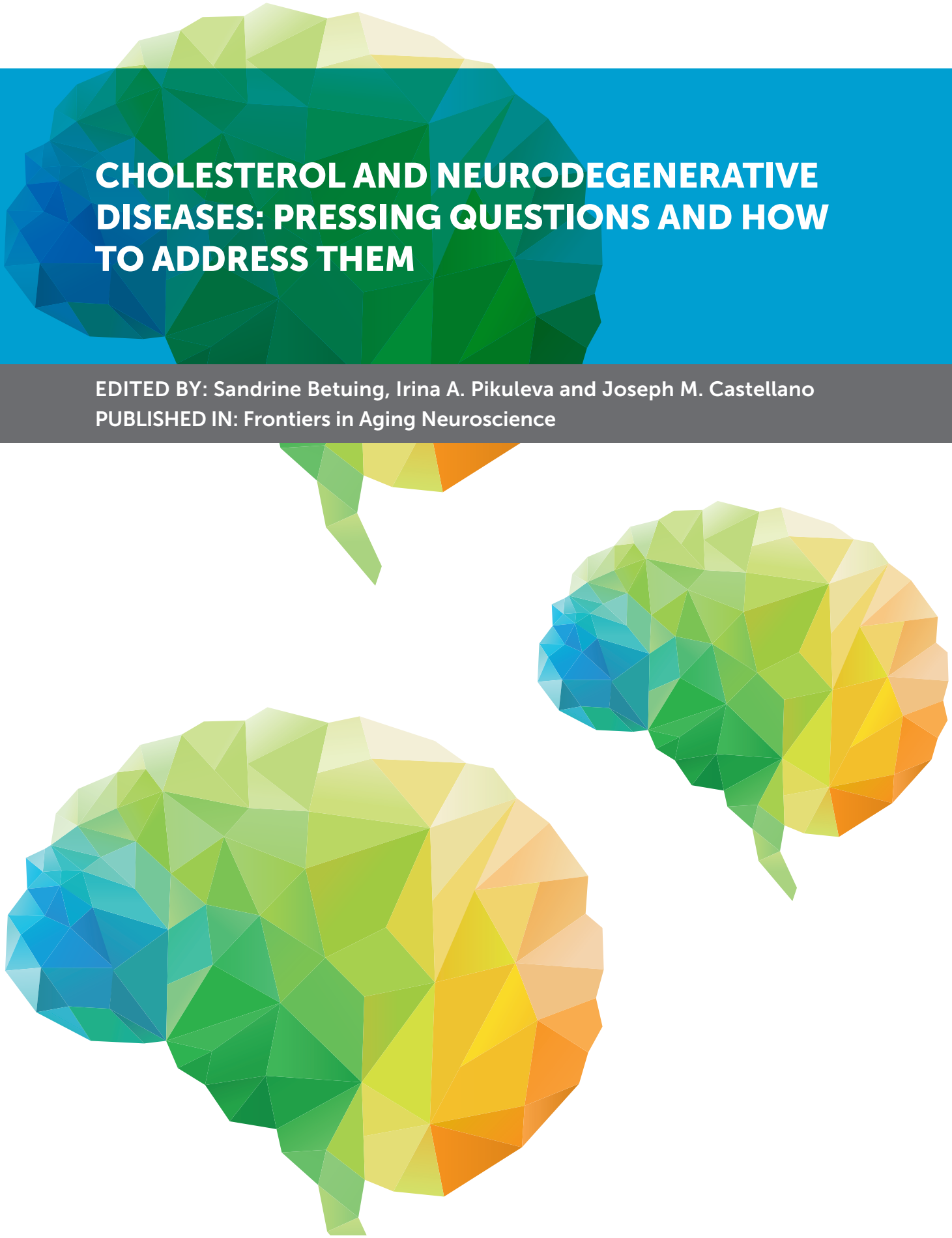

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CHOLESTEROL AND NEURODEGENERATIVE DISEASES: PRESSING QUESTIONS AND HOW TO ADDRESS THEM

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EDITORIAL
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Editorial: Cholesterol and Neurodegenerative Diseases - Pressing Questions and How to Address Them

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Keywords: cholesterol, APOE, Alzheimer's disease, Parkinson's disease, neurodegeneration, CYP46A1, Huntington's disease (HD)

Editorial on the Research Topic

Cholesterol and Neurodegenerative Diseases: Pressing Questions and How to Address Them

Within the central nervous system, maintenance of normal cholesterol homeostasis is important for various processes, including brain development, myelination, and neuronal signaling. These functions depend critically on *de novo* synthesis within the brain, as apolipoprotein particles in the systemic circulation are sequestered from the brain by the blood-brain barrier. While maintenance of whole-body cholesterol levels is well-understood, brain cholesterol homeostasis has emerged as an important topic of study given our rapidly evolving understanding of the interactions among neural cells that govern circuits and behavior, as well as the brain's response to aging neurodegeneration. During brain development, cholesterol is critical for neural differentiation and support across brain regions—needs that are met by high levels of cholesterol synthesis. Into adulthood, cholesterol generation shifts from neurons to predominantly astrocytes, and overall synthesis decreases as the brain ages in specific brain regions (Thelen et al., 2006). Age-related changes in cholesterol homeostasis are hypothesized to underlie reduced neural plasticity and function with age. Moreover, changes in cholesterol metabolism may increase risk for certain forms of neurodegenerative disease (Feringa and van der Kant), as exemplified by several cholesterol-related genes, including *APOE*, *APOJ*, *ABCA7*, and *SORL1* (Karch and Goate, 2015), that were found to be associated with Alzheimer's disease (AD) risk. These observations have prompted important questions in recent years related to brain cholesterol homeostasis: what are the causal links between cholesterol dysregulation and age-related neurodegenerative diseases? Are there potential peripheral biomarkers of this dysregulation that may be useful for diagnosing neurodegeneration? What methodological advances can push this field forward? In this Research Topic, "Cholesterol and Neurodegenerative diseases: pressing questions and how to address them," we present ten studies that collectively address these important questions, which we hope will create a framework for further exploration.

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The involvement of cholesterol in the pathogenesis of neurodegenerative diseases is summarized in four review papers for this topic. Feringa and van der Kant present a broad overview of cholesterol function within the brain, as well as putative mechanisms by which cholesterol metabolism may modify pathobiology of AD. The review explores how Alzheimer's risk alleles linked to cholesterol homeostasis may regulate pathogenesis and proposes emerging methods that may shed light on these associations (Feringa and van der Kant). Kacher et al. address a topic of altered brain cholesterol homeostasis in Huntington's disease (HD) and how cholesterol dyshomeostasis could be a determinant factor in neuronal degeneration and HD progression. The pathways and major mechanisms by which cholesterol and sterols are regulated in the CNS are presented alongside the main clinical strategies for restoring cholesterol metabolism in the CNS in HD. Pikuleva and Cartier focus on CYP46A1, a key enzyme for cholesterol elimination from the brain, and provide a summary of seminal research that led to the identification of CYP46A1 as a therapeutic target for major neurodegenerative (including AD and HD) and non-neurodegenerative brain disorders. The authors describe CYP46A1 involvement in critical cellular pathways beyond cholesterol homeostasis (e.g., gene transcription, endocytosis, misfolded protein clearance, vesicular transport, and synaptic transmission) and propose how a single enzyme can exert central control of essential brain functions. Duong and colleagues review vascular contributions to cognitive impairment (VCID) and dementia and link cholesterol, atherosclerosis, APOE, and VCID into a model. The authors then discuss potential future therapies for both atherosclerosis and dementia as a result of vascular pathology (Duong et al.). The review portion of the topic is concluded by an intriguing analysis of the research workforce studying neurodegeneration and cholesterol. Pfrieger uses a novel bibliometric TeamTree approach to identify key players in this research area, while demonstrating how the field has developed since the 1950s.

The topic's experimental papers focus on the identification of potential biomarkers for PD as well as cognitive function: four studies used patient serum or plasma and one used cerebrospinal fluid. For example, Bakeberg et al. examined serum HDL, LDL, cholesterol, and triglycerides in subjects from the Australian PD Registry, finding a sex-specific elevation in HDL that associates with worse cognitive function in female PD subjects compared to males. Griffiths et al. conducted sterol profiling in the cerebrospinal fluid of PD patients to identify cholesterol metabolites or pathways linked to PD. This work highlights the potential clinical significance of the bile acid biosynthesis pathway in PD and defines a methodology that can be used to measure the pathway intermediates within a clinical laboratory setting. Simeone et al. investigated the association of cardiovascular risk with cognitive function, reporting that serum levels of PCSK9 are associated with short-term memory only in females with high cardiovascular risk. Liu et al. analyzed whether long-term increase or decrease in plasma cholesterol levels is associated with cognitive decline. The study found that the long-term increase in non-high-density lipoprotein cholesterol was associated with decreased risks of both global cognitive decline and memory decline in females and participants without any cardiovascular disease. Finally, McFarlane et al. assessed plasma or serum lipids as biomarkers of early cognitive decline in aging adults (McFarlane et al.). Significant differences were found only for the serum levels of total cholesterol and low-density lipoprotein cholesterol with both being the highest in the mild cognitive impairment group and lowest in the mild dementia and cognitively normal groups.

Overall, this special topic highlights the key molecular pathways involved in each major aspect of cellular cholesterol metabolism, the connection to pathogenesis of neurodegenerative diseases, and points to new directions for the field. Since 2000, research in this field has expanded considerably, and therapeutic strategies have begun to emerge. However, pressing questions remain to be answered. In particular, how is cholesterol metabolism affected in specific neural cells, and what new tools can provide insights? Generation of various neural cell types from human induced pluripotent stem cells, combined with introduction of specific risk mutations by CRISPR/Cas9 gene-editing will provide a comprehensive new tool with which to study cholesterol metabolism in the setting of disease. Single-nuclei or single-cell RNA-sequencing of post-mortem tissues will discriminate the specific imprinting of neural cells related to cholesterol metabolism in pathological contexts. Finally, study of the biological role of cholesterol derivatives would broaden our knowledge of neurodegeneration and could lead to potential biomarkers. Combined, these future studies will require significant efforts but will likely have a significant impact on targeting cholesterol homeostasis to slow down neurodegenerative processes.

AUTHOR CONTRIBUTIONS

All authors conceptualized and wrote the manuscript. All authors approved the submitted version.

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ORIGINAL RESEARCH
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Blood Lipids and Cognitive Performance of Aging Polish Adults: A Case-Control Study Based on the PolSenior Project

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Background: The demand for effective strategies for maintaining cognitive capableness and establishing early dementia diagnosis has been tremendous, especially in the context of population aging. However, studies on the elderly population and neurocognitive impairment had provided ambiguous results throughout, while potential blood biomarkers of cognitive decline are yet to be clearly understood.

Objectives: The present study is aimed at assessing the relationship between blood lipids—especially in the context of their usefulness as biomarkers of an early cognitive decline—and cognitive functioning of aging adults.

Materials and Methods: The study sample consisted of 230 participants—(109 women, 121 men) aged 65+ years. Plasma 24(S)-hydroxycholesterol [24(S)-OHC], serum total cholesterol (TC), high-density lipoprotein cholesterol (HDL), and low-density lipoprotein cholesterol (LDL) were assessed. The analyses were conducted in three groups of cognitive performance: cognitively normal, mild cognitive impairment (MCI), and mild dementia, of which the subjects were divided with the Mini-Mental State Examination (MMSE).

Abbreviations: 24(S)-OHC, 24(S)-hydroxycholesterol; 27-OHC, 27(S)-hydroxycholesterol; 7α-OHC, 7α-hydroxycholesterol; 7β-OHC, 7β-hydroxycholesterol; AD, Alzheimer's disease; ADL, Activities of Daily Living; APP, amyloid-precursor protein; BBB, blood-brain barrier; CSF, cerebrospinal fluid; CYP46A1, 24-hydroxylase; GDS-15, 15 item version of the Geriatric Depression Scale; HDL, high-density lipoprotein; IADL, Instrumental Activities of Daily Living; LDL, low-density lipoprotein; MCI, mild cognitive impairment; MMSE, Mini-Mental State Examination; TC, total cholesterol; VaD, vascular dementia.

Results: No significant differences in 24(S)-OHC plasma concentrations for different levels of cognitive performance were found. Significant differences were found in serum TC (p = 0.026) and LDL (p = 0.007) concentrations for different levels of cognitive performance. Concentrations of both parameters were highest in the MCI group and lowest in mild dementia and cognitive norm, respectively. No significant differences between serum HDL concentrations and cognitive performance were found.

Conclusions: To fully assess the potential of research on blood lipids in regards to a cognitive decline, cross-sectional or epidemiological studies aimed at further exploring blood lipid roles in both the early and advanced MCI and dementia, are needed.

Keywords: neurodegeneration, mild cognitive impairment, dementia, cognition, cholesterol, biomarker, 24(S)-hydroxycholesterol, plasma

HIGHLIGHTS

- No differences in 24(S)-OHC plasma concentrations for different levels of cognitive performance were observed
- Concentrations of both total cholesterol and LDL fraction were highest in the MCI group
- 24(S)-OHC cannot be qualified as a blood biomarker of an early neurodegeneration

INTRODUCTION

Cholesterol is a pivotal constituent of neurons, and the brain is its most substantial source, sustaining about 25% of all cholesterol in the body (Dietschy and Turley, 2001). About 70% is found in myelin (Petrov et al., 2016), and around 30% in membranes of neuronal and glial cells, where it is metabolically active and undergoes recycling for neuron repair and remodeling (Dietschy and Turley, 2004). It constitutes a crucial component of cell membranes (Herz and Bock, 2002), and myelin sheath which provides insulation for the transmission of nerve impulses, where its loss inevitably causes neurological difficulties. It influences the functioning of brain synapses and is pivotal in both production, and secretion of neurotransmitters. A connection allying cholesterol metabolism deficiencies and neurodegenerative processes has been acknowledged (Petrov et al., 2016).

The initial studies on cholesterol and cognition by Sparks et al. (1990, 1994) sparked numerous research on cholesterol and cognitive impairment, some suggesting that hypercholesterolemia may be an important risk factor of neurodegenerative diseases (Liu et al., 2016). Research showed that demented or cognitively impaired aging adults show an increase in plasma total cholesterol (TC) or low-density lipoprotein cholesterol (LDL), as compared to non-demented individuals of the same gender and age (Evans et al., 2000; Lesser et al., 2001; Yaffe et al., 2002). Several, although—in most cases—retrospective epidemiological studies, mentioned a smaller rate of Alzheimer's disease (AD) amongst patients taking statins (Jick et al., 2000; Wolozin et al., 2000, 2007; Cramer et al., 2008; Haag et al., 2009). Stronger evidence, causally connecting cholesterol with AD was revealed by experimental studies indicating that controlling the amount of this lipid, altered the concentrations of amyloid precursor proteins (APP) and beta-amyloid (Friedhoff et al., 2001). Nevertheless, several uncertainties regarding the supposition causally connecting cholesterol with AD have emerged. Some studies failed to observe the discrepancies or indicated that cholesterol concentrations in AD patients were lower than non-demented individuals (Romas et al., 1999; Slooter et al., 1999; Kalmijn et al., 2000; Knittweis and McMullen, 2000; Tan et al., 2003), reporting negative correlations of serum lipid values with AD (or with all dementias; Solfrizzi et al., 2002; Mielke et al., 2005). Also, prospective studies on statins and AD have not entirely supported those associations (Rea et al., 2005; Zandi et al., 2005; McGuinness et al., 2013). Clinical interventions manipulating statins to lower cholesterol levels to prevent and cure neurodegeneration proved unsuccessful (McGuinness et al., 2014). Even experimental data is open to different interpretations, as modifying cholesterol levels impacts various proteins, not confined to APP and beta-amyloid (Wood et al., 2014). Additionally, peripheral cholesterol cannot cross the blood-brain barrier (BBB; Björkhem and Meaney, 2004). The aforementioned presumptions do not validate the causing role of elevated cholesterol concentrations in AD or mild cognitive impairment (MCI; Liu et al., 2016). At present, molecular mechanisms constituting neurodegeneration remain unclear and we have no valid blood indicators for initial diagnosis. The inconsistency of the findings of numerous up-to-date scientific investigations suggests that added efforts aimed at the observation and analysis of lipid measurements are needed, especially as blood-based biomarkers are easily acquired, relatively inexpensive, and more feasible for extensive application than cerebrospinal fluid (CSF) markers.

Over the years, the variable that began being associated with neurodegenerative processes is the modified metabolism of brain cholesterol, with research suggesting that oxidized derivatives of cholesterol—oxysterols (OHC)—might be amongst the most important determinants of AD. They are biologically active cholesterol metabolites circulating in plasma that may be formed enzymatically or by autoxidative mechanisms (Dias et al., 2018). Oxysterols, such as 27-OHC, 24S-OHC, 7α-OHC i 7β-OHC not only penetrate the BBB but exhibit cytotoxic and proapoptotic

potential. Among those, 24S-OHC is the most prevalent in the brain. It modulates cholesterol homeostasis and aids neuronal activity through the activation of liver X receptors (Okabe et al., 2013). The elevation of 24S-OHC in plasma in the course of neurodegenerative disorders is believed to show neuronal atrophy and growing secretion to the circulation; therefore, it is possibly mostly related to neurocognitive disorder pathogenesis (Testa et al., 2016).

24(S)-hydroxycholesterol [24(S)-OHC]

The conversion of cholesterol to 24S-OHC is catalyzed by cholesterol 24-hydroxylase (CYP46A1), abundantly expressed in the brain (Boussicault et al., 2016). Plasma concentration of 24(S)-hydroxycholesterol [24(S)-OHC] depends on various determinants, including cholesterol turnover factors, liver oxysterol elimination, plasma lipoprotein metabolism, genetics, and behavioral patterns (Leoni and Caccia, 2013). It has been hypothesized that plasma levels of 24(S)-OHC can serve as the first biochemical indicators of modified homeostasis of cholesterol in the central nervous system (CNS; Lütjohann et al., 2000). Further research delivered contradictory outcomes, reporting normal (Schönknecht et al., 2002) or lowered (Bretillon et al., 2000; Kölsch et al., 2004; Solomon et al., 2009) levels of 24(S)-OHC in demented vs. cognitively normal individuals. Some studies indicate that plasma concentrations of 24(S)-OHC may be higher in initial AD and Vascular dementia (VaD), possibly due to cholesterol turnover elevation associated with neuronal degradation or a defect in the BBB, present in neurodegenerative disorders, including AD (Zuliani et al., 2011). BBB defects, the occurrence of inflammation, or elevated cholesterol turnover can all counterbalance this notion, causing an increase in, or—occasionally—modification of 24(S)-OHC plasma concentrations. Similarly, reduction of those levels in severe dementia can be associated with the deficit of metabolically active neurons and the atrophy level of cell membranes (Schönknecht et al., 2002). Scarce work has focused on the evaluation of the associations between oxysterols and MCI (Liu et al., 2016). Initial reports suggest that increased concentrations of 24(S)-OHC (Leoni et al., 2006) or 27(S)-hydroxycholesterol (27-OHC; Liu et al., 2016) in CSF can pose a neurodegeneration indicator in individuals with MCI.

MATERIALS AND METHODS

The study was conducted with retrospective use of selected data and biological material from a nationwide cross-sectional PolSenior project carried out between 2007 and 2012. The project originally involved 5,695 respondents, of which 4,979 were aged 65 years and over, and 716 aged 55–59 years. Participants had been subject to randomized selection from 16 administrative zones of Poland by a multi-stage, proportional, age-group stratified process, as outlined previously (Bledowski et al., 2011). The study fully complied with all applicable institutional and governmental regulations concerning the ethical use of human volunteers, and with the terms of the Helsinki Declaration. The institutional review board approved the study protocol (the Bioethics Committee of the Medical University of Silesia in Katowice, Poland; no KNW-6501-38/I/08) and all the recruited subjects gave their written informed consent.

Participants

The study sample consisted of 230 subjects, including 109 women and 121 men, aged 65+ years selected amongst the PolSenior project respondents from the kujawsko-pomorskie voivodeship. The sampling followed strict exclusion criteria: (1) statin ingestion; (2) symptoms of depression—Geriatric Depression Scale 15-item version (GDS-15) >5 points; (3) moderate or severe dementia; (4) brain stroke; and (5) other pathological states that could severely influence cognitive functioning. To ensure comparability, analyses were conducted in three age- and gender-matched groups of cognitive performance: cognitively normal, MCI, and mild dementia. Cognitive function was screened using the Mini–Mental State Examination (MMSE; Folstein et al., 1975). The total score of MMSE is 30 points, of which 30–28 points were considered as the cognitive norm, 27–24 points as MCI, and 23–20 points as mild dementia. The test had been administered by trained nurses. The overall cognitive score was based on the MMSE result alone.

Biochemical Parameters

Blood samples were obtained from each participant during a home visit by vacuum venipuncture to ensure transport safety. Samples were delivered by nurses within a maximum of 2 h to project local laboratories, where after separating serum and plasma they were stored at −20°C until analysis. Serum concentrations of TC (measurement range: 3–800 mg/dl; error: <1.7%), LDL (measurement range: 3–550 mg/dl; error: <1.2%) and high-density lipoprotein (HDL; measurement range: 3–120 mg/dl; error: <1.3%) were assayed in the Central Laboratory in Warsaw with the use of an enzymatic colorimetric method (Modular PPE, Roche Diagnostics, Mannheim, Germany). Plasma concentrations of 24(S)-OHC (test sensitivity: 0.78 ng/ml) were assayed in the Department and Clinic of Geriatrics Collegium Medicum in Bydgoszcz with the use of the ELISA immunoenzyme assay.

Functional Assessment

We conducted a retrospective analysis of respondent data, including chosen clinical parameters (age, gender, socioeconomic data, health, and lifestyle), and results of GDS-15 (Sheikh and Yesavage, 1986), Katz Index of Activities of Daily Living (ADL; Katz et al., 1970), and Lawton Instrumental Activities of Daily Living Scale (IADL; Lawton and Brody, 1969), which is reported in the sample description below.

Statistical Analyses

The main analyzed variable regarding cognitive functioning level was the MMSE test result. It was explored regarding both plasma 24(S)-OHC and serum lipid levels, with the use of appropriate statistical methods, as detailed below. The functional assessment (e.g., chosen clinical parameters, ADL, and IADL test results) was used to describe the study sample. Statistica

10.0 (StatSoft, Inc., 2016), R statistical packet (R Core Team, 2016) and RStudio environment (RStudio Team, 2016) were used for all analyses. Normally distributed data (serum lipid levels) are presented as mean ± standard deviation and were analyzed using the independent-samples *t*-test or ANOVA with the RiR Turkey test, as appropriate. Not-normally distributed data (plasma lipid levels) are presented as median with one and three quartiles and were analyzed using the Mann–Whitney *U* test or the Kruskal–Wallis test, as appropriate. Correlations were assessed using the Spearman Rank correlation test for nonparametric distribution. *P*-value <0.05 was considered to indicate statistical significance.

RESULTS

Demographic Characteristics and Functional Assessment

Cognitively normal, referred to as control group, consisted of 71 subjects (33 women and 38 men, with the average age of 77.8); MCI group consisted of 85 participants (43 women and 42 men, with the average age of 78.8); mild dementia group consisted of 74 subjects (33 women and 41 men, with the average age of 80.7). The study sample was age- and gender-matched. The sample was homogenous regarding the assessed socioeconomic, lifestyle, and health factors that could affect cognitive performance. The vast majority (78%) achieved the primary or vocational education level. Ninety-seven percentage were employed at some part of their lives, where 71% were physical laborers or farmers. Seventy-six percentage received their financial situation as good. The sample was very homogenous in terms of having children—95% had them. Seventy-six percentage reported undertaking activities requiring physical activity in the last 12 months, however, the group was not physically active in general. Current participation in exercise or rehabilitation was declared by only 13% of the respondents, and exercising or undertaking sports activities in the past–14%. The ability to climb the 1st floor was declared by 89%, while 13% thought they could swim 10 m. The majority declared good health; 18% had the legal title of being disabled; 87%—normal thyroid function, and the remaining—subclinical thyroid disease; 73%—no vision problems. Ninety-five percentage did not report a history of depression treatment. Eighty-four percentage declared their alcohol intake to be not more than a few servings a year. Eighty-four percentage of respondents were completely independent in basic ADLs. The most differentiating variables were the declared ability to ride a bike (59% non-riders and 41% riders), smoking (43% of former/current smokers vs. 57% of non-smokers), and independence in instrumental ADL, declared by only 53% of the respondents (**Tables 1**, **2**).

TABLE 1 | Activities of daily living (ADL) results of the study sample.

ADL questionnaire points	Number	Percent
0–2	3	1.3
3–4	3	1.3
5–6	224	97.4

ADL interpretation: 5–6 points: no disorders. 3–4 points: moderate impairment. 0–2 points: severe impairment.

TABLE 2 | Instrumental activities of daily living scale (IADL) results of the study sample.

IADL questionnaire points	Number	Percent
8–18	44	19.3
19–23	63	27.6
24	121	53.1

IADL interpretation: 8–18 points: severe dependence. 19–23 points: moderate dependence. 24 points: full independence.

Plasma Concentrations of 24(S)-OHC

No statistically significant differences were found in 24(S)-OHC concentrations for different groups of cognitive performance. No statistically significant differences were found in 24(S)-OHC concentrations based on gender (**Tables 3**, **4**).

TABLE 3 | Plasma 24(S)-OHC levels [median (1 quartile–3 quartile)] and cognitive performance measured with the Mini-Mental State Examination (MMSE).

	Cognitive functioning level			K–W
	Norm	MCI	Mild dementia	*p* level
24(S)-OHC	199.7 [184–222.1]	209.8 [193.3–224.3]	200.2 [178.6–216.3]	0.225

TABLE 4 | Plasma 24(S)-OHC levels [median (1 quartile–3 quartile)] and gender.

	Gender		U M–W
	Women	Men	*p* level
24(S)-OHC	206.0 [182.2 – 225.1]	201.9 [184.0 – 218.4]	0.503

Serum Concentrations of TC, LDL, and HDL

Statistically significant differences were found in serum TC ($p = 0.026$) and LDL ($p = 0.007$) concentrations for different levels of cognitive performance. No statistically significant differences between serum HDL concentrations and cognitive performance were found (**Table 5**). We found no statistically significant correlations between 24(S)-OHC and TC, LDL, HDL.

TC was the highest in the MCI group (222.4 ± 47.1), and lowest in mild dementia (205.0 ± 38.0). In the cognitive norm, it was 208,6 ± 42,3. LDL concentration was the highest in the MCI group (142.1 ± 38.4), and lowest in cognitive norm (125.5 ± 37.1). In the mild dementia group, the LDL level was 125.9 ± 32.6. Multiple regression revealed statistically significant differences in LDL levels between the cognitive norm and MCI ($p = 0.020$), and MCI and mild dementia ($p = 0.014$). For TC, statistically important differences occurred between MCI and mild dementia only ($p = 0.029$; **Table 6**, **Figures 1–3**). We found no statistically significant correlations between 24(S)-OHC and TC, LDL, HDL (**Table 6**).

TABLE 5 | Serum total cholesterol (TC), low-density lipoprotein cholesterol (LDL), and high-density lipoprotein cholesterol (HDL) concentrations (mean ± SD) with cognitive performance measured with the MMSE.

	Cognitive functioning level			ANOVA
	Norm	MCI	Mild dementia	*p*-level
TC	208.6 ± 42.3	222.4 ± 47.1	205.0 ± 38.0	0.026
LDL	126.5 ± 37.1	142.1 ± 38.4	125.9 ± 32.6	0.007
HDL	48.6 ± 12.3	48.6 ± 12.5	52.5 ± 15.1	0.109

TABLE 6 | Spearman correlations between 24(S)-OHC and TC, LDL, HDL.

Parameter	24(S)-OHC
TC	−0.01494
LDL	0.01217
HDL	0.02951

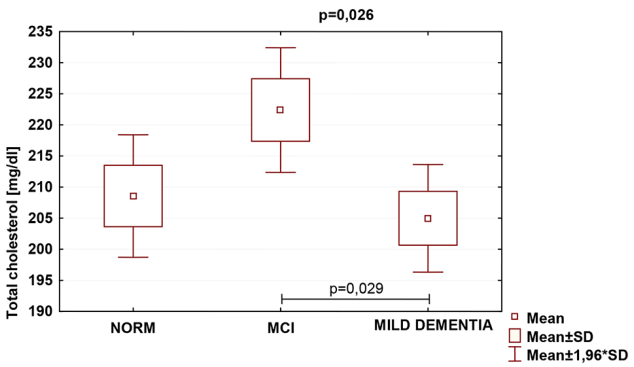

FIGURE 1 | Serum total cholesterol (TC) concentrations and cognitive performance.

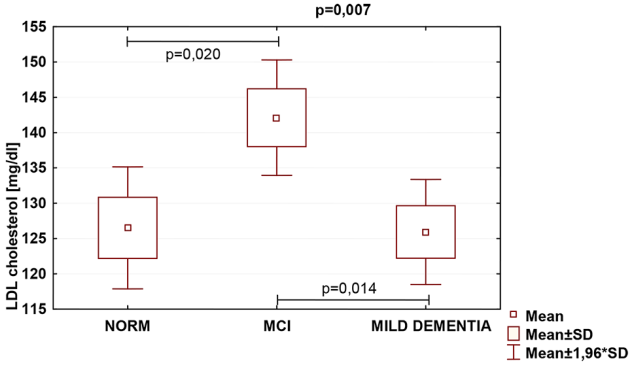

FIGURE 2 | Serum low-density lipoprotein cholesterol (LDL) concentrations and cognitive performance.

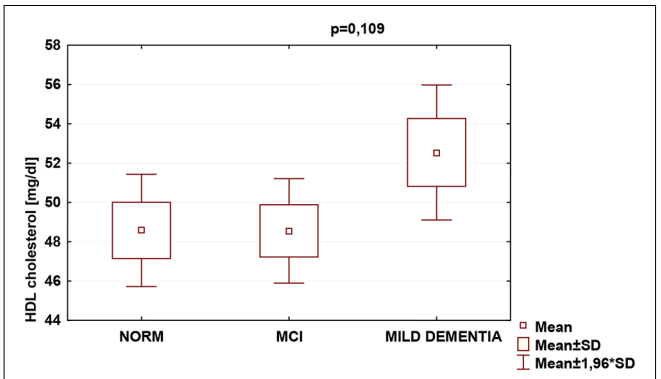

FIGURE 3 | Serum high-density lipoprotein cholesterol (HDL) concentrations and cognitive performance.

DISCUSSION

Cholesterol homeostasis disorders can impact dementia progression (Liu et al., 1998; Björkhem, 2006). As mentioned before, plasma levels of 24(S)-OHC can reflect brain cholesterol catabolism and in consequence—ongoing neurodegeneration. Therefore, possibly the plasma concentrations of this parameter could be a candidate biochemical marker of altered homeostasis of cholesterol in CNS (Lütjohann et al., 2000). Studies on plasma 24(S)-OHC levels in the context of dementia have brought conflicting results throughout (Bretillon et al., 2000; Lütjohann et al., 2000; Schönknecht et al., 2002; Kölsch et al., 2004; Solomon et al., 2009) with an interesting hypothesis of higher concentrations of brain cholesterol when neurodegeneration markers are elevated, but neuron loss and brain atrophy degree remains small (Zuliani et al., 2011). This is following other clinical observations (Kölsch et al., 2004; Solomon et al., 2009), also indicating that lowered plasma 24(S)-OHC levels are typical for dementia (Liu et al., 1998; Leoni and Caccia, 2013), but they lower gradually with disease progression (Björkhem and Meaney, 2004). According to this hypothesis, we expected 24(S)-OHC levels to be elevated in the mild dementia group in comparison with the cognitive norm; however, this assumption was not confirmed. Perhaps more than one measurement in the course of the disease is required to capture its progression reflected by plasma lipid levels. It was previously demonstrated that plasma levels of both 24-OHC and 7-OHC, but not 27-OHC were higher in demented vs. non-demented individuals, and might be lowered by simvastatin (Vega et al., 2004; Vega and Weiner, 2007).

Few studies assessed correlations between oxysterols and MCI (Tan et al., 2003; Liu et al., 2016), providing conflicting results of either elevated or lowered plasma 24(S)-OHC levels (Lütjohann et al., 2000; Papassotiropoulos et al., 2000). Again, the hypothesis of higher plasma levels of 24(S)-OHC in an early MCI, compared to significant neuron loss stage has been raised (Hughes et al., 2013), for contradictory results might have been caused by research groups with varying MCI duration. Advanced disease was associated with neuronal atrophy and decrease of 24S–OHC, while its onset—with the elevated level of this oxysterol. Possibly the differences resulted from cholesterol release due to the myelin breakdown (Hughes et al., 2013). It seems plausible, since conflicting research, e.g., study by Liu et al. (2016) that found no significant differences in plasma concentrations of 24(S)-OHC between individuals with and without MCI, did not allow to observe the impairment progression. Similarly,

our case-control study showed no significant differences in 24(S)-OHC concentrations amongst cognitively normal, MCI and mild dementia patients, thus failing to demonstrate an indicative role of this oxysterol for MCI. There are, therefore, certain premises that lowering plasma levels of 24(S)-OHC can be related to cognitive impairment progression, characterized by loss of metabolically active neurons. According to this hypothesis, elevated cholesterol turnover would counteract this tendency (Schönknecht et al., 2002). However, to test it, prospective studies with multiple measurements of this oxysterol are needed.

Blood lipids can directly affect neurodegeneration (Bretillon et al., 2000; Schönknecht et al., 2002; Kölsch et al., 2004; Li et al., 2005; Fischer et al., 2006; Hall et al., 2006; Reitz et al., 2008; Solomon et al., 2009). Serum TC levels were significantly higher in MCI than in mild dementia, and serum LDL levels were significantly higher in MCI than in cognitively normal and mild dementia groups. HDL levels did not vary significantly between the study populations. Our findings of concentrations of both TC and LDL fraction highest in the MCI group might indicate that this diagnostic entity should be further explored in concerning blood lipid levels. Because those can be altered by eating habits, physical activity, pharmaceuticals, and modification of harmful behaviors, such as tobacco use, the above results might carry major public health implications. Approaches aiming at modification of blood lipid levels may therefore pose feasible large-scale interventions intended for preserving brain function over the years.

So far, findings from investigations on serum lipid levels and cognitive performance have lacked consistency (Kivipelto et al., 2001; Michikawa, 2003; Li et al., 2004; Mielke et al., 2005), similarly to the results of studies on relationships between advanced age lipids with cognitive decline or dementia. We found TC levels significantly different between the study populations; they were lowest in mild dementia, which supports earlier reports on lower TC levels in demented patients (Kuusisto et al., 1997; Panza et al., 2006), as compared to non-demented individuals, even upon completion of a 26-year follow-up (Stewart et al., 2007). The prevailing view, however, has been that elevated cholesterol constitutes a risk factor for dementia. Substantial data have confirmed that both high risk of AD development (Patterson et al., 2008), and considerably increased likelihood of MCI (Piguet et al., 2003), are associated with high TC (Patterson et al., 2008) regardless of possible interfering factors, being indicative of playing a role in developing cognitive impairment (Piguet et al., 2003). In our sample, the highest TC levels were indeed found in the MCI group, which seems to favor this notion. Nonetheless, the process by which high TC could contribute to cognitive decline remains uncertain. Previous data suggested both that modifications of brain cholesterol homeostasis might be associated with the core pathological characteristics of AD, specifically beta-amyloid (Burns and Duff, 2002; Sponne et al., 2004), and that elevated TC in advanced age does not correlate with any form of cognitive decline or dementia (Kivipelto et al., 2001; Beydoun et al., 2011; Anstey et al., 2017). On the contrary, it seems to be linked to a reduced probability of incident AD in older adults (Kuusisto et al., 1997), acting as a preventive measure against cognitive impairment (Piguet et al., 2003). Rather, mid-age elevated levels of TC are connected with an increased odds ratio for MCI and cognitive deterioration in old age (Anstey et al., 2017). The explanation of those discrepancies might partly lie in the moment of cholesterol level assessment regarding the clinical onset of dementia, indicating that TC starts decreasing years before the manifestation of the symptoms, possibly due to ongoing neurodegeneration. This study, providing a one–time measurement of blood lipids in advanced-age individuals, and thus not allowing to observe cholesterol dynamics in the sample over years, warns against drawing bold conclusions regarding the potential of research on TC regarding cognitive decline. However, our findings of lowest TC in mild dementia, medium in the cognitive norm and highest in MCI seem to partly support the above interpretation.

Regarding cholesterol fractions, an Indian study, assessing lipid profiles in similar groups of cognitive functioning, reported AD patients with higher levels of LDL, and lower HDL (Vasantharekha et al., 2017). Additionally, a longitudinal study on 1,159 elderly Chinese revealed correlations of both high TC and LDL concentrations with rapid cognitive deterioration (Ma et al., 2017). It is partially consistent with our findings of the highest levels of both TC and LDL in the MCI group, and lowest LDL in the cognitive norm, which was expected. As to HDL levels, they did not differ significantly between the groups. We find it surprising, a majority of transverse investigations which involved individuals aged 75 plus, did recognize a link between high HDL levels and improved results of cognitive ability examinations (van Vliet et al., 2009). Some recent studies, however, failed to demonstrate both advanced age HDL and triglyceride correlation with a high probability of developing VaD, and HDL with MCI, AD, or other dementia disorders, respectively (Anstey et al., 2017). It ought to be noted that conflicting findings might be derivatives of differences in study and population type selected, age of participants, sampling, diagnostic procedures, methods and measures used, scientific rigor applied, making the results inappropriate for direct comparisons. Consequently, even though research on cholesterol and cognition was initiated decades ago, the associations between blood lipids and cognition are not yet fully understood (Li et al., 2018). We believe that this study with its stringent exclusion criteria, allowed to eliminate the plethora of confounding factors regarding cognitive functioning, thus constituting a valuable reference for further debate.

The study sample was homogenous concerning the majority of the analyzed socioeconomic, lifestyle, and health factors which could impact cognitive functioning (except for smoking and riding a bike). Therefore, it seems justified to test a hypothesis of cognitive functioning level differences resulting from other uncontrolled variables, such as diet or psychological factors. Limitations to this study, mainly resulting from the nature and design of the PolSenior project, have to be taken into consideration; as a cross-sectional, multicenter, community-based project involving nearly 6,000 participants, only routine screening examination of cognition, mood, and functional dependence was performed. Although providing a multi-aspect

assessment to a sample this size, representative for the elderly population of a country, definitely constitutes a significant scientific achievement, it also contributes to certain problems, including those of a methodological nature. No results of further detailed assessments are available, including brain imaging data. Findings of this case-control study on aging Polish adults need to be confirmed in further longitudinal studies, and cannot be generalized to other populations. Even though discrepancies amongst study populations have been noted for various lipid parameters, the numerical differences are small. Perhaps population type, diagnostic procedure, or statistical significance could influence data precision. To seize the overall impact of blood lipids on cognitive capabilities more accurately, longitudinal studies spanning over middle-aged individuals and seniors, on several constant lipoproteins and cognitive measures, are necessary. Also, usage of MMSE, not entirely adjusted to the Polish population of the older adults, lacking norms for under-educated individuals, can potentially lead to research bias of underestimating the cognitive performance in a part of the sample. Under-educated individuals might have scored lower as a result of their lack of general knowledge and overall poor level of cognitive performance, which, for them is normal functioning, not indicative of cognitive impairment. This may apply to 26 respondents, constituting 12% of the study sample, who achieved only incomplete primary education, including two people with no education, being probably self-educated in terms of reading, writing, and counting. An interesting for the analyses is the fact that, to our best knowledge, there are no MMSE norms for the Polish population, defined by the sum of points on the scale, constituting cut-off points for subsequent levels of cognitive impairment severity. Determining the ceiling for the under-educated—mostly due to the historical factors—the older population of Poland requires further discussion and research. Application of a full battery of neuropsychological tests would enable a comprehensive cognitive assessment and improve its accuracy, however, the nature and range of the project impeded it. Therefore, although the understanding of blood lipid profiles in the study population is not thorough, they may serve as a base for further exploration, setting out the framework and reference for future research. Despite limitations, it is one of few studies thoroughly investigating blood lipid roles of both circulating and brain lipids concerning the cognitive functioning of the elderly in Poland, also addressing potential blood biomarkers of an early cognitive decline among aging adults.

CONCLUSION

Following the criteria for potential blood biomarkers of early neurodegeneration, we can assume that 24(S)-OHC cannot aspire to a biomarker of an early cognitive decline in the elderly. To fully assess the potential of research on cholesterol in the context of a cognitive decline, longitudinal cross-sectional studies aimed at further exploring blood lipid profiles in both the initial and advanced MCI and dementia, are needed. Upcoming studies dedicated to determining the processes that constitute the foundation of the influence of plasma lipids on cognition are hoped to deliver a pivotal view on the triggers and interrelationships of MCI and dementia, as well as prompt original approaches for managing these conditions.

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 the Bioethics Committee of the Medical University of Silesia in Katowice, Poland. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

OM contributed to the design, analysis of data, and wrote the main manuscript text. MK contributed to the design of work and assayed biochemical parameters. KK-K made substantial contributions to drafting and revising the work. DG contributed to acquisition and interpretation of data. AS made substantial contributions to drafting the manuscript. MS made substantial contributions to data acquisition. AK-R made substantial contributions to revising the work. All authors contributed to the article and approved the submitted version.

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BRIEF RESEARCH REPORT
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Sex-Specific Association of Endogenous PCSK9 With Memory Function in Elderly Subjects at High Cardiovascular Risk

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Background: Growing evidence indicates that cognitive decline and cardiovascular diseases (CVDs) share common vascular risk factors. Protease proprotein convertase subtilisin/kexin type 9 (PCSK9) is associated with CV disease risk and has been also involved in neuronal differentiation.

Aim: Evaluate whether in patients at high CV risk cognitive function is related to PCSK9 levels.

Methods. One hundred sixty-six patients (67 female) were enrolled. A detailed neuropsychological (NP) assessment was performed. PCSK9 levels were measured with ELISA.

Results: Men had significantly higher short-term memory, executive function, and praxic and mental representation skills, as reflected by Forward Digit Span (FDS) ($p = 0.005$), Trail Making Test-A (TMT-A) ($p = 0.047$), Clock Drawing Test (CDT) (0.016). Endogenous PCSK9 levels were higher in female ($p = 0.005$). On linear regression analysis PCSK9 predicts short term memory only in females (Beta = 0.408, $p = 0.001$), with an interaction between PCSK9 and gender ($p = 0.004$ for interaction PCSK9 by sex). The association of PCSK9 with FDS in female was partially mediated by waist circumference (mediation effect 8.5%).

Conclusions: In patients at high CV risk short term memory was directly related to PCSK9 levels only in women, revealing the relevance of sex in this relationship. The association of PCSK9 with memory function may be mediated, at least in part, by waist circumference.

Keywords: cognitive impairment, PCSK9 (proprotein convertase subtilisin kexin type 9), gender, cardiovascular risk, waist circumference, short term memory

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INTRODUCTION

Growing evidence indicates that cognitive decline and cardiovascular diseases (CVDs) share common vascular risk factors, such as smoking, hypertension, high cholesterol, and diabetes mellitus and similar pathogenetic processes such as atherosclerosis and ischemia (Qiu and Fratiglioni, 2015).

Protease proprotein convertase subtilisin/kexin type 9 (PCSK9) is a regulator of low-density lipoprotein (LDL)-cholesterol clearance (Horton et al., 2009), is associated with CVD risk (Seidah et al., 2014) and may have a role in the central nervous system and in neuropsychiatric disorders. PCSK9 has been detected in the brain and in the cerebral spinal fluid (CSF) and has been involved in neuronal differentiation, apoptosis, and inflammation in the brain (O'Connell and Lohoff, 2020).

Few and controversial data are available about the potential role of PCSK9 in Alzheimer Disease (AD): In APOE$^{(-/-)}$ mice fed with a high-fat diet, the hippocampal neuronal apoptosis was associated with an increase of PCSK9 expression (Zhao et al., 2017). Consistently, silencing of PCSK9 attenuated the neuronal apoptosis induced by cerebral ischemia reducing brain damage in mice (Wang et al., 2018). Conversely, PCSK9 may prevent neuronal apoptosis through the decrease in amyloid beta generation (Wu et al., 2014). PCSK9 in the brain is thought to interfere with the cholesterol uptake by neurons, by targeting the VLDL and ApoE receptors, and to impair the amyloid beta clearance, by targeting and degrading the LDL receptor-related protein 1 (LRP1), expressed in microglia, neurons, astrocytes and pericytes, and CD36, mainly present in microglia (Adorni et al., 2019). In contrast, few genetic studies available focused only on PCSK9 genetic variants leading to loss-of-function mutations were not able to show an association between PCSK9 and AD risk (Mefford et al., 2018; Paquette et al., 2018).

Recently, some concerns have been raised about the potential neurological side effects of PCSK9 inhibitors, a class of drugs used as cholesterol-lowering treatments (Mannarino et al., 2018). A meta-analysis found a non-significant trend toward adverse neurocognitive effects for PCSK9 inhibitors in the outcome studies with a larger sample size and longer follow-up (Khan et al., 2017). However, in a randomized trial of the PCSK9 inhibitor evolocumab, no significant between-group difference in cognitive function was observed over a median of 19 months (Giugliano et al., 2017) and 2.2 years of treatment (Gencer et al., 2020), but long-term follow-up studies are required to draw final conclusions.

An additional unexplored issue is the relevance of sex in PCSK9-related cognitive function. Sex-differences have been observed in prevalence and rates of cognitive decline. Namely, women show faster cognitive decline and brain atrophy rates and patterns after diagnosis of mild cognitive impairment (MCI) or AD dementia (Hua et al., 2010; Sundermann et al., 2016). The majority of studies in which cognitive data were stratified by sex in AD dementia indicate that women score lower than men in verbal memory and fluency tasks (Pusswald et al., 2015; Gale et al., 2016).

In a large European cohort (n = 3,673), sex has been shown as a strong predictor of PCSK9 and different variables have been associated with PCSK9 in a sex-specific way, e.g., mean corpuscular hemoglobin concentration and smoking habits are PCSK9-independent predictors in women, whereas hypercholesterolemia and physical activity are independent predictors in men (Ferri et al., 2020). Moreover, PCSK9 is regulated by sex hormones and in women PCSK9 correlates inversely with estradiol (E2) (Ooi et al., 2015).

The aim of this study was to evaluate whether, in patients at high CV risk, cognitive function may be related to circulating PCSK9 levels and the possible influence of sex in this association.

Abbreviations: CV, cardiovascular; PCSK9, protease proprotein convertase subtilisin/kexin 9; NP, neuropsychological; FDS, Forward Digit Span; TMT-A, Trail Making Test-A; LDL, low-density lipoprotein; CSF, cerebral spinal fluid; MCI, mild cognitive impairment; AD, Alzheimer Disease; T2DM, type 2 diabetes mellitus; ADA, American Diabetes Association; OGTT, Oral glucose tolerance test; ELISA, enzyme-linked immunosorbent assays.

MATERIALS AND METHODS

Patients Recruitment

One hundred sixty-six patients (67 female and 99 male) were enrolled in this observational study. For both gender median age was 68 years. Eighty-six patients (32 female and 54 male) had T2DM. T2DM diagnosis was made according to the ADA criteria (fasting plasma glucose $\geq$ 126 mg/dL or 2-h plasma glucose $\geq$ 200 mg/dL during OGTT or HbA1c $\geq$6.5 or a random plasma glucose $\geq$ 200 mg/dL) (American Diabetes Association, 2020).

Patients were referred to the Clinical research Center of the Center for Advanced Studies and Technology (CAST), "G. D'Annunzio" University Foundation, by primary care physicians or enrolled at the Diabetes Clinic of Chieti University Hospital. Each subject signed written informed consent to participate, and the Protocol was approved by the Ethics Committee of the University of Chieti (Prot.1129 18.07.2013). All the patients were in treatment with low-dose aspirin (100 mg/die) for cardiovascular prevention.

Exclusion criteria were: uncontrolled hypertension, uncontrolled dyslipidemia, significant comorbidities such as kidney or liver disease, pregnancy or lactation, chronic inflammation, cigarette smoking; clinically significant cardiac and/or pulmonary insufficiency; history of malignant neoplasms (diagnosed and treated within the past 5 years); history of malabsorption; regular (daily) alcohol consumption; regular (i.e., more than 3 days per week) non-steroidal anti-inflammatory drug intake.

This study was performed under the Good Clinical Practice regulations (Good Clinical Practice for Trial on Medicinal Product-CPMP/European Commission-July 1990; Decreto Ministeriale 27.4.1992-Ministero della Sanità) and the Declaration of Helsinki (Hong Kong 1989). By signing the protocol, the participants in the study committed to adhere to local legal requirements.

PCSK9 Levels

All samples were collected at 8 a.m. after an overnight fasting to avoid PCSK9 variations due to its diurnal rhythm (Persson et al., 2010). Blood collected into EDTA-containing vacuum tubes (vacutainer, Becton Dickinson) was centrifuged at 1,200 × g for 10 min at RT to separate plasma. Plasma was aliquoted and frozen at −80°C. PCSK9 levels were measured with commercial enzyme-linked immunosorbent assays (ELISA) kit (#DPC900, R&D) according to the Manufacturer's instructions.

Neurocognitive Examination

Cognitive function evaluation was evaluated through a short neuropsychological (NP) battery including Forward Digit span (FDS: short-term memory), memory test with 10-s interference (MI: working memory with distracting tasks) Trail Making test part A (TMT-A: executive function and selective attention), Trail Making test part B (TMT-B: executive function and attention switching), Clock Drawing Test (CDT: praxic and mental representation skills). Cognitive tests were taken from the ENB-2 neuropsychological battery, standardized for the Italian population.

The raw scores (raw scores) reported by patients to individual neuropsychological tests were transformed into zeta scores (z-scores) by using averages and standard deviations of the Italian regulatory population.

Trail Making Test (Part a and b)

Trail Making Test part A (TMT-A) is a commonly used measure of attention and information processing speed (Lezak, 1995), and already used previously as a measure of attention (Beavers et al., 2017; Tu et al., 2018).

The TMT-B is a well-known instrument for describing the attentive function but at the same time it evaluates sets witching ability working memory and inhibition control (i.e., executive functions); thus, it requires the involvement of executive functions, making it a valid measure of this function (Lezak, 1995; Kemp et al., 2016). Both parts (A and B) of the Trail Making Test consist of 25 circles distributed over a sheet of paper.

Forward Digit Span (FDS)

Digit span is the standard test of verbal short-term memory performance that is routinely used in psychological studies, either as a stand-alone test or as part of a number of psychological assessment batteries. Although various other measures of verbal short-term memory capacity exist, the digit span task is the most widely used one in scientific works (Jones and Macken, 2015).

Clock Drawing Test (CDT)

The CDT is used for screening for cognitive impairment and dementia and as a measure of spatial dysfunction and neglect. It was originally used to assess visuo-constructive abilities but abnormal clock drawing may occur in other cognitive impairments. Doing the test requires verbal understanding, memory, and spatially coded knowledge in addition to constructive skills (Shulman, 2000).

Interference Memory Test (IMT: dual task)

Interference memory test is a test that evaluates working memory by memorizing triplets of letters along with a distracting activity (an activity that prevents subvocal repetition) (Mondini et al., 2011).

STATISTICAL ANALYSIS

Comparisons of variables between men and women were performed by χ^2 tests or Mann-Whitney U tests. Spearman rank correlation test was used to assess univariable relationships among continuous variables. Multivariable linear regression analysis was performed: (a) to assess the association of memory function (FDS z-score) with PCSK9, separately in men and women; (b) to test if a different association between FDS z-score and PCSK9 occurs in men and women (this hypothesis was tested by adding the interaction term PCSK9*sex in the model), and (c) if the association is mediated by waist circumference. We performed causal mediation analysis to verify whether waist circumference is related to the observed PCSK9-memory relationship. Indeed, a test of mediation examines whether the effect of the independent variable (x) on the dependent variable (y) occurs via a third, intervening variable (z). This analysis was suggested by the observation that waist circumference is associated both with PCSK9 and memory function and is a variable likely to be a mediator of the association between PCSK9 and memory function; the hypothesis of mediation was tested and quantified by adding waist circumference in the multivariable model, and evaluating the percentage change in the beta coefficient of the association of PCSK9 with memory function.

Because of a positive skewed distribution, PCSK9 was natural-log-transformed for the analysis. Only 2-tailed probabilities were used for testing statistical significance, and $P < 0.05$ was considered statistically significant. All calculations were carried out using SPSS (SPSS, Chicago, IL, USA).

RESULTS

Baseline Characteristics

One hundred sixty-six patients (67 female and 99 male) were enrolled. Baseline characteristics of patients by sex are listed in **Table 1**.

Median age was 68 years and median BMI was 29. Seventy-seven patients (46%; 28 female) had overt CV disease (chronic coronary syndrome, PAD, previous MI, stroke or TIA or revascularizations). Eighty-six patients (52%; 32 female) had T2DM, according to the initial protocol design. Thus, in our population the prevalence of diabetes was higher than in the general population with comparable age (Cho et al., 2018).

In this population at high CV risk, male and female patients displayed differences in a few clinical variables as well in cognitive function parameters and PCSK9 levels. Men had higher weight ($p < 0.001$), waist circumference ($p = 0.010$), WHR ($p < 0.001$), systolic blood pressure ($p = 0.038$), diastolic blood pressure ($p = 0.048$), and creatinine ($p < 0.001$).

Endogenous PCSK9 levels ($p = 0.005$), total cholesterol ($p < 0.001$), HDL ($p = 0.001$), and LDL ($p = 0.001$), circulating leptin ($p = 0.038$) were higher in female (**Table 1**). Women were less treated with metformin ($p = 0.044$), in line with the literature reporting a higher risk of adverse drug reactions, and possible drug discontinuation, with metformin in women (de Vries et al., 2020) and were more frequently treated with β-blockers ($p = 0.028$), consistently with a recent cohort study (Walli-Attaei et al., 2020). Male had higher executive function, short-term memory, and praxic and mental representation skills, as reflected by TMT-A z-score ($p = 0.047$), FDS z-score ($p = 0.005$), and CDT z-score (0.016) (**Table 1**).

Circulating PCSK9 levels were directly related to FDS z-score (Rho = 0.377, $p = 0.002$) only in female patients (**Table 2** and

TABLE 1 | Clinical characteristics of study patients by sex.

Variables	Female (*N* = 67)	Male (*N* = 99)	*p*-value*
Age (years)	68 (62–71)	68 (63–73)	0.932
Weight (kg)	71 (64–80)	84 (75–92)	**<0.001**
BMI (kg/m^2)	28.8 (25.7–32.0)	29.0 (26.0–31.2)	0.728
Waist circumference (cm)	99 (91–108)	104 (96–111)	**0.010**
Hip circumference (cm)	108 (102–114)	104 (99–110)	0.067
WHR	0.91 (0.87–0.95)	0.98 (0.94–1.01)	**<0.001**
Systolic BP (mmHg)	139 (130–150)	145 (134–160)	**0.038**
Diastolic BP (mmHg)	74 (68–80)	79 (70–85)	**0.048**
Fasting plasma glucose (mmol/L)	5.44 (5.06–6.56)	5.78 (5.17–7.11)	0.154
HbA1c (%)	6.10 (5.70–6.70)	6.10 (5.60–7.00)	0.986
HbA1c (mmol/mol)	43 (39–50)	6.10 (38–53)	0.986
Creatinine (mg/dl)	53.4 (53.4–61.0)	68.6 (61.0–76.3)	**<0.001**
eGFR (ml/min)	88.6 (68.9–97.7)	89.0 (77.0–98.0)	0.318
Total bilirubin (μmol)	10.3 (8.5–13.7)	12.0 (8.5–17.1)	**0.006**
AST (U/L)	23 (20–28)	25 (20–31)	0.266
ALT (U/L)	28 (24–36)	31 (25–39)	0.167
hs-C-reactive protein (nmol/L)	0.23 (0.11–0.51)	0.18 (0.08–0.34)	0.114
Uric acid (mg/dl)	5.3 (4.4–6.4)	5.6 (4.8–6.7)	0.146
PCSK9 (ng/ml)	308 (251–394)	267 (231–340)	**0.005**
Leptin (ng/ml)**	31.0 (15.4–43.0)	18.0 (11.7–34.1)	**0.038**
Lipid Profile			
Total cholesterol (mg/dl)	5.15 (4.3–5.6)	4.5 (3.7–5.0)	**<0.001**
Triglycerides (mg/dl)	1.3 (1.1–1.8)	1.4 (1.0–1.9)	0.707
HDL cholesterol (mg/dl)	1.4 (1.1–1.7)	1.22 (1.0–1.4)	**0.001**
LDL cholesterol (mg/dl)	3.0 (2.3–3.4)	2.5 (1.9–3.0)	**0.001**
Disease			
T2DM, *n* (%)	32 (47.8%)	54 (54.5%)	0.431
Hypertension, *n* (%)	58 (86.6%)	81 (81.8%)	0.522
Dyslipidemia, *n* (%)	41 (61.2%)	52 (52.5%)	0.339
CVD, *n* (%)	28 (41.8%)	49 (49.5%)	0.346
MS ATP III, *n* (%)	48 (71.6%)	65 (65.7%)	0.498
Stable CAD, *n* (%)	1 (1.5%)	10 (10.1%)	0.052
Previous MI, or revascularization, *n* (%)	6 (9.0%)	18 (18.2%)	0.119
Previous TIA/stroke, o revascularization, *n* (%)	4 (6.0%)	14 (14.1%)	0.128
PAD, *n* (%)	2 (3.0%)	4 (4.0%)	1.000
Microvascular disease, *n* (%)	1 (1.5%)	7 (7.1%)	0.145
Medications			
Metformin, *n* (%)	16 (23.9%)	40 (40.4%)	**0.044**
Glinides, *n* (%)	6 (9.0%)	5 (5.1%)	0.354
Sulfonylureas, *n* (%)	1 (1.5%)	3 (3.0%)	0.648
PPAR-gamma, *n* (%)	6 (9.0%)	5 (5.1%)	0.354
GLP1RA, *n* (%)	2 (3.0%)	0 (0%)	0.161
DPP-IVi, *n* (%)	1 (1.5%)	4 (4.0%)	0.649
Acarbose, *n* (%)	0 (0%)	1 (1.0%)	1.000
Insulin, *n* (%)	1 (1.5%)	2 (2.0%)	1.000
SGLT2i, *n* (%)	1 (1.5%)	1 (1.0%)	1.000
ACE-I, *n* (%)	16 (23.9%)	35 (35.4%)	0.126
ARBs, *n* (%)	22 (32.8%)	31 (31.3%)	0.866
Diuretics, *n* (%)	22 (32.8%)	28 (28.3%)	0.606
B-block, *n* (%)	28 (41.8%)	25 (25.3%)	**0.028**
CCA, *n* (%)	16 (23.9%)	26 (26.3%)	0.856

(Continued)

TABLE 1 | Continued

Variables	Female (*N* = 67)	Male (*N* = 99)	*p*-value*
Statins, *n* (%)	27 (40.3%)	47 (47.5%)	0.427
Fibrates, *n* (%)	1 (1.5%)	0 (0%)	0.404
Ezetimibe, *n* (%)	7 (10.4%)	7 (7.1%)	0.571
Proton pump inhibitors, *n* (%)	25 (37.3%)	39 (39.4%)	0.871
ASA, *n* (%)	67 (100%)	99 (100%)	1.000
Neuropsychological Tests Battery Z-Score			
Trail making test A	0.59 [0.04–0.95]	0.76 [0.36–1.07]	**0.047**
Trail making test B	0.37 [−0.20–0.70]	0.44 [0.01–0.90]	0.120
Forward digit span	−0.62 [−1.35–0.39]	0.24 [−0.68–0.55]	**0.005**
Clock drawing test	−0.87 [−2.23–0.08]	−0.27 [−1.32–0.47]	**0.016**
Interference memory test	−0.42 [−1.31–0.39]	−0.10 [−0.91–0.69]	0.184

BMI, body mass index; WHR, waist-hip ratio; BP, blood pressure; MS, metabolic syndrome; CVD, cardiovascular disease; MI, myocardial infarction; TIA, transient ischemic attack; PAD, peripheral artery disease; ACE-I, ACE-inhibitors; ARBs, angiotensin receptor blockers; B-block, beta-blockers; CCA, calcium channel antagonists; ASA, acetylsalicylic acid; MS, Metabolic Syndrome; ATP III, National Cholesterol Education Program—Adult Treatment Panel III.

***86 missing, 34 in female and 52 in male.*

*Data are median (25–75th percentile). *Determined by Mann-Whitney or X^2 test, as appropriate. Significant values ($p < 0.05$) are indicated in bold.*

Figures 1A,B). None of the other tests z-scores were significantly correlated to PCSK9 in either sex. Among the biochemical and anthropometrical variables analyzed, waist circumference was the only variable associated with both FDS z-score and PCSK9 in females (Rho=-0.292, $p = 0.017$ and Rho=-0.305, $p = 0.012$, respectively) (**Figures 2A,C**). No significant correlation was observed between waist circumference and either FDS z-score or PCSK9 in male (**Figures 2B,D**).

Linear regression analysis with FDS z-score as the dependent variable revealed an independent association between LnPCSK9 and FDS z-score only in female (Beta = 0.408, $p = 0.001$) but not in male (Beta=-0.073, $p = 0.478$). The modification of the effect by sex in this association was confirmed by an appropriate test for interaction between PCSK9 and sex, and indeed a significant interaction was found ($p = 0.002$). These findings were independent of a large panel of potential covariates; in fact, they were virtually unchanged following the inclusion in the regression model of total cholesterol or triglycerides, HDL, LDL, systolic and diastolic blood pressure, creatinine, metabolic syndrome, circulating leptin, or ongoing medications affecting PCSK9 levels (**Table 3**).

We finally tested the hypothesis that the association between FDS z-score and PCSK9 in female was, at least in part, mediated by waist circumference. We observed that the introduction of waist circumference in the model led to a weakening of the association between LnPCSK9 and FDS z-score in female (Beta from 0.408 to 0.376), thus we quantified that the mediating effect of waist circumference was 8.5%.

DISCUSSION

In this manuscript we explored the relationship between endogenous PCSK9 levels and cognitive performance in patients at high cardiovascular risk. Since different variables are associated with PCSK9 in a sex-specific way (Picard et al., 2019), and sex-differences have been reported in rates and patterns of cognitive decline, we investigated the influence of sex in this relationship.

Several lines of evidence indicate that PCSK9 may play a role in the pathophysiology of AD both in the pre-symptomatic and symptomatic phases of the disease (O'Connell and Lohoff, 2020; Picard et al., 2019). In autopsy-confirmed human brains, a significant increase in cortical *PCSK9* gene expression and protein levels has been reported in AD patients when compared to age-matched control subjects (Picard et al., 2019). Of note, CSF PCSK9 correlated with CSF tau protein, suggesting that PCSK9 may influence tau metabolism and neurofibrillary tangles accumulation rather than amyloid plaques deposition, at least in the pre-symptomatic phase of late-onset AD (Picard et al., 2019).

A still unanswered question is whether PCSK9 exerts a local effect on the brain or a systemic effect in peripheral tissues thereby affecting the brain, and what is the relationship between circulating and brain PCSK9 concentrations. Neither cholesterol nor PCSK9 cross the blood-brain barrier (BBB) under normal conditions (Dietschy, 2009; Nieweg et al., 2009; Chen et al., 2014); however, several disease states can cause BBB permeability and leakage that might affect brain cholesterol homeostasis. For instance, serum hypercholesterolemia may promote inflammation that damages the BBB and allows passage of LDL, pro-inflammatory cytokines, and other factors into the brain that increase amyloid beta aggregation (Altman and Rutledge, 2010).

PCSK9 is detectable in the CSF of healthy subjects without the typical diurnal pattern of plasma PCSK9, indicating a different regulation in the two body compartments (Chen et al., 2014). Increased CSF concentrations of PCSK9, with a positive correlation with ApoE4 levels, have been reported in AD subjects, suggesting a pathophysiological link between PCSK9, apoE4, and AD (Zimetti et al., 2017).

Thus, circulating PCSK9 can cross the BBB in conditions characterized by inflammation, highly prevalent in our high CV risk population, and modulate cholesterol homeostasis, beta amyloid accumulation, and neuroinflammation. Alternatively,

TABLE 2 | Correlations between circulating PCSK9 and cognitive parameters.

			TMT-A	TMT-B	FDS	CDT	IMT
Female	PCSK9	Rho	0.145	0.051	**0.377***	0.053	0.118
		p-value	0.244	0.727	**0.002**	0.673	0.352
		N	66	49	**67**	67	64
Male	PCSK9	Rho	−0.078	−0.058	−0.124	0.094	0.036
		p-value	0.448	0.601	0.225	0.364	0.734
		N	96	85	97	95	93

*TMT-A, Trail making test A; TMT-B, Trail making test B, FDS, Forward digit span, CDT, Clock drawing test; IMT, Interference Memory Test. *p < 0.05. Significant values (p < 0.05) are indicated in bold.*

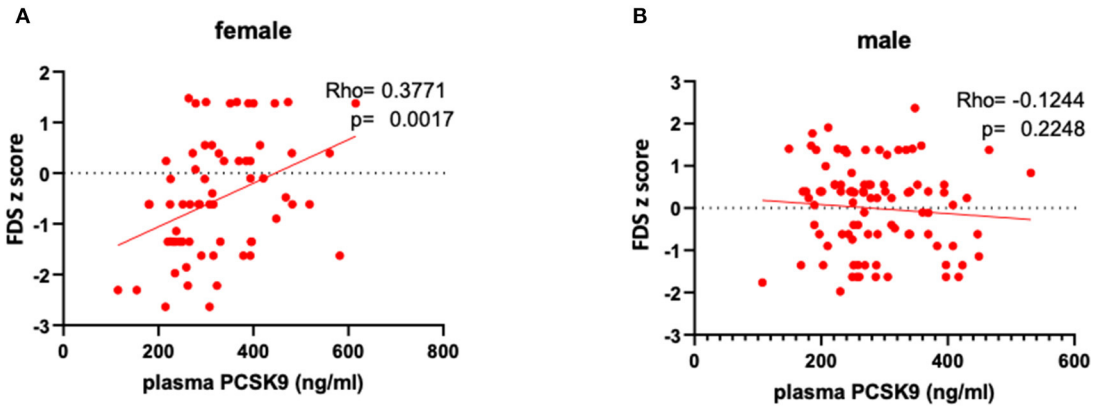

FIGURE 1 | Correlations between circulating PCSK9 levels and short-term memory, as reflected by FDS z-score in female **(A)** and male **(B)** patients.

circulating PCSK9 may exert its effects on peripheral tissues in turn contributing to neurocognitive changes. As expected and previously reported (Ooi et al., 2015; Ruscica et al., 2017; Ferri et al., 2020), endogenous PCSK9 levels were higher in female than male. We also found gender differences in patterns of cognitive test performance, as previously observed in other studies (Jorm et al., 2004; De Frias et al., 2006; Munro et al., 2012).

Interestingly, we observed for the first time that PCSK9 and short memory function were directly associated only in female. Consistently, PCSK9 regulation seems to be under tight genetic control, with specific variants of PCSK9 that may predispose to increased AD risk in females only (Picard et al., 2019). In addition, further investigation of these variants in two independent cohorts showed a female specific association with AD risk and with CSF Tau levels in cognitively impaired individuals (Picard et al., 2019).

The pathophysiology underlying the association between PCSK9 and memory function in female is largely uncharacterized. Altered PCSK9 activity in the central nervous system may contribute to the reported deterioration of brain cholesterol homeostasis and indirectly, to lipoprotein dysfunction and AD pathophysiology. Although PCSK9 is a known regulator of LDL cholesterol (Horton et al., 2009) and high LDL and total cholesterol levels have been associated with cognitive impairment in old women (Yaffe et al., 2002), the observation of no correlation between short-term memory and cholesterol levels in our group of patients (data not shown) prevented us to support the hypothesis of an effect mediated by cholesterol lowering.

The influence of sex hormones has been advocated to explain sex differences in both PCSK9 levels (Peticca et al., 2013; Ancelin et al., 2014; Ghosh et al., 2015) and cognitive function, including memory (Mordecai et al., 2008; Joseph et al., 2012). Estrogen levels were inversely correlated to circulating PCSK9 in pre-menopausal females (Ghosh et al., 2015). Maternal serum PCSK9 levels at parturition were significantly elevated in comparison to controls (Peticca et al., 2013). Variation of endogenous estrogen levels during the menstrual cycle likely contributes to the inter-individual variation in PCSK9 and LDL-C in normal females (Ghosh et al., 2015). On the other hand, a longitudinal cohort study revealed gender-specific associations between lipids and cognitive decline in the elderly, involving hormonal status in women (Ancelin et al., 2014). Increased estradiol levels in the late follicular phase in pre-menopausal women was associated with increased activation in left frontal circuitry and decrements in working memory performance (Joseph et al., 2012).

Finally, several lines of evidence including genetic studies indicate that circulating PCSK9 is directly associated with depression, a condition with higher prevalence in women and related to insulin resistance (Nelson et al., 2019; Macchi et al., 2020a).

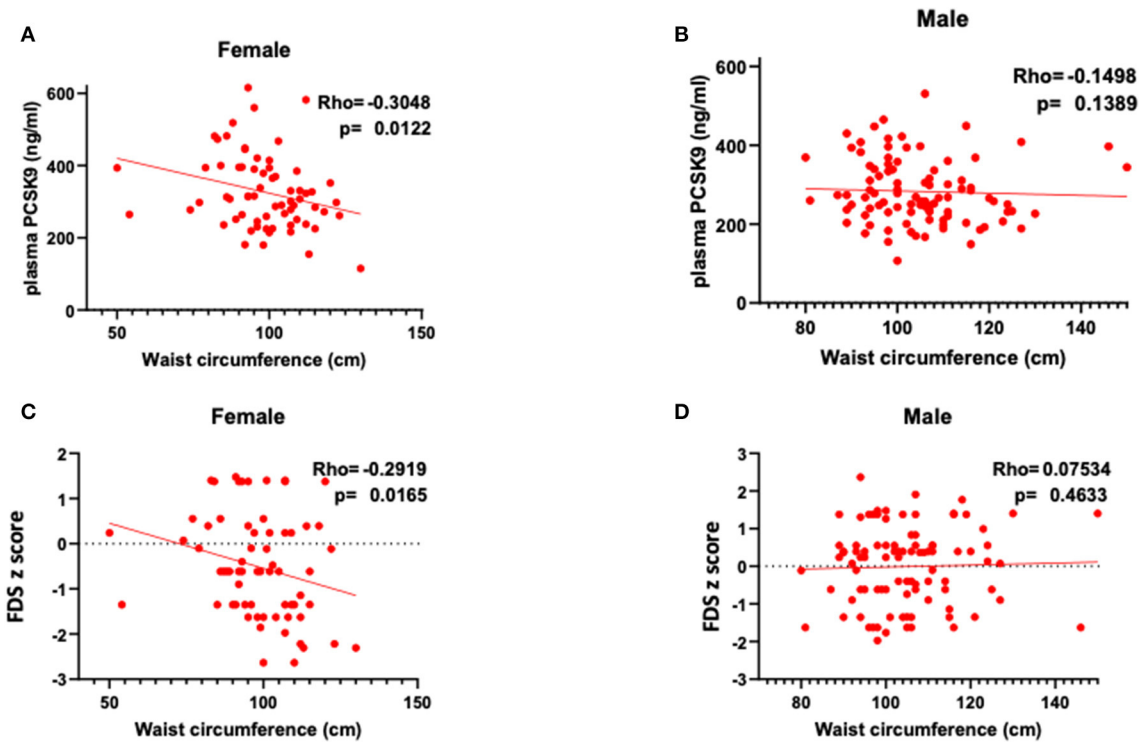

FIGURE 2 | Correlations between waist circumference, circulating PCSK9 levels and short-term memory, as reflected by FDS z-score in female **(A,C)** and in male **(B,D)**.

TABLE 3 | Sex-specific, multivariable association of cognitive performance in the memory domain with PCSK9 in subjects at high cardiovascular risk.

Variable added to the model	Association between LnPCSK9 and FDS z-score in female		*P* for interaction lnPCSK9*gender
	Beta	*P*	
–	0.408	0.001	0.002
Triglycerides	0.411	0.000	0.002
Total cholesterol	0.401	0.001	0.002
HDL cholesterol	0.360	0.002	0.003
LDL cholesterol	0.410	0.001	0.001
Leptin**	0.523	0.002	0.000
Blood pressure (SAP and DAP)	0.387	0.001	0.003
Creatinine	0.412	0.001	0.002
MS ATPIII	0.405	0.000	0.003
ACE-I	0.427	0.000	0.001
Statins	0.395	0.002	0.002
Ezetimibe	0.397	0.002	0.003

Data are presented as standardized regression coefficient β. HDL, high density lipoprotein; LDL, low density lipoprotein; SAP, systolic arterial pressure; DAP, diastolic arterial pressure; MS ATPIII, Metabolic Syndrome National Cholesterol Education Program—Adult Treatment Panel III; ACE-I, ACE-inhibitors.
***86 missing for leptin concentrations, 34 in female and 52 in male.*

More intriguing is the hypothesis that the association of PCSK9 with cognitive function in female patients is mediated, at least in part, by its effect on waist circumference. In our cohort, male and female patients, otherwise comparable for most of the clinical characteristics, were significantly different in weight-related anthropometric indices. This was expected since there are large differences in body composition and fat distribution in men vs. women, with women having more body fat and men having a relatively more central distribution of fat (Stevens et al., 2010). This is also reflected by the use of separate waist cut-points by gender (Huxley et al., 2007).

Diverse sources of evidence support a female-specific association between obesity and cognitive impairment. In a longitudinal study obesity has been associated with dementia

more strongly in women (Whitmer et al., 2005) and another study showed that, only in women, for every 1.0 increase in BMI at age 70 years, Alzheimer Disease risk increased by 36% (Gustafson et al., 2003). A negative correlation between PCSK9 and central obesity has been previously described in female (Hasan et al., 2017) and both in experimental models and in humans, PCSK9 deficiency results in increased ectopic fat accumulation (Baragetti et al., 2017).

PCSK9 limits visceral adipogenesis likely via adipose VLDLR regulation (Roubtsova et al., 2011).

Vice versa, in conditions of dysfunctional visceral fat depots PCSK9 is induced by leptin and resistin through the involvement of the inflammatory pathway of STAT3. In HepG2 cells, leptin and resistin up-regulated PCSK9 gene and protein expression (Macchi et al., 2020b). However, at least in our cohort, circulating leptin, although higher in women than men, was not related to either PCSK9 or memory function. Similarly, in our population, the prevalence of metabolic syndrome, although previously associated with PCSK9 (Hasan et al., 2017), is comparable between sexes and does not influence the sex-specific relationship between PCSK9 and memory.

PCSK9 induces CD36 degradation thus limiting fatty acid uptake and triglyceride accumulation in tissues such as adipocytes and mouse liver (Demers et al., 2005). PCSK9 regulates the degradation of VLDLR and ApoER2 too, two receptors implicated in both lipid metabolism and neuronal development (Poirier et al., 2008). A role for PCSK9 on sex- and tissue-specific subcellular distribution of VLDLR, has been described (Roubtsova et al., 2015). These data are consistent with our findings showing a mediation effect by waist circumference on the sex-specific association between PCSK9 circulating levels and short-term memory.

Notably, although men and women differed for several cognitive domains, including executive function and praxic and mental representation skills, short term memory was the only cognitive domain significantly associated with PCSK9 circulating levels, consistent with the experimental data indicating a role for PCSK9 in the pathogenesis of AD (Adorni et al., 2019). While sex-differences in most of cognitive functions may be attributed to sex hormones, as discussed above, or to historical differences in education and cognitive reserve (Bloomberg et al., 2021), memory trajectories appear to be independent of education, and diverse mechanisms should be advocated to explain reported differences. Within the limits of our cross-sectional study, we propose that PCSK9 may play a role in this regard.

Limitations of the present study include the small sample size and the cross-sectional nature of our study, which prevented to assess a cause-and effect relationship between PCSK9 and memory function in female. However, the multivariable analysis allowed us to adjust for potential confounders such as the lipid profile and ongoing medications, which are known to affect PCSK9 circulating levels (Macchi et al., 2019). We also have to acknowledge lack of neuro-imaging analysis to detect and characterize cognitive function and the limited neuropsychological battery used to explore cognitive domains. Lack of a genetic analysis for the identification of PCSK9 mutations is an additional limitation, although loss of function and gain of function mutations are relatively rare in Caucasians. Strengths include the study population comprising a well-characterized sample where most of the clinical, anthropometrical, and biochemical features have been addressed in the analysis.

CONCLUSIONS

In conclusion, our results unraveled a previously unappreciated female-specific relationship between PCSK9 circulating levels and short-term memory in patients at high CV risk largely treated with preventive strategies including aspirin and statins. These findings may shed light on the controversial relationship between PCSK9-inhibitors and cognitive function in previous trials. Indeed, the absence of significant between-group difference in changes in cognitive function in randomized trials involving patients who received either PCSK9 inhibitors or placebo may be due to the high prevalence of male patients (approximately 70%) and to lack of sex-disaggregated analyses (Giugliano et al., 2017), while our results reveal a link between PCSK9 and cognition only in female. Thus, sex-specific sub-analyses may be warranted.

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 University of Chieti (Prot.1129 18.07.2013). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

FS and FV designed and set up the study. PS and FS were involved in patients enrolment. RT, RL, and SC were involved in sample collection and/or analysis. RT and AD performed statistical analysis. FS, FV, RT, PS, and FC were involved in data analysis and interpretation. FS and RT wrote the first draft of the paper. FS, FV, and FC made a critical revision for important intellectual content. FS is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. All authors contributed to the article and approved the submitted version.

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MINI REVIEW
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Cholesterol, Atherosclerosis, and APOE in Vascular Contributions to Cognitive Impairment and Dementia (VCID): Potential Mechanisms and Therapy

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Vascular contributions to cognitive impairment and dementia (VCID) are a common cause of cognitive decline, yet limited therapies exist. This cerebrovascular disease results in neurodegeneration *via* acute, chronic, local, and systemic mechanisms. The etiology of VCID is complex, with a significant impact from atherosclerosis. Risk factors including hypercholesterolemia and hypertension promote intracranial atherosclerotic disease and carotid artery stenosis (CAS), which disrupt cerebral blood flow and trigger ischemic strokes and VCID. Apolipoprotein E (APOE) is a cholesterol and phospholipid carrier present in plasma and various tissues. APOE is implicated in dyslipidemia and Alzheimer disease (AD); however, its connection with VCID is less understood. Few experimental models for VCID exist, so much of the present information has been drawn from clinical studies. Here, we review the literature with a focus on the clinical aspects of atherosclerotic cerebrovascular disease and build a working model for the pathogenesis of VCID. We describe potential intermediate steps in this model, linking cholesterol, atherosclerosis, and APOE with VCID. APOE4 is a minor isoform of APOE that promotes lipid dyshomeostasis in astrocytes and microglia, leading to chronic neuroinflammation. APOE4 disturbs lipid homeostasis in macrophages and smooth muscle cells, thus exacerbating systemic inflammation and promoting atherosclerotic plaque formation. Additionally, APOE4 may contribute to stromal activation of endothelial cells and pericytes that disturb the blood-brain barrier (BBB). These and other risk factors together lead to chronic inflammation, atherosclerosis, VCID, and neurodegeneration. Finally, we discuss potential cholesterol metabolism based approaches for future VCID treatment.

Keywords: cholesterol, atherosclerosis, APOE, vascular dementia, inflammation, glia, macrophage

INTRODUCTION

Vascular contributions to cognitive impairment and dementia (VCID) are defined by cognitive impairment secondary to acute and/or chronic cerebral ischemia and encompass the classical term, vascular dementia (Gorelick et al., 2011; Iadecola et al., 2019). VCID is the most common form of secondary neurodegeneration worldwide; one out of six people with dementia have VCID (van der Flier and Scheltens, 2005). The four subtypes of VCID are: (1) post-stroke, manifesting within 6 months after infarct; (2) subcortical ischemia, including small-vessel occlusion; (3) multi-infarct, including medium-to-large vessel disease; and (4) mixed, incorporating vascular and protein aggregate pathologies (Skrobot et al., 2018). The most common etiology of dementia is Alzheimer disease (AD), a form of cognitive impairment with amnestic-predominant phenotype and the presence of cerebral amyloid and tau protein aggregates (Jack et al., 2018). Compared to the distinct anatomical pattern of AD neurodegeneration originating within the medial temporal lobe, neurodegeneration linked to VCID occurs secondary to focal or global insufficiency of cerebral blood supply. Mixed dementia includes contributions from vascular and other pathologies (often AD) and comprises about 20% of all dementia cases (van der Flier and Scheltens, 2005; Suemoto et al., 2017). Several VCID animal models exist, though they are limited in their capacity to comprehensively recapitulate mixed pathologies/phenotypes in humans (Gooch and Wilcock, 2016). This review article focuses on the clinical aspects of certain risk factors for VCID, i.e., cholesterol, atherosclerosis, and APOE4, as well as the relationship of these risk factors for AD. We hope to offer new insight into disease mechanisms and future therapies (**Figure 1**).

In 1894, Otto Binswanger provided the first clinicopathologic description of VCID. His patients demonstrated focal neurodegeneration where cerebral arteries displayed "extensive atherosclerotic changes," "fatty degeneration," and "thickening of inner and middle vascular membranes." Strikingly, he noted a "primary proliferation of the glial parts" surrounding lipid-filled vessels (Binswanger, 1894; Blass et al., 1991). Thus, VCID is strongly associated with cerebral **atherosclerosis**, the chronic dysfunction of lipid homeostasis, and local inflammation caused by the accumulation of **cholesterol**, **cholesteryl esters (CEs)**, other lipids, and activated stromal cells, including lipid-laden foamy macrophages, endothelial, and smooth muscle cells of vessel walls (Glass and Witztum, 2001; Hansson et al., 2006). Since perivascular astrocytes and pericytes modulate local blood vessel diameter to reflect real-time neuronal activity through "neurovascular coupling" (Attwell et al., 2010), it is reasonable to consider how activated glia and pericytes respond and contribute to intracranial atherosclerosis and ischemia (Binswanger, 1894; MacVicar and Newman, 2015; Price et al., 2018; Fernandez et al., 2019), particularly regarding lipid regulation (Koizumi et al., 2018). Atherosclerosis causes vessel stenosis and occlusion, thereby reducing cerebral blood flow. VCID is often associated with hypercholesterolemia, wherein elevated serum cholesterol promotes a cascade of cerebrovascular cholesterol deposition, inflammation, ischemia, neuronal injury, and cognitive impairment (Solomon et al., 2009; Appleton et al., 2017). We frame atherosclerosis as a condition associated with cerebrovascular lipid deposition and disease of large, medium, and small vessels, including arteriosclerosis and lipohyalinosis. While this review focuses on atherosclerotic influences on VCID, it is important to note that additional vascular etiologies contribute to VCID, including ischemia due to thromboembolism (cardiogenic sources, coagulopathy), chronic hypoperfusion, hypertension, et cetera (Qiao et al., 2017). These factors are covered in several reviews (Gorelick et al., 2011; Santos et al., 2017; Wolters and Ikram, 2019).

Apolipoprotein E (APOE) is a lipid-carrier protein tightly linked to dementia (Strittmatter et al., 1993). The *APOE* ε4 allele (**APOE4**), a minor allele of the *APOE* gene, is associated with a higher risk for AD (>2-fold increased risk for heterozygotes, >9-fold risk for homozygotes) and an elevated risk for VCID (2-fold increased risk for heterozygotes, 3-fold risk for homozygotes) (Rasmussen et al., 2018). Generally, APOE4 is linked to early memory impairment (Caselli et al., 2007), limbic dysfunction (Wolk and Dickerson, 2010), white matter (WM) ischemia (Koizumi et al., 2018), aberrant lipid metabolism, and neuroinflammation (Liu et al., 2013; Rasmussen, 2016; Tzioras et al., 2019). We then explore APOE4 in atherosclerosis and VCID.

WORKING MODEL OF ATHEROSCLEROSIS, APOE, AND VCID

Here, we synthesize clinical data on cholesterol, atherosclerosis, APOE, and VCID into a working model; we posit that atherosclerosis and APOE4 promote reoccurring occlusion and ischemia that eventually lead to VCID. Then, we discuss potential intermediate steps involved, based on emerging research.

Atherosclerosis Is Associated With VCID and AD

Atherosclerosis can be divided into several categories: cerebral atherosclerosis that affects distal microscopic vessels, intracranial atherosclerotic disease (ICAD) that affects cerebral arteries, and large-vessel disease such as carotid artery stenosis (CAS) affecting carotid arteries supplying the brain (**Box 1**, **Supplementary Figure 1**). Myriad clinical data support the link between atherosclerosis and VCID as well as AD. For instance, cerebral autopsies (Beach et al., 2017) and Doppler ultrasound investigations of internal carotid arteries (Hofman et al., 1997) reveal vessel stenosis is more frequently observed in VCID and AD than in normal cognition, with a stronger association between VCID and atherosclerosis than AD with atherosclerosis. Additional studies support the association of atherosclerosis with dementia (Dolan et al., 2010; Dearborn et al., 2017), and with AD particularly (Roher et al., 2011; Yarchoan et al., 2012). In *APOE4* carriers, ICAD/CAS is associated with greater cognitive decline (Haan et al., 1999).

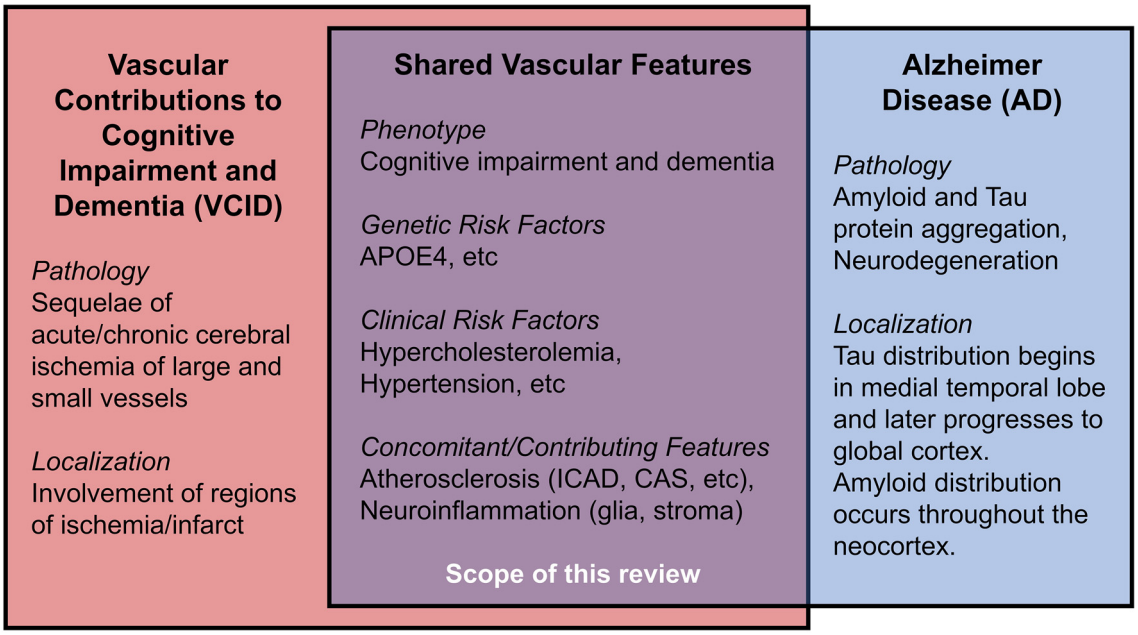

FIGURE 1 | Shared features in Vascular Contributions to Cognitive Impairment and Dementia (VCID) and Alzheimer Disease (AD).

Relationships Between Atherosclerosis, Amyloid, and Tau

Amyloid and tau pathologies are the two distinct criteria for AD; they can also be present in VCID. Patients with suspected VCID and appreciable amyloid/tau biomarkers may have mixed dementia (Skrobot et al., 2018). Alterations in lipid deposition and cholesterol metabolism may modulate amyloid and/or tau pathology (Pappolla et al., 2002). Recent evidence from *in vivo* and *ex vivo* studies suggests that vascular lipid dysregulation and atherosclerosis may be independent dementia risk factors, in addition to their effects on amyloid/tau pathology. Several studies support this view: autopsies indicate ICAD does not correlate with amyloid/tau in either aging patients (Dolan et al., 2010), or in AD cohorts (Kosunen et al., 1995). ICAD severity on magnetic resonance angiography does not correlate cross-sectionally or spatially with *in vivo* cerebral amyloid burden based on positron emission tomography imaging (Gottesman et al., 2020). ICAD and AD elicit similar proteomic alterations in human dorsolateral prefrontal cortex glia and oligodendrocytes (Wingo et al., 2020). Interestingly, ICAD but neither amyloid nor tau pathology was associated with neurodegeneration, as measured by neurofilament light elevation (Iadecola, 2020; Wingo et al., 2020). Thus, amyloid may exert vascular changes through distinct paths from those caused by hypercholesterolemia and ICAD. Cerebral amyloid angiopathy (CAA), the deposition of amyloid aggregates in cerebral vessels, is associated with AD and VCID; moreover, both APOE4 and ICAD are associated with CAA and neurodegeneration (Premkumar et al., 1996; Tian et al., 2004; Yarchoan et al., 2012). While the *APOE*ε2 allele (APOE2) is associated with lower AD risk, it raises CAA risk (Nelson et al., 2013), demonstrating a complex interplay between cholesterol, APOE, and parenchymal vs vascular amyloid. *These data suggest vascular atherosclerosis and AD pathologies are two dissociable yet interacting contributions to neurodegeneration and cognitive decline.*

BOX 1 | Glossary.

Carotid Artery Stenosis (CAS): Narrowing of intracranial, extracranial, and/or common carotid arteries that supply the head, neck, and Circle of Willis of the brain.

Circle of Willis (CoW): A circular network of cerebral arteries fed from carotid and vertebral arteries that deliver blood to the brain.

Intracranial Atherosclerotic Disease (ICAD): Deposition of lipids, debris, and inflammatory cells in the walls of arteries in the skull that supply the brain. This condition is closely associated with VCID.

Ischemia: Loss of blood and oxygen supply to the tissue that can be chronic (atherosclerosis), or acute (sudden occlusion).

Occlusion: Blockage of a vessel leading to ischemia. In the brain, arterial occlusion causes a stroke, the occurrence of sudden neurologic deficit(s) due to inadequate vascular supply.

Stromal Activation: Proliferation and reaction of non-neuronal cells responding to and often exacerbating the neuronal injury. This includes activation of astrocytes, microglia, oligodendrocytes, vessel endothelial cells, pericytes, smooth muscle cells, macrophages, and other immune cells.

Vascular Contributions to Cognitive Impairment and Dementia (VCID): Cognitive impairment or dementia due to acute and/or chronic ischemia, secondary to a stroke, intracranial atherosclerosis, carotid artery stenosis, et cetera or a combination of vascular disease, Alzheimer neuropathologic changes (amyloid/tau), and/or other pathologies.

APOE4 Is Associated With VCID and AD by Promoting Atherosclerosis

There is a clear association between *APOE* genotype and elevated VCID risk, validated through population studies (Slooter et al.,

1997; Chang et al., 2010; Beach et al., 2017; Rasmussen et al., 2018; Pendlebury et al., 2020) and meta-analyses (McCarron et al., 1999). Studies investigating APOE4 in dementia after acute ischemic infarcts suggest that APOE4 impedes stroke recovery and promotes post-stroke VCID (Slooter et al., 1997; Pendlebury et al., 2020; Montagne et al., 2020b). Likewise, *APOE4* carriers have an elevated risk of severe cardiac, extracranial and intracranial atherosclerosis (Mahley and Rall, 2000; Bennet et al., 2007; Granér et al., 2008). These findings are corroborated by post-mortem analyses of atherosclerosis in the Circle of Willis (CoW; **Box 1**; Kosunen et al., 1995; Abboud et al., 2008), and by *in vivo* internal carotid artery imaging studies (Terry et al., 1996; Cattin et al., 1997; Haan et al., 1999; Elosua et al., 2004; Volcik et al., 2006). A meta-analysis of 490 case-control studies also supports the impact of APOE4 on ICAD risk (Wei et al., 2017). Notably, CAS risk may be greater in middle-aged asymptomatic *APOE4* carriers (Cattin et al., 1997), and ICAD may be associated more with male *APOE4* carriers (Elosua et al., 2004; Abboud et al., 2008). The latter finding is distinct from AD pathology, where APOE4 is more strongly linked to AD risk in females (Neu et al., 2017). Overall, the association between APOE and cerebrovascular disease is affected by age and sex (Liu et al., 2013; Beach et al., 2017; Hohnman et al., 2018; Lamar et al., 2019). It should be noted that other studies suggest that APOE4 may not be associated with CoW ICAD severity (Premkumar et al., 1996; Yarchoan et al., 2012; Beach et al., 2017). However, on balance, a substantial plurality of studies strongly indicates a close association exists between APOE4 and ICAD.

REFINING THE MODEL: INTERMEDIATE STEPS

The mechanisms leading from APOE4 to atherosclerosis to VCID are complex. Here, we consider candidate steps that may act as mediators, including serum/brain cholesterol, neurological/systemic inflammation, blood-brain permeability, and vascular aging. This requires the integration of clinical evidence with insight from animal and *in vitro* studies. It is important to note that additional vascular risk factors such as hypertension and smoking also influence dementia risk *via* multiple mechanisms to affect ICAD, stroke, and WM ischemia (Skoog et al., 1998; Kivipelto et al., 2001; Qiao et al., 2017; Koizumi et al., 2018; Nasrallah et al., 2019).

Hypercholesterolemia, APOE4, and Dementia

Hypercholesterolemia is associated with atherosclerotic cardiovascular and cerebrovascular disease (Duncan et al., 2019). Indeed, cohort studies and meta-analyses reveal that hypercholesterolemia at various times in the lifespan significantly increases the risk for ICAD (Ritz et al., 2014) and VCID (Moroney et al., 1999; Reitz et al., 2004). Elevated serum cholesterol may also promote AD, likely through mixed pathologies (Kivipelto et al., 2001; Solomon et al., 2009; Anstey et al., 2017; Wingo et al., 2019; Zhou et al., 2020). Mid-life cholesterol elevation is significantly associated with AD and trended towards elevated risk in VCID (Solomon et al., 2009). Furthermore, patients with familial hypercholesterolemia have a higher risk of mild cognitive impairment (Zambón et al., 2010). It is worth noting that most, but not all studies (Slooter et al., 1999; Mielke et al., 2010), show that hypercholesterolemia significantly raises dementia risk. Atherosclerosis risk and hypercholesterolemia are linked by mechanisms that involve APOE. APOE is present in plasma lipid particles as well as various cell types in the body and brain, as an essential lipid-carrier. It binds to low-density lipoprotein (LDL) receptors and other related receptors (Herz, 2009; Liu et al., 2013). While APOE2 is associated with elevated very-low-density lipoprotein (VLDL) and lower dementia risk (Reiman et al., 2020), APOE4 is linked to higher LDL cholesterol (Beilby et al., 2003; Saito et al., 2004; Hall et al., 2006; Bennet et al., 2007) and increased dementia risk.

Lipid Load in Astrocytes and Microglia, Neuroinflammation, and APOE4

The relationship between glia and lipid deposition traces to the original discoveries of VCID and AD, wherein Alzheimer observed that "many glial cells show adipose saccules" (Alzheimer, 1907; Stelzmann et al., 1995), and Binswanger noted the presence of rich "glial coating" of atherosclerotic vessels displaying "fatty degeneration" (Binswanger, 1894; Blass et al., 1991). Yet, only recently has local lipid deposition regained attention. Post-mortem lipidomic analyses of AD brains reveal deposits of a few selected lipid species, including cholesteryl esters (CEs), sphingomyelin, and ganglioside GM3. These species were enriched in AD-vulnerable regions (i.e., entorhinal and prefrontal cortex), but not cerebellum (Chan et al., 2012).

Astrocytes play essential roles in neuroinflammation and lipid deposition. Cumulatively, evidence indicates that astrocyte activation is linked to intracellular brain lipid accumulation, and APOE4 may exacerbate this response. For instance, human stem cell-derived APOE4 astrocytes display higher intracellular and extracellular cholesterol load, and compromised cholesterol efflux (Lin et al., 2018; Julia et al., 2019), as well as impaired endocytosis of lipids, amyloid, and other proteins, relative to astrocytes without APOE4 (Fernandez et al., 2019; Narayan et al., 2020). Similarly, cholesterol metabolism disruption was observed in human astrocytes expressing AD mutations, and these abnormalities were associated with tau hyperphosphorylation and neuronal toxicity (van der Kant et al., 2019). Lipid accumulation in AD astrocytes might be associated with aberrant acetate/acetyl-CoA metabolism, fatty-acid oxidation, and oxidative stress, which are markers for astrocyte activation (Wyss et al., 2011; Fernandez et al., 2019).

Microglia likely act as neuroinflammatory intermediaries between lipid overload and neurodegeneration. Lipid-laden microglia trigger oxidative stress and release proinflammatory cytokines (Marschallinger et al., 2020). Strikingly, numerous dementia-associated genes (i.e., *APOE*, *ABCA1*, *ABCA7*, *CLU*, *PLCγ2*, *TREM2*) are critical to lipid homeostasis and selectively expressed in microglia (Verheijen and Sleegers, 2018). Studies in mouse models demonstrate that deficiency of either *APOE* or *TREM2* impairs phagocytosis, abrogates clearance of myelin-derived lipids, and promotes CE buildup

(Nugent et al., 2020). ABCA1 is a key mediator of cellular lipid efflux. Variants in *ABCA1* are implicated in cerebrovascular disease and AD (Nordestgaard et al., 2015). Further, *PLCγ2* and *TREM2* form a vital microglial lipid-sensing axis; genetic knockout of either gene in human stem cell-derived microglia models cause accumulation of cholesterol, CEs, and myelin-derived lipids, and perturbed inflammatory response (Andreone et al., 2020). APOE4 contributes to an inflammatory cascade of the neurovascular milieu (Lathe et al., 2014; Fernandez et al., 2019; Tzioras et al., 2019), likely in response to amyloid and lipid load. In APOE4 human microglia models, immunity/inflammation transcriptional pathways are dysregulated (Lin et al., 2018). Interestingly, in AD mouse models, *Apoe* expression occurs in the "late response" phase of microglial activation, possibly indicating a role for microglial *APOE* in chronic neuroinflammation and dementia (Keren-Shaul et al., 2017; Mathys et al., 2017).

Macrophages in Atherosclerosis and Inflammation

Macrophages are innate immune phagocytes found in both systemic and brain tissue. Macrophages display phenotypic plasticity in gene expression and cell function. In mouse models and in human atherosclerotic plaques, macrophages can be categorized into several distinct categories, including resident, proinflammatory, and foamy macrophages (Cochain et al., 2018). The cholesterol-rich, lipid-laden foamy macrophages are hallmarks of the early stages of atherosclerosis (Glass and Witztum, 2001). Surprisingly, foamy macrophages are distinct from other proinflammatory macrophages and even share transcriptional similarities to activated smooth muscle cells in atherosclerotic lesions (Winkels et al., 2018; Zernecke et al., 2020). These foamy macrophages express high levels of *APOE* and *TREM2*, suggesting crucial roles for APOE and TREM2 in maintaining macrophage cholesterol homeostasis (Zernecke et al., 2020). These studies illustrate the diversity and complexity of responses to lipid deposition in various immune and stromal cells. Foam cells in atherosclerosis may be analogous to the aging, cholesterol-rich, lipid-laden glia in the brain; *APOE* and *TREM2* may play key roles in maintaining proper cholesterol homeostasis in the foamy microglia and astrocytes. Importantly, macrophages and glia regularly interact at key interfaces in glymphatic pathways, meningeal lymphatic vessels, and perivascular spaces (Louveau et al., 2017). Such crosstalk is integral to understanding inflammatory and vascular contributions to neurodegeneration.

Commonalities Between VCID and AD: Cholesterol, APOE4, and Inflammation

Lipid deposition (both intracellular and extracellular) and chronic inflammation are shared between atherosclerosis, VCID, and AD. In VCID, cholesterol, CEs, and other lipids accumulate in macrophages and cerebral blood vessels and impair blood flow (**Figure 2A**). In AD, similar lipid species accumulate mainly in glia and impede clearance of amyloid/tau aggregates (**Figure 2B**). In VCID and AD, crosstalk of systemic and neuroinflammatory pathways leads to stromal dysfunction (Holmes et al., 2009; Tao et al., 2018). *Hence, lipid-associated inflammation alters glial, myeloid, and stromal cell interactions, hampers the turnover of accumulated lipid and protein aggregates, and promotes neurodegeneration*. It is important to determine how/when proposed inflammatory cascades occur and how/when lipid overload and APOE4 spur local/systemic inflammation and worsen ICAD, VCID, and AD. Together, cholesterol accumulation, subsequent neuroinflammation, and impaired phagocytosis may be common features across the neurodegenerative spectrum.

Blood-Brain Barrier

Endothelial cells line cerebral capillaries and are linked to a vast network of pericytes and astrocyte end-feet, forming a blood-brain barrier (BBB). This structure selectively restricts the passage of substances from plasma into brain parenchyma. Dysfunction of the BBB is a potential APOE4-mediated pathway toward dementia. Indeed, loss of BBB integrity and subsequent vasogenic edema are common consequences of acute ischemia (Yang et al., 2019). Moreover, chronic activation and retraction of pericytes and endothelial cells may perpetuate pre-existing neurodegeneration in humans (Lau et al., 2020; Montagne et al., 2020a) and animal models (Bell et al., 2012). Various vascular risk factors are also associated with BBB damage (Cortes-Canteli and Iadecola, 2020). Dynamic contrast-enhanced magnetic resonance imaging (MRI) studies illustrate associations between APOE4 and limbic BBB breakdown in AD (Montagne et al., 2020a) and pericyte dysfunction in human stem cell-derived BBB models of CAA (Blanchard et al., 2020). Akin to atherosclerosis, mediation between APOE4 and dementia by the BBB may be unique to amyloid/tau response (Montagne et al., 2020a). Hence, VCID and AD likely share intermediate mechanisms, including ICAD and BBB dysfunction.

Vascular Aging

Aging may contribute to vessel disease and VCID (Ungvari et al., 2018). The relationship between age and VCID is partially attributable to age-related reduction in cholesterol metabolism/clearance, promoting hypercholesterolemia, atherosclerosis, and cognitive changes (Wang and Bennett, 2012; Zlokovic et al., 2020). Additionally, aging may promote hypertension, vessel stiffness, and disease *via* lipid peroxidation, oxidative stress, mitochondrial dysfunction, and senescence of stroma, vasculature, and brain parenchyma (Gustaw-Rothberg et al., 2010; Tarantini et al., 2019; Kiss et al., 2020). These metabolic changes could be exacerbated by APOE4 (Yin et al., 2019).

POTENTIAL FUTURE THERAPIES

Targeting Cholesterol in Cerebral Arteries: Statins and Atherosclerosis Therapy

We now address potential therapeutic targets for both atherosclerosis and dementia. Atherosclerosis management and research have overwhelmingly been guided by stroke outcomes rather than cognition. Nevertheless, because atherosclerosis is a strong risk factor for VCID, cholesterol optimization is a current

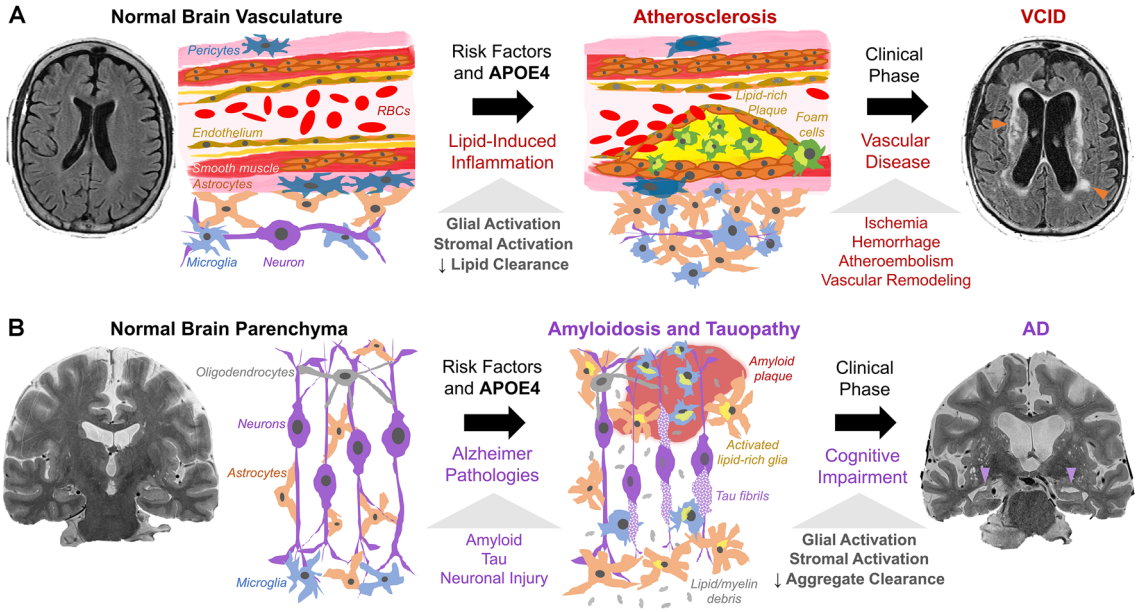

FIGURE 2 | Working model of cholesterol, APOE4, and stromal activation in (**A**) VCID and (**B**) AD. (**A**) Risk factors (hypercholesterolemia, hypertension, smoking, APOE4) trigger atherosclerosis *via* cholesterol-rich plaques with local/systemic inflammation and stromal dysfunction (glia, macrophages, smooth muscle, endothelium, pericytes). Lipid-induced inflammation impairs lipid turnover and exacerbates plaque formation. This leads to ischemia (arrowheads) and dementia as detected by fluid-attenuated inversion recovery MRI. (**B**) Amyloid and tau pathology, with risk factors and APOE4, trigger neuronal injury. Subsequent proteinopathy-associated glial activation and lipid deposits (from glial proliferation and myelin debris) hinder amyloid/tau aggregate clearance and promote accumulation. This leads to aggregate spread and neurodegeneration (arrowheads) as seen on T2-weighted MRI.

treatment strategy for cerebrovascular diseases. Statins lower LDL by inhibiting hydroxymethyl glutaryl-CoA reductase, thus increasing LDL receptor expression through sterol-mediated regulatory response in the liver (Brown and Goldstein, 1986). Though statins are an essential treatment for ICAD, CAS, and stroke, the results for VCID and AD treatments are mixed. While robust randomized controlled trials had not yet been performed for VCID and statins, cohort studies imply that statins may significantly reduce the incidence of VCID in participants with vascular risk factors such as hypercholesterolemia or diabetes (Hajjar et al., 2002; Fei et al., 2013; Giannopoulos et al., 2014). Moreover, statins may mitigate cognitive progression in adults with normal cognition and MCI (Steenland et al., 2013). *APOE4* homozygotes emerged from re-analysis of statin trials with the greatest benefit, including slower cognitive decline and lower dementia incidence (Geifman et al., 2017). A meta-analysis found that statins may significantly lessen the risk of AD and MCI (Chu et al., 2018), and supporting studies trended toward benefit (Smith et al., 2017). However, numerous investigations report that statins do not prevent dementia while those studies that do support usage are often limited by smaller sample sizes (Shepardson et al., 2011a,b; McGuinness et al., 2016). Despite unclear evidence, the clinical co-occurrence of cerebrovascular disease and VCID often favors cholesterol management with statins.

To reduce infarcts that trigger/exacerbate VCID, current ICAD treatment includes cholesterol management, antiplatelet therapy (i.e., aspirin or $P2Y_{12}$ inhibitors), and interventional methods (Chabriat and Bousser, 2006; Flusty et al., 2020). Yet, patients with ICAD on maximum medical therapy have non-negligible vascular risks, i.e., intracranial hemorrhage. One standard approach to treat cerebral ischemia with endovascular stenting even worsens outcomes significantly in ICAD (Chimowitz et al., 2011). Due to mixed findings, current neuro-interventional treatments are indicated for severe, symptomatic CAS only, but not for ICAD or VCID (Flusty et al., 2020). More research is required to better evaluate the possible benefit of ICAD treatment for VCID.

Targeting Cholesterol in Glia and Macrophages: LXR Agonists and ACAT1 Blockade

Statins act mainly in the liver. Beyond statins, additional strategies that act in local tissues exist to modulate cholesterol metabolism in-principle. We cite two examples. Liver X Receptors (LXRs) are essential membrane receptors that regulate cholesterol efflux in macrophages (LXRα) as well as in astrocytes and glia (LXRβ). LXRs represent possible targets for ICAD and VCID. Indeed, activation of LXRs reduces serum cholesterol and increases ABCA1-mediated cholesterol transport in humans and mouse/primate models of atherosclerosis (Calkin and Tontonoz, 2011; Muse et al., 2018), mainly by modulating lipid load in macrophages (Zhang et al., 2014). Further, LXR agonist attenuates amyloid pathology and microglial inflammation in transgenic AD models (Zelcer et al., 2007). However,

current LXR agonists also adversely upregulate fatty-acid and triglyceride syntheses in mice and humans (Kirchgessner et al., 2016; Muse et al., 2018). Therefore, clinical translation may require investigation of the net impact of these opposing effects.

CEs are storage forms of cholesterol. In foamy macrophages, like those found in atherosclerotic lesions, CEs accumulate by acyl-CoA:cholesterol acyltransferase (ACAT, aka sterol O-acyltransferase, SOAT) converting cholesterol to CEs, and by cholesterol hydrolases cleaving CEs back to cholesterol (Brown et al., 1980). ACAT1 and ACAT2 enzymes are encoded by *SOAT1* (Chang et al., 1993) and *SOAT2* genes (Anderson et al., 1998; Cases et al., 1998; Oelkers et al., 1998). Both enzymes are integral endoplasmic reticulum membrane proteins; both are allosterically activated by cholesterol or oxysterols, and act on various sterols and long-chain fatty-acyl-CoAs as substrates (reviewed in Rogers et al., 2015). In healthy humans, ACAT1 is ubiquitously expressed across peripheral and brain tissue, whereas ACAT2 is mostly expressed in enterocytes and hepatocytes (reviewed in Chang et al., 2009). Cell models show that unlike statins (which inhibit cholesterol efflux), ACAT1 blockade promotes cholesterol efflux *via* ABCA1 mediated lipid efflux (Yamauchi et al., 2004). Mouse studies portray that reduction in myeloid ACAT1 alleviates diet-induced atherosclerosis (Huang et al., 2016; Melton et al., 2019). In AD studies, ACAT inhibitors significantly reduced amyloid accumulation in cell culture (Puglielli et al., 2001) and mouse models (Hutter-Paier et al., 2004). ACAT1/*SOAT1* gene knockout in mice reduces amyloid (Bryleva et al., 2010) and tau burden (Shibuya et al., 2015a), by stimulating autophagy in microglia and neurons (Shibuya et al., 2014, 2015b). Additionally, ACAT1 inhibition rescues CE accumulation in human AD neurons (van der Kant et al., 2019) and human stem cell-derived microglia with *TREM2* gene ablation (Nugent et al., 2020). These studies suggest that ACAT1 is a potential target to address atherosclerosis and proteinopathy in VCID or mixed dementia. Moreover, in a mouse model for the Niemann-Pick type C disease, a neurological disease caused by primary genetic defects in cholesterol homeostasis, ACAT1 blockade diverts cholesterol from storage to promote more efficient utilization in neural and peripheral tissues, suggesting that targeting ACAT1 is a potential strategy for multiple neurological diseases that involve cholesterol dyshomeostasis (Chang et al., 2020). Humans ACAT1/*SOAT1* genetic variant analyses also support the role of ACAT1 in modulating dementia susceptibility (Wollmer et al., 2003; Alavez-Rubio et al., 2021).

APOE-Based Therapy

Investigational dementia therapies include targeting APOE directly, such as reducing APOE4 expression, with antibodies (Xiong et al., 2021), anti-sense oligonucleotides (Huynh et al., 2017), gene therapy, and gene editing (Liu et al., 2013; Yamazaki et al., 2016). In fact, an individual harboring a frameshift mutation ablating *APOE* expression had relatively normal cognition (Mak et al., 2014), possibly indicating treatment safety in altering *APOE* expression. To this end, adenoviral APOE2 knock-in approaches have already been tested in mice and in non-human primates (Williams et al., 2020). Small-molecule approaches include inhibiting deleterious APOE functions and correcting aberrant APOE structure induced by ε4 polymorphisms and associated with elevated amyloid, tau, and lipids (Mahley and Huang, 2012; Wang et al., 2018). Although it is still unclear whether APOE4 raises dementia risk by a gain of toxic function and/or loss of protective function (Kim et al., 2009; Serrano-Pozo et al., 2021), APOE-based therapies might address both mechanisms at local and systemic levels.

CONCLUSION

Neurodegeneration due to vascular etiologies is multifactorial and complex. Here, we describe a working model linking cholesterol load with atherosclerosis, APOE4, and VCID. At the cellular level, we posit that cholesterol load causes chronic neuroinflammation in microglia and astrocytes and triggers systemic inflammation in macrophages. Crosstalk between neuroinflammatory and systemic inflammatory signaling leads to stromal dysfunction. APOE4 may exacerbate local cellular cholesterol dyshomeostasis and stimulate glial/stromal inflammation, metabolic dysfunction, and BBB breakdown. At the macroscopic level, these changes promote atherosclerosis, ischemia, and cerebrovascular disease, ultimately contributing to VCID. Moreover, vascular changes, cholesterol accumulation, inflammation, and impaired turnover of protein and/or lipid aggregates are shared across neurodegeneration. We review potential therapies targeting cholesterol metabolism in astrocytes, microglia, and macrophages, though our understanding is far from complete. Future research may delineate VCID pathophysiology towards effective treatment.

AUTHOR CONTRIBUTIONS

MD, T-YC, and CC conceived the project. MD wrote the initial version of the review and received inputs from IN, DW, CC, and T-YC for revisions. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnagi.2021.647990/full#supplementary-material.

SUPPLEMENTARY FIGURE 1 | Anatomy of cerebral arteries, including the (**A,B**) carotid arteries and (**C,D**) Circle of Willis (CoW), also shown on computed tomography arteriography (**B,D**). Illustrations adapted from Henry Gray's *Anatomy of the Human Body* (1918), www.bartleby.com.

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Elevated HDL Levels Linked to Poorer Cognitive Ability in Females With Parkinson's Disease

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Introduction: Cholesterol levels have been associated with age-related cognitive decline, however, such an association has not been comprehensively explored in people with Parkinson's disease (PD). To address this uncertainty, the current cross-sectional study examined the cholesterol profile and cognitive performance in a cohort of PD patients.

Methods: Cognitive function was evaluated using two validated assessments (ACE-R and SCOPA-COG) in 182 people with PD from the Australian Parkinson's Disease Registry. Total cholesterol (TC), high-density lipoprotein (HDL), low-density lipoprotein (LDL), and Triglyceride (TRG) levels were examined within this cohort. The influence of individual lipid subfractions on domain-specific cognitive performance was investigated using covariate-adjusted generalised linear models.

Results: Females with PD exhibited significantly higher lipid subfraction levels (TC, HDL, and LDL) when compared to male counterparts. While accounting for covariates, HDL levels were strongly associated with poorer performance across multiple cognitive domains in females but not males. Conversely, TC and LDL levels were not associated with cognitive status in people with PD.

Conclusion: Higher serum HDL associates with poorer cognitive function in females with PD and presents a sex-specific biomarker for cognitive impairment in PD.

Keywords: Parkinson's disease, cognitive decline, cognitive impairment, domain-specific, cholesterol, HDL-cholesterol, sex-specific

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INTRODUCTION

Parkinson's disease (PD) is the world's second most common neurological disease and is associated with a variety of motor and non-motor symptoms. Non-motor symptoms, including cognitive impairment and dementia, are commonly reported as being equally as debilitating as cardinal motor symptoms (Triantafyllou et al., 2008; Tarolli et al., 2019). Longitudinal studies indicate that nearly 50% of people with PD develop some degree of mild cognitive impairment (MCI) after

10 years of the disease. This proportion rises to over 80% after 20 years of disease course, which is particularly disturbing as PD-MCI itself is associated with impaired quality of life, increased caregiver burden, and increased risk of progression to dementia (PDD) (Hely et al., 2008; Aarsland and Kurz, 2010; Cosgrove et al., 2015). As such, developing objective biological measures associated with the progression of disease-associated cognitive decline will greatly assist in clinical practice.

As a well-established risk factor for cerebrovascular and cardiovascular disease (CVD) (Goldstein and Brown, 2015; Leritz et al., 2016; Mansoor et al., 2016a), cholesterol profiles and metabolism are being increasingly implicated in age-related cognitive impairment, vascular dementia, and Alzheimer's disease. Interestingly, within the context of PD, a number of studies have indicated that higher serum cholesterol levels are associated with reduced risk of PD, and that certain lipid subfractions are lower in individuals with PD when compared to healthy controls (Huang et al., 2011; Rozani et al., 2018; Kummer et al., 2019; Choe et al., 2021). Serum cholesterol is comprised of a number of lipid subfractions including low-density lipoproteins (LDLs) and high-density lipoproteins (HDLs). When in excess, unused LDL subfractions are commonly deposited in the arteries (Goldstein and Brown, 2015; Mansoor et al., 2016a,b) and for this reason have been associated with poor cardiovascular outcomes. However, in more recent larger studies, lower LDL levels have been associated with age-associated dementia, indicating a possible protective effect of this form of cholesterol (Zhou et al., 2018).

In contrast, HDL is thought to have anti-oxidative, anti-inflammatory and cardioprotective capabilities (Hottman et al., 2014). For instance, some studies have tied elevated HDL levels with superior cognitive function (Henderson et al., 2003; Reitz et al., 2010; Crichton et al., 2014; Svensson et al., 2019) and low HDL levels being associated with decreased hippocampal volume (Wolf et al., 2004; Ledreux et al., 2016). A recent cross-sectional study investigated the relationship between serum lipid profile and cognitive function in healthy ageing women, with elevated HDL level being identified as a marker for improved cognition, better verbal memory and superior learning ability (Bates et al., 2017). While associated with disease risk of a number of neurodegenerative diseases (Martín et al., 2014), there is a paucity of studies of this nature within the context of cognitive decline in PD. While one recent study examined the association between lipid levels and PD-related symptoms, no significant associations were noted (Choe et al., 2021). However, an increasing body of literature indicates notable sex-differences among various aspects of the clinical presentation of PD (Augustine et al., 2015; Liu et al., 2015; Bakeberg et al., 2020a), and cognitive impairment specifically (Bakeberg et al., 2019, 2021). Thus, it is of utmost importance to examine these associations in light of such sex-differences.

As an individual's lipid profile is known to be a relatively modifiable target for intervention and prevention through non-invasive means, coupled with growing evidence implicating cholesterol levels in cognition, exploring the association between lipid profile and cognitive impairment in PD is of major clinical significance and therapeutic potential. Although an individual's sex is identified to significantly change the trajectory of cognitive decline (Song et al., 2014; Nicoletti et al., 2017; Cholerton et al., 2018; Reekes et al., 2020), it is often disregarded (Leritz et al., 2016; de Oliveira et al., 2017), and rarely controlled for Reitz et al. (2010); Crichton et al. (2014), Sterling et al. (2016); Svensson et al. (2019). Here, we focussed on cholesterol markers (TC, LDL, and HDL levels) that have previously been examined in the context of cognitive impairment, and whether or not there was a sex-specific effect of these lipid fractions on cognitive ability in people with PD.

MATERIALS AND METHODS

Participants

One hundred and eighty-two community-based individuals with PD (114 males and 68 females) were sequentially recruited, as previously described (Evans et al., 2017; Riley et al., 2018; Bakeberg et al., 2019). In brief, participants were recruited from Movement Disorders Clinics at the Perron Institute for Neurological and Translational Science (Perth, WA, Australia) and St. Vincent's Hospital (Melbourne, VIC, Australia), between 2012 and 2015. All individuals with PD were examined by a movement disorder neurologist prior to inclusion in the study for verification of the diagnosis in accordance with the United Kingdom Brain Bank criteria for idiopathic PD (Hughes et al., 1992). This study was approved by a Human Research and Ethics Committee (Approval number 2006/073), and written informed consent was obtained from all participants, in accordance with the National Health and Medical Research Council guidelines.

Clinical Assessments of Participants

Clinical evaluations included collection of patient demographic variables and medication dosage, assessments of motor and cognitive function, and other disease-related features (**Table 1**). All PD medications were converted to a total levodopa equivalent daily dose (LEDD), based on a previously reported conversion equation (Parkin et al., 2002; Tomlinson et al., 2010). Motor symptoms were evaluated in the "ON" state using the Movement Disorder Society-Unified Parkinson's Disease Rating Scale (MDS-UPDRS) Part III (Goetz et al., 2007). In addition, each participant was evaluated by a clinical psychologist and completed a panel of neuropsychological assessments, as previously described (Evans et al., 2017; Bakeberg et al., 2019). Briefly, global cognitive function was assessed using the Scales of Outcomes in Parkinson's disease – Cognition (SCOPA-Cog), and global and domain-specific cognition was also assessed using the revised "Addenbrooke's Cognitive Examination" (ACE-R 2004). The SCOPA-Cog is a reliable and validated tool to assess cognitive function, specifically within PD (Marinus et al., 2003), whereby scores can range between 0 and 43, with higher scores representing superior cognitive performance. Studies have determined that scores of 30 or less may be considered accurately representative of MCI (Marras et al., 2013). Whereas, the ACE-R provides an evaluation of global cognitive function, as well as domain-specific assessment of attention and orientation,

TABLE 1 | Clinical characteristics of the PD cohort used in this study, when combined and when compared by sex.

Clinical characteristics		Mean (SD) or *n* (%)			
		Combined (*n* = 182)	Males (*n* = 114)	Females (*n* = 68)	Significance [a] (*p*-value; Cohen's *d*)
Age (years)		64.5 (9.5)	64.9 (10.4)	63.9 (7.8)	*p = 0.236; d* = 0.11
Age at onset (years)		55.4 (10.5)	55.4 (11.4)	55.3 (8.8)	*p = 0.997; d* = 0.01
Disease duration (years)		9.2 (6.0)	9.5 (6.4)	8.6 (5.6)	*p = 0.473; d* = 0.15
LEDD (mg/day)		942.1 (622.6)	932.9 (618.4)	957.3 (633.7)	*p = 0.883; d* = 0.04
DBS	Yes	26 (14.3%)	19 (16.7%)	7 (10.3%)	***p < 0.001***
	No	156 (85.7%)	95 (83.3%)	61 (89.7%)	
DA	Yes	87 (47.8%)	52 (45.6%)	32 (47.1%)	*p = 0.603*
	No	94 (51.6%)	63 (54.4%)	35 (51.5%)	
MDS-UPDRS	III	20.6 (14.9)	22.5 (14.7)	17.4 (14.7)	***p = 0.005****; d* = 0.35
ACE-R	Total	85.3 (13.4)	84.2 (14.7)	87.0 (11.0)	*p = 0.274; d* = 0.22
SCOPA-Cog	Total	26.5 (8.1)	25.8 (8.4)	27.3 (7.6)	*p = 0.324; d* = 0.19

[a] *Independent Samples test, Mann–Whitney U test or Spearman's Chi Square test conducted for between group analyses, based on sex.*
LEDD, Levodopa Equivalent Daily Dose; DBS, deep brain stimulation; DA, dopamine agonists; MDS-UPDRS III, Movement Disorder Society-Unified Parkinson's Disease Rating Scale III; ACE-R, Addenbrooke's Cognitive Examination – Revised; SCOPA-Cog, Scales for Outcomes in Parkinson's Disease Cognition.
Bolding to highlight statistical significance. These italic values are stylistic.

memory, verbal fluency, language and visuospatial-perceptual ability (Mioshi et al., 2006; Bakeberg et al., 2020b). Cut-off ACE-R scores of ≤88 out of 100 have been used previously as an indicator for probable cases of MCI (Marras et al., 2013).

Serum Analysis

Fasted participant blood samples were collected prior to clinical and psychological assessments. For blood collection, 10 ml of whole blood was taken from a median cubital vein, and stored in a standard BD EDTA vacutainer® (Becton Dickinson and Company, Franklin Lakes, NJ, United States). Serum TC, LDL, HDL, and triglyceride (TRG) levels were assessed via routine lipid biochemistry, as conducted at State Pathology centres. Normal/recommended serum subfraction levels are as follows: TC, 2.9–5.5 mmol/L; LDL, 1.7–3.5 mmol/L; HDL, 1.0–2.9 mmol/L; and TRG, 0.2–2.0 mmol/L (Tonkin et al., 2005; Bates et al., 2017).

Statistical Methods

Data was analysed using IBM-SPSS (v. 26, IBM Corporation). Variables were described using mean ± standard deviation (SD), or frequency and percent (%), as appropriate. A significant nominal *p*-value of <0.05 was employed for all statistical tests. Continuous variables distributions were assessed using the Shapiro–Wilk test of normality. Where appropriate, univariate regression analysis or Mann–Whitney *U* analysis was performed to identify differences between groups. Cohen's *d* ES were calculated for the mean differences, with an ES of 0.20 considered small, 0.50 medium and 0.80 large.

To investigate associations between cholesterol and cognitive scores, male and female participants were analysed separately. Generalised linear models (GLMs) were used as univariate models to investigate association between cognitive outcome measures and cholesterol serum markers. Multivariable adjusted GLMs were used for analysing relationships between lipids and cognitive function, while taking covariates into account. Variables considered as covariates included those identified in grouped analyses (being DBS and MDS-UPDRS III), as well as other variables known to influence cognitive performance, such as age at time of assessment, age of onset, disease duration, and parkinsonian medications. All variables were included in GLMs to determine whether they were significantly associated with cognitive outcome measures, prior to inclusion as a covariate in multivariable GLMs. Residual plots were examined for all models and no violations were noted.

RESULTS

Cohort Information

Clinical characteristics of the PD cohort are summarised in **Table 1**. This cohort was predominantly male (62.6%), with mean age at assessment of 64.5 years, mean disease duration of 9.2 years and mean age at PD onset of 55.4 years, and these did not vary significantly when separated by sex. Males had a significantly higher mean MDS-UPDRS part III score (22.6 ± 15 vs. 17.4.9 ± 15; $p = 0.005$, males vs. females) and were also more likely to have received DBS treatment (16.7% vs. 10.3%; $p = 0.001$) (**Table 1**). There were no significant sex-specific differences in PD medication treatment, specifically no differences in LEDD or DA dosage. Importantly, there were no mean differences in cognitive performance between male and female cohorts, as determined by ACE-R or SCOPA-Cog assessments (**Table 1**). Subsequent analyses of relationships between lipids and cognitive function therefore included MDS-UPDRS III scores and DBS status as covariates, as well as other variables which influence cognitive performance (age at assessment, age of PD onset, and disease duration).

Sex-Based Differences Within Lipid Profile of PD Cohort

To explore the association of lipids with cognitive performance in light of recognised sex differences in lipid profiles, a total of 114 males and 68 females with PD were included. When

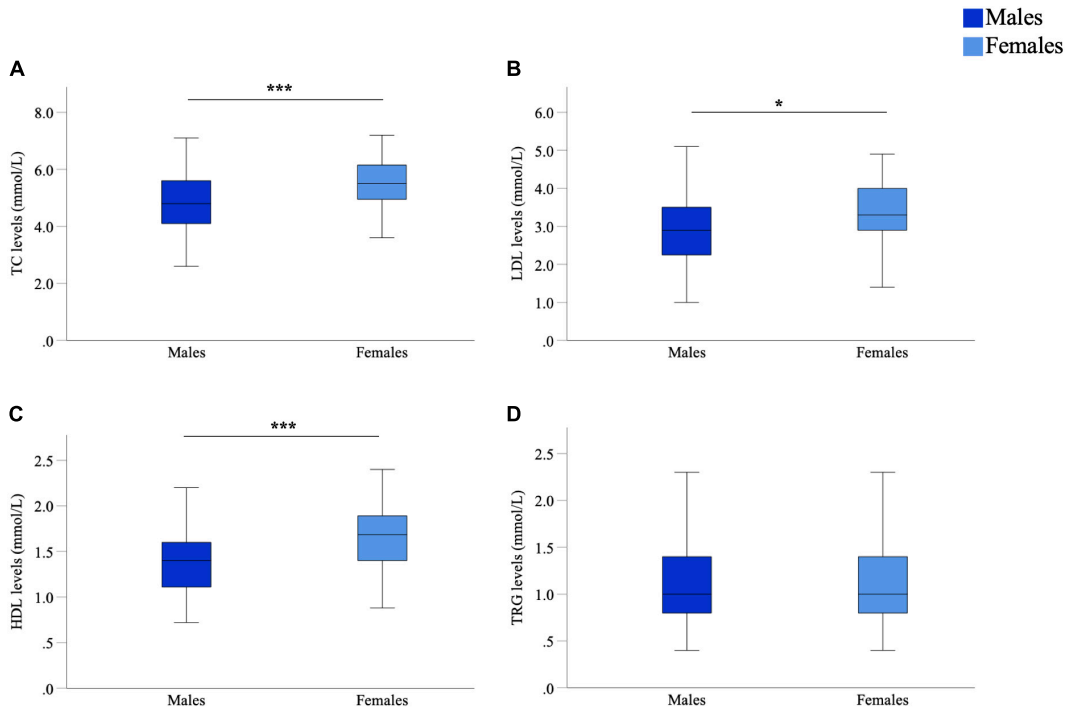

FIGURE 1 | Comparison of serum marker levels between males and females in a Parkinson's disease (PD) cohort. Mean TC **(A)**, LDL **(B)**, and HDL **(C)** levels were significantly higher in females than males, mean TRG **(D)** levels were not significantly different. Data are presented as mean ± SEM. *$p < 0.05$; ***$p < 0.001$. TC, total cholesterol; LDL, low-density lipoprotein; HDL, high-density lipoprotein; TRG, triglyceride.

examining mean lipid profiles of the whole cohort, TC was 5.1 mmol/L (±1.0), LDL was 3.1 (±0.9), HDL levels were 1.5 (±0.4), and TRG was 1.1 mmol/L (±0.5); all of which fall within normal reported ranges of serum lipid profile levels (TC, 2.9–5.5 mmol/L; LDL, 1.7–3.5 mmol/L; HDL, 1.0–2.9 mmol/L; and TRG, 0.2–2.0 mmol/L) (Tonkin et al., 2005; Bates et al., 2017).

As sex-specific differences in lipid profiles are often reported in healthy populations, we compared sex differences in lipid profiles of this cohort of people with PD. We observed significant differences between male and female participants in TC ($p < 0.001$, $d = 0.62$), LDL ($p = 0.012$, $d = 0.35$) and HDL ($p < 0.001$, $d = 0.83$) levels (**Figure 1**). Such a difference could not be said for TRG levels ($p = 0.455$, $d = 0.06$) (**Figure 1D**). Specifically, males had lower mean lipid levels than females for TC (4.92 vs. 5.53 mmol/L, male vs. female), LDL (3.01 vs. 3.33 mmol/L) and HDL levels (1.40 vs. 1.71 mmol/L). As such, to accurately explore the effects of lipids on cognitive performance in people with PD, separated analyses based upon participant sex was considered imperative and were employed for further statistical analyses concerning TC, LDL and HDL levels. Whereas, analysis of TRG levels was conducted with males and females being combined.

Cognitive Performance and TC Levels

Regardless of sex, levels of TC were not significantly associated with measures of cognition (total scores or subdomains) in naïve GLMs in most instances, although there was a significant association within the domain of attention and orientation in female participants ($p = 0.030$; **Table 2**). When considering covariates, TC was significantly associated with the ACE-R subdomains of attention and orientation ($p = 0.017$) and visuospatial domain ($p = 0.011$) within females with PD (**Table 2**). Conversely, TC levels were not significantly associated with cognitive status in males, in either unadjusted or covariate adjusted comparisons. Whereas, in females, certain significant associations were evident between TC levels and cognitive status in adjusted comparisons.

Cognitive Performance and LDL Levels

Levels of LDL were not significantly associated with any measure of cognition in naïve GLMs, regardless of sex (**Table 3**). However, LDL levels of females with PD were significantly associated with the visuospatial domain of the ACE-R assessment, in a model adjusting for covariates ($p = 0.013$). On the other hand, LDL levels were not significantly associated with cognitive status of males, in either unadjusted or adjusted comparisons.

Cognitive Performance of Females, but Not Males, Is Significantly Affected by HDL Levels

In males, univariate analyses revealed HDL was not associated with cognition scores. In models adjusted for covariates,

TABLE 2 | Cognitive performance in participants based on TC levels, when separated by sex.

Variable	Unadjusted		Adjusted	
Males (*n* = 114)	**β-Co (SEM)**	***p*[a] value**	**β-Co (SEM)**	***p*[b] value**
Total ACE-R	1.678	0.227	0.693	0.611
Attention and orientation	0.121	0.506	−0.039	0.822
Memory	0.521	0.348	0.184	0.734
Fluency	0.554	0.154	0.385	0.310
Language	0.212	0.338	0.089	0.689
Visuospatial	0.173	0.575	0.043	0.891
Total SCOPA-Cog	0.837	0.273	0.312	0.687
Females (*n* = 68)	**β-Co (SEM)**	***p*[a] value**	**β-Co (SEM)**	***p*[b] value**
Total ACE-R	−1.253	0.374	−2.086	0.135
Attention and orientation	−0.543	**0.030**	−0.611	**0.017**
Memory	−0.050	0.938	−0.567	0.382
Fluency	0.092	0.844	−0.096	0.844
Language	−0.282	0.231	−0.165	0.448
Visuospatial	−0.473	0.088	−0.650	**0.011**
Total SCOPA-Cog	−0.531	0.586	−0.616	0.500

[a]*p-value taken from GLM without adjustment for covariates.*
[b]*p-value taken from GLM adjusted for DBS, MDS-UPDRS III, age at time of assessment, age of onset and disease duration.*
ACE-R, Addenbrooke's Cognitive Examination – Revised; SCOPA-Cog, Scales for Outcomes in Parkinson's Disease Cognition.
Bolding to highlight statistical significance.

TABLE 3 | Cognitive performance in participants based on LDL levels, when separated by sex.

Variable	Unadjusted		Adjusted	
Males (*n* = 114)	**β-Co (SEM)**	***p*[a] value**	**β-Co (SEM)**	***p*[b] value**
Total ACE-R	2.150	0.164	1.187	0.433
Attention and orientation	0.204	0.311	0.065	0.736
Memory	0.645	0.297	0.275	0.648
Fluency	0.557	0.199	0.396	0.349
Language	0.324	0.188	0.214	0.389
Visuospatial	0.283	0.410	0.169	0.631
Total SCOPA-Cog	1.068	0.207	0.515	0.550
Females (*n* = 68)	**β-Co (SEM)**	***p*[a] value**	**β-Co (SEM)**	***p*[b] value**
Total ACE-R	0.204	0.891	−0.648	0.667
Attention and orientation	−0.276	0.308	−0.311	0.271
Memory	0.453	0.500	−0.131	0.850
Fluency	0.423	0.387	0.248	0.633
Language	0.066	0.792	0.224	0.330
Visuospatial	−0.461	0.115	−0.676	**0.013**
Total SCOPA-Cog	0.569	0.580	0.418	0.668

[a]*p-value taken from GLM without adjustment for covariates.*
[b]*p-value taken from GLM adjusted for DBS, MDS-UPDRS III, age at time of assessment, age of onset and disease duration.*
ACE-R, Addenbrooke's Cognitive Examination – Revised; SCOPA-Cog, Scales for Outcomes in Parkinson's Disease Cognition.
Bolding to highlight statistical significance.

there remained no association between HDL and cognitive scores (**Table 4**). In contrast, in females, univariate analysis revealed HDL levels were significantly associated with ACE-R subdomains, attention and orientation, memory, fluency, language and total SCOPA-Cog, but not with visuospatial cognition. In models adjusting for covariates, HDL levels in females were significantly associated with total ACE-R score ($p = 0.001$), ACE-R subdomain scores of attention and orientation ($p = 0.001$), memory ($p = 0.026$), fluency ($p = 0.023$) and language ($p < 0.001$); and total SCOPA-Cog score ($p = 0.004$). However, the visuospatial domain did not exhibit a significant association with HDL levels in females, regardless of covariate adjustment ($p = 0.351$, **Table 4**).

TABLE 4 | Cognitive performance in participants based on HDL levels, when separated by sex.

Variable	Unadjusted		Adjusted	
Males (*n* = 114)	**β-Co (SEM)**	***p*[a] value**	**β-Co (SEM)**	***p*[b] value**
Total ACE-R	0.808	0.849	−1.169	0.776
Attention and orientation	−0.222	0.687	−0.584	0.261
Memory	0.664	0.695	−0.076	0.963
Fluency	1.468	0.215	0.857	0.454
Language	−0.416	0.538	−0.428	0.524
Visuospatial	−0.229	0.808	−0.470	0.622
Total SCOPA-Cog	1.670	0.489	0.848	0.712
Females (*n* = 68)	**β-Co (SEM)**	***p*[a] value**	**β-Co (SEM)**	***p*[b] value**
Total ACE-R	−9.998	**0.001**	−9.571	**0.001**
Attention and orientation	−1.781	**0.002**	−1.900	**0.001**
Memory	−3.187	**0.029**	−3.165	0.026
Fluency	−2.667	**0.011**	−2.401	**0.023**
Language	−1.779	**0.001**	−2.053	**<0.001**
Visuospatial	−0.594	0.371	−0.558	0.351
Total SCOPA-Cog	−5.879	**0.007**	−5.545	**0.004**

[a]*p-value taken from GLM without adjustment for covariates.*
[b]*p-value taken from GLM adjusted for DBS, MDS-UPDRS III, age at time of assessment, age of onset and disease duration.*
ACE-R, Addenbrooke's Cognitive Examination – Revised; SCOPA-Cog, Scales for Outcomes in Parkinson's Disease Cognition.
Bolding to highlight statistical significance.

TABLE 5 | Cognitive performance in participants based on TRG levels, not separated by sex.

Variable	Unadjusted		Adjusted	
Males and Females (*n* = 182)	**β-Co (SEM)**	***p*[a] value**	**β-Co (SEM)**	***p*[b] value**
Total ACE-R	0.466	0.825	0.287	0.888
Attention and orientation	0.047	0.876	0.029	0.922
Memory	−0.020	0.982	−0.069	0.935
Fluency	0.251	0.685	0.272	0.655
Language	−0.140	0.681	−0.161	0.631
Visuospatial	0.041	0.928	−0.022	0.962
Total SCOPA-Cog	−1.083	0.390	−0.832	0.490

[a]*p-value taken from GLM without adjustment for covariates.*
[b]*p-value taken from GLM adjusted for DBS, MDS-UPDRS III, age at time of assessment, age of onset and disease duration.*
ACE-R, Addenbrooke's Cognitive Examination – Revised; SCOPA-Cog, Scales for Outcomes in Parkinson's Disease Cognition.

Cognitive Performance and TRG Levels

Levels of TRG were not significantly associated with any measure of cognition in both unadjusted and adjusted GLMs (**Table 5**).

DISCUSSION

Previous studies have indicated a link between cholesterol levels and metabolism, and age-associated cognitive impairment and dementia. Sex-specific differences in cholesterol levels complicate studies on cognitive performance, with only a few studies considering males and females separately (Walhovd et al., 2014; Bates et al., 2017; Zhao et al., 2019) or controlling for sex as a covariate (Reitz et al., 2010; Crichton et al., 2014; Sterling et al., 2016; Svensson et al., 2019; Choe et al., 2021). In the current study, females with PD showed higher LDL, HDL, and TC levels when compared to males with PD. Such differences are consistent with studies on healthy aged populations (Bates et al., 2017; Zhou et al., 2018; Zhao et al., 2019) and individuals with PD (Sterling et al., 2016). However, the current study is the first to investigate sex-specific differences in lipids and cognition in people with PD, measuring multiple lipid fractions and using two validated cognitive assessment protocols.

The present study reports a novel association between higher HDL cholesterol levels and poorer cognitive function among females with PD, but not males. Furthermore, in females, HDL cholesterol was associated with poorer performance in all global and domain-specific assessments of cognition except one, being total SCOPA-Cog, total ACE-R, and ACE-R subdomains of attention and orientation, memory, fluency and language. Such consistent associations are suggestive of a robust association between HDL cholesterol and cognition in females with PD. However, conflicting findings exist within this area of research, as multiple healthy elderly cohort studies report that plasma HDL levels are protective and are associated with retained cognitive function (Van Exel et al., 2002; Reitz et al., 2010; Crichton et al., 2014; Bates et al., 2017; Kinno et al., 2019; Svensson et al., 2019), while others exhibit no link between HDL levels and incidence of cognitive impairment and dementia (Yaffe et al., 2002; Li et al., 2005; Zhou et al., 2018). Our findings therefore point to a disease-specific influence of serum cholesterols on cognition. Coupled with the current finding of a sex-specific influence of HDL on cognition in a cohort of PD, serum cholesterols appear to be mediators of cognition in PD, though the disease-specific and sex-specific mechanisms are still unclear.

As aforementioned, sex is an important factor to take into consideration when studying cognitive ability (Song et al., 2014; Nicoletti et al., 2017; Cholerton et al., 2018; Reekes et al., 2020; Bakeberg et al., 2021). In general, it is widely accepted that non-verbal and verbal reasoning skills such as language and fluency, decision-making and memory are cognitive strengths of females when compared to males in healthy cohorts (Li and Singh, 2014), and among people with PD (Bakeberg et al., 2021), which may be the result of sex-specific structural and functional connectivity networks (Lin et al., 2018). Such sex-specific differences in neurobiology likely underpin the novel finding reported herein, though sex-specific mechanisms underlying the differential influence of lipid profile on cognition are still largely unknown. Literature suggests a number of potential mechanisms, including sex differences in lipid transportation and age-related lipid changes (Knopp et al., 2006; Casiglia et al., 2008), as well as genetic differences in lipid metabolism and steroid hormone synthesis in males and females (Sowers et al., 2006), and an association between elevated cholesterol levels and α-synuclein-related cognitive impairment (Allinquant et al., 2014; Lu et al., 2017; Jin et al., 2019). Notably, another plausible mechanism relates to the neuroprotective effects of female sex hormones, outlined below.

Within this cohort, our findings were specific to females likely to be in the peri- or post-menopausal period. While the neuroprotective properties of oestrogen are well-established (Miller and Cronin-Golomb, 2010; Lin et al., 2018), such effects are known to be altered during the peri-menopausal transition, and recent studies indicate that the transition to menopause can also trigger chronic low-grade inflammation (Yin et al., 2015; El Khoudary et al., 2018) and elevated systemic levels of inflammatory cytokines (e.g., IL-2, IL-4, and IL-6) (Giuliani et al., 2001; Yasui et al., 2007; Mishra and Brinton, 2018). Additionally, it is increasingly thought that low-grade, chronic inflammation occurs within people with PD, and that it is central in the genesis and pathophysiology of PD (Houser and Tansey, 2017; Gorecki et al., 2019, 2020). Importantly, it has been reported that the generally accepted "good" HDL cholesterol may become dysfunctional in instances of elevated inflammation, losing its anti-inflammatory and cardioprotective properties (Van Lenten et al., 2001; Ansell et al., 2007; Navab et al., 2009; Hb et al., 2011; El Khoudary et al., 2018; Nazir et al., 2020). As such, we propose that within females in this PD cohort, HDL cholesterols may have become dysfunctional and lost their protective effects. While the current study reports an inverse association between HDL-cholesterol and cognitive performance in females, Bates et al. (2017) reported a positive association between HDL-cholesterol and cognition, though this was in healthy ageing females (Bates et al., 2017). We believe that the inflammatory state, and resultant dysfunctional HDL cholesterol, proposed in individuals with PD, and to a greater degree in females with PD, may provide an explanation for these contradicting findings. Moreover, given their propensity to an enhanced peri- and post-menopausal inflammatory state, females with PD may lack the positive HDL effects to a greater degree than their male counterparts. Such literature is often overlooked and should be considered in future studies assessing markers of inflammation coupled with lipidomic profiles and cognition in PD cohorts.

Contrasting with the strong association between HDLs and cognitive performance in females with PD in the current study, associations between cognition and TC or LDL levels were less robust. While LDL levels were associated with poorer performance in the visuospatial domain in females, no significant associations were found for any other cognitive domains, or among males with PD. Conversely, one other study investigating serum cholesterol in people with PD found executive function and fine motor control were significantly associated with LDL cholesterol in people with PD, but not healthy controls (Sterling et al., 2016), whereas other studies in healthy populations do

not report significant findings between cognition and LDL levels (Rej et al., 2016) or cholesterol-lowering medications (Giugliano et al., 2017; Gencer et al., 2020). Thus, the current study adds to a growing body of literature indicating that serum lipid profiles may mediate cognitive ability differently in healthy and diseased states.

LIMITATIONS

A number of limitations of the current study must be noted. Firstly, participants were assessed in two different movement disorder centres, however, possible scoring variability was mitigated by the use of standardised clinical assessment protocols which were administered by trained clinician-researchers. Secondly, the home-based recruitment excluded patients with more advanced PD, which may contribute to higher cognitive scores among the current cohort that do not represent the full spectrum of cognitive impairment in PD. Thirdly, as the study was cross-sectional in nature, the relationship between changes in cholesterol levels and cognitive decline over time was not assessed. Furthermore, other vascular risk factors, menopausal state/hormone status, and inflammatory marker information was not available for analysis. Therefore, the present findings should be confirmed using more comprehensive cognitive testing protocols in larger longitudinal studies, to further examine how vascular and inflammatory risk factors and cholesterol levels associate with disease course in PD. Lastly, the effects of cholesterol-lowering medications were not accounted for in the current study due to a lack of retrospective availability of information regarding the use of non-PD medications among participants. However, statins are known to primarily influence LDL levels and to have only relatively minor effects on HDL levels (Chapman, 2004; Baigent et al., 2010). Moreover, recent studies, as well as a very large longitudinal systematic review, do not report a significant influence of LDL cholesterol-lowering medications on cognitive status nor risk of cognitive decline and dementia (McGuinness et al., 2016; Giugliano et al., 2017; Gencer et al., 2020). Therefore, the findings reported herein concerning HDL levels and cognitive impairment in females with PD are considered valid. However, further studies are required to confirm the present findings, and to investigate the mechanisms underlying the specific association of HDL-cholesterol levels and poorer cognitive performance in females with PD.

CONCLUSION AND FUTURE DIRECTIONS

While the sex-specific association between HDL and cognition evident here may be explained by peri-menopausal changes in inflammatory status and altered HDL properties, this requires further study. Furthermore, inflammation is known to induce cholesterol oxidation and production of cholesterol aldehydes (Wentworth et al., 2003; Bosco et al., 2006), such as the blood brain barrier-traversing metabolite 27-hydroxycholesterol (27-OHC) (Bosco et al., 2006; Shafaati et al., 2011; Marwarha and Ghribi, 2015; Schommer et al., 2018), which may be relevant in this context, as 27-OHC has been shown to increase α-synuclein levels, as well as aggregation and fibrilization of the protein (Bosco et al., 2006; Koob et al., 2010; Thanan et al., 2014; Marwarha and Ghribi, 2015; Schommer et al., 2018). As the accumulation of α-synuclein in the cortex is the hallmark of PD and has long been associated with cognitive decline in people with the disease (Braak et al., 2003; Lewis et al., 2010; Diógenes et al., 2012; Twohig and Nielsen, 2019; Cinar et al., 2020), future studies should examine circulating levels of this metabolite and α-synuclein levels in relation to cognition to elucidate whether this underpins the current findings.

The novel findings of this cross-sectional study provide further support for the role of cholesterol as a disease-modifying factor for cognitive dysfunction within PD (Ferri et al., 2005; Cheng et al., 2014; Cooper et al., 2015), supporting the notion that elevated levels of cholesterol may aggravate the pathophysiology of the disease (Paul et al., 2015, 2017; Jin et al., 2019). Furthermore, the identified sex-specific effect of HDL in relation to cognition suggests the presence of differing mechanisms underlying cognitive decline in males and females with PD. Vascular factors, including lipid profile, may be a relevant disease-modifying factor for cognition in females with PD, and therefore present as modifiable lifestyle factors. Future studies should therefore consider dietary interventions to delay, slow or prevent the progression to cognitive impairment and dementia in females with PD. In light of the robust association between HDL levels and cognition in females with PD, further studies should investigate the underlying mechanisms in order to maximise the therapeutic and diagnostic potential of these findings.

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 Sir Charles Gairdner Hospital Human Research and Ethics Committee (Approval number 2006/073). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MCB executed the research project, designed and executed the statistical analysis, and wrote the first draft of the manuscript. AG executed the research project, and reviewed and critiqued the statistical analysis and manuscript preparation. JK executed the research project, and reviewed and critiqued the manuscript preparation. AJ and SM organized and executed the research project. MB executed the research project. SG and MH conceptualised the research project and reviewed and critiqued the manuscript preparation. RS reviewed and critiqued the

manuscript preparation. SW organized the research project. FM conceptualised the research project, and reviewed and critiqued the statistical analysis and manuscript preparation. RA conceptualised and organized the research project, designed the statistical analysis, executed the statistical analysis, and reviewed and critiqued the manuscript preparation. All authors contributed to the article and approved the submitted version.

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Long-Term Increase in Cholesterol Is Associated With Better Cognitive Function: Evidence From a Longitudinal Study

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Background: Higher visit-to-visit cholesterol has been associated with cognitive decline. However, the association between long-term increase or decrease in cholesterol and cognitive decline remains unclear.

Methods: A total of 4,915 participants aged ≥45 years with normal cognition in baseline were included. The participants were divided into four groups, namely low–low, low–high, high–low, and high–high, according to the diagnostic thresholds of total cholesterol (TC), non-high-density lipoprotein cholesterol (NHDL-C), low-density lipoprotein cholesterol, and high-density lipoprotein cholesterol (HDL-C) after 4 years of follow-up. Cognitive function was assessed by episodic memory and mental intactness. Binary logistic regression was used to analyse the association of cholesterol variation with cognitive decline.

Results: Among the participants, 979 (19.9%) experienced global cognitive decline. The odds ratio (OR) of global cognitive and memory function decline were remarkably lower in participants in the low–high NHDL-C group than those in the low–low group [OR and 95% confidence interval (CI): 0.50 [0.26–0.95] for global cognitive decline, 0.45 [0.25–0.82] for memory function decline]. The lower OR was also significant in females (OR [95% CI]: 0.38 [0.17–0.87] for global cognitive decline; 0.44 [0.19–0.97] for memory function decline) and participants without cardiovascular disease (OR [95% CI]: 0.31 [0.11–0.87] for global cognitive decline; 0.34 [0.14–0.83] for memory function decline). The increases in other cholesterol were also negatively associated with the risk of cognitive decline although not significantly.

Conclusions: A longitudinal increase in NHDL-C may be protective for cognition in females or individuals without cardiovascular disease.

Keywords: cognitive decline, episodic memory, mental intactness, cholesterol, longitudinal study

INTRODUCTION

Cognitive decline is one of the greatest causes of disability (Lee et al., 2018). It is also a critical period for the prevention of neurodegenerative diseases, such as dementia and amyotrophic lateral sclerosis (Anderson, 2019). However, cognitive decline has always been a primary public health issue globally. Approximately 30% of Americans and 20% of Chinese aged over 65 years have cognitive decline, and the prevalence of cognitive decline is gradually increasing worldwide (Alzheimer's Association, 2016; Li et al., 2020). Thus, it is imperative to prevent cognitive decline.

Cholesterol is a well-established risk factor of cardiovascular disease that further promotes cognitive decline. Higher total cholesterol (TC) or low-density lipoprotein cholesterol (LDL-C) is associated with worse cognitive function (Stough et al., 2019; McFarlane et al., 2020). However, current studies do not yield consistent results. In the Longitudinal Aging Study Amsterdam, a lower TC level is associated with worse general cognition and information processing speed (van den Kommer et al., 2009). Chinese studies find that a high LDL-C level is associated with lower risks of dementia and cognitive decline, but the protective effect of high-density lipoprotein cholesterol (HDL-C) is only observed in women (Lv et al., 2016; Zhou et al., 2018). The gender-specific association is also validated in a French study, in which higher TC and LDL-C and lower HDL-C are associated with an increased risk of cognitive decline in French men but not women (Ancelin et al., 2014). These discrepant results add to the complexity of developing a prevention strategy for cognitive decline.

Long-term variation in cholesterol has become an interesting indicator for cognitive decline in recent years. Previous literature has documented that higher visit-to-visit cholesterol variation, an intraindividual variation index measured by the coefficient of variation (CV), variability independent of the mean (VIM), or standard deviation (SD), is correlated with worse cognitive performance regardless of baseline cholesterol levels (Smit et al., 2016; Lee et al., 2018). However, high intraindividual variation may be observed in the two variation patterns of cholesterol determined by diagnostic threshold: from low to high and high to low levels. Little is known about which variation direction of cholesterol is associated with cognitive decline.

The purpose of this study was to investigate whether the long-term variation in cholesterol from low to high or high to low levels is associated with cognitive decline. The variation patterns of cholesterol consist of a persistent low level, from low to high levels, from high to low levels, and a persistent high level based on two blood tests in 2011 and 2015. Given that cholesterol has a gender-specific association with cognition and a close association with cardiovascular disease, we also conducted subgroup analyses according to sex and the presence or absence of cardiovascular disease.

MATERIALS AND METHODS

Study Population

The China Health and Retirement Longitudinal Study (CHARLS) is a nationally representative survey initiated in 2011 that aimed to collect health-related information among Chinese who are over 45 years old and their spouses. The investigation areas consist of 30 provinces in which 450 villages/urban areas and 10,287 households were randomly selected by probabilities proportional to size. A total of 17,714 participants were initially recruited, and biennial follow-up was carried out. Only 7,463 individuals provided blood samples and were tested for blood lipids in 2011 and 2015, and 333 individuals did not complete the cognitive test in 2011 or 2015. We excluded 77 individuals with Alzheimer's disease, brain atrophy, or Parkinson's disease and 124 individuals who were under 45 years old at baseline. We additionally excluded 2014 participants with cognitive impairment at baseline. Eventually, 4,915 individuals with normal baseline cognition were included (characteristics between included and excluded participants are presented in **Supplemental Table 1**; the flowchart of participant selection is presented in **Figure 1**). The survey was approved by the institutional review board of Peking University, China (IRB00001052-11015). All subjects provided written informed consent at the baseline and follow up.

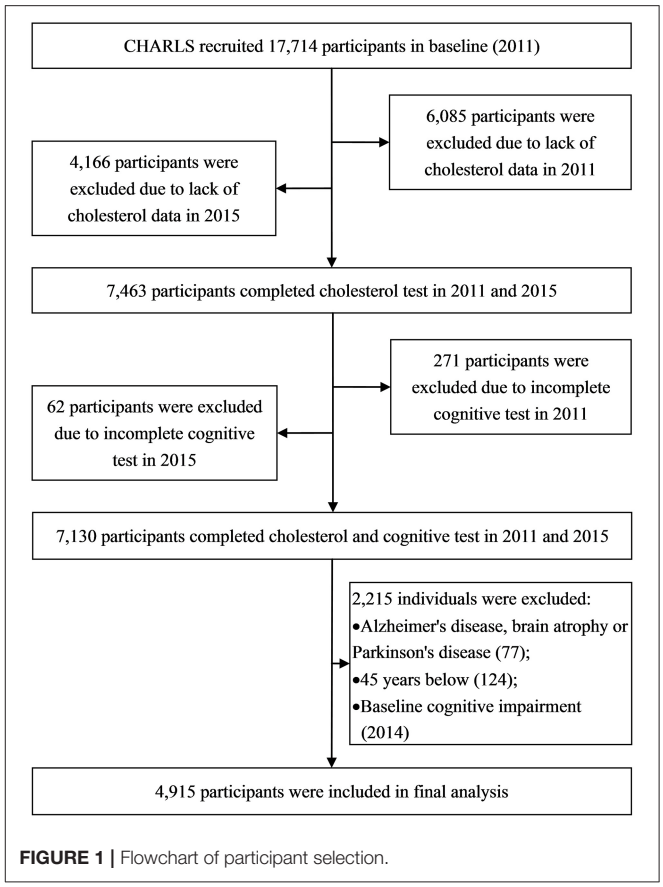

FIGURE 1 | Flowchart of participant selection.

Cholesterol Measurement and Assessment

A blood sample was collected after an overnight fast by medically trained staff of the local Centres for Disease Control and Prevention (CDC) at the survey site. Whole blood (4 mL) was centrifuged into plasma and buffy coat and then stored in cryovials and frozen at −20°C. Plasma and buffy coat were

transported within 2 weeks to the Chinese CDC in Beijing, where they were placed in a deep freezer and stored at −80°C. Plasma cholesterol was determined by an enzymatic colorimetric test in the Youanmen Center for Clinical Laboratory of Capital Medical University. Non-high-density lipoprotein cholesterol (NHDL-C) was calculated by subtracting HDL-C from TC. The high levels of TC, NHDL-C, LDL-C, and HDL-C were defined as >240, >190, >160, and >50 mg/dL, respectively, according to the guidelines for the prevention and treatment of dyslipidaemia in Chinese adults (revised in 2016) (Opoku et al., 2019). Blood tests were only carried out in 2011 and 2015. Variation in cholesterol was classified into four groups: low–low, low levels in 2011 and 2015; low–high, low level in 2011 and high level in 2015; high–low, high level in 2011 and low level in 2015; and high–high, high levels in 2011 and 2015. Participants in the low–high and high–low groups both had higher intraindividual CV and SD, and participants in the low–low and high–high groups both had lower intraindividual CV and SD (**Supplementary Figure 1**).

Cognitive Assessment

In CHARLS, cognitive function was evaluated through episodic memory and mental intactness. For episodic memory, each participant was asked to repeat as many words as possible immediately after the investigator read a list of 10 words (immediate word recall) and after 5 min (delayed word recall). One correct word recall was coded as one point. Episodic memory score was 20 points in total. The method of episodic memory measurement in this survey has good reliability and validity (Baars et al., 2009).

Numerical ability, time orientation, and picture drawing were used to assess mental intactness. For numerical ability, each participant was asked to subtract 7 from 100 serially five times. Time orientation was assessed by asking the participant to provide the date of the investigation day (month, day, and year), day of the week, and season of the year. For picture drawing, the participants were shown a picture of two overlapping pentagons and asked to redraw it. Same as episodic memory, one correct answer or successful redraw of the picture was coded as one point. The mental intactness score ranged from 0 to 11 points. Mental intactness is a well-established and valid measure as the Mini-Mental State Examination used to screen cognitively impaired individuals (Seo et al., 2011).

Global cognitive score was the summation of episodic memory and mental intactness scores and ranged from 0 to 31. Cognitive impairment was defined as a score of fewer than 11 points according to previous studies (Crimmins et al., 2011; Zhou et al., 2020). In addition, the participants with the lowest quartiles of difference in episodic memory or mental intactness score from 2011 to 2015 were considered to have a remarkable decline in memory function and mental intactness, respectively. Cognitive decline was, thus, determined by global cognitive decline, episodic memory decline, or mental intactness decline in 2015.

Covariates

Covariates were collected by questionnaires in the baseline survey. Education level was categorized as illiterate, primary school, primary or private school, middle school, high school, or above. Marital status was grouped as married and living together, married but separated and single (divorced, widowed, or never married). Smoking and drinking were arranged as all the time, former, and never. Active exercise was defined as light intensity physical activity at least twice a week. According to Chinese body mass index (BMI) standards, underweight, normal weight, and overweight/obesity were defined as BMI <18.5, 18.5–23.9, and ≥24.0 kg/m^2, respectively. Physical diseases were based on self-report as a response to the question, "Has a doctor ever told you that you had hypertension, diabetes, disability, heart problems, or stroke?" Medication use was also investigated by self-report. In addition, systolic pressure ≥140 mmHg or diastolic pressure ≥90 mmHg at baseline was also considered hypertension, and fasting blood glucose ≥126 mg/dL or glycosylated haemoglobin ≥6.5% was also considered as diabetes.

Statistical Analyses

Baseline characteristics between participants with and without global cognitive decline were presented. Unordered categorical variables were compared using χ^2-test, and continuous variables with normal distribution were compared by Student's *t*-test. The non-parametric test Kruskal–Wallis was used for comparing the differences in ordinal, categorical, and continuous variables with non-normal distribution between groups.

Binary logistic regression was used to evaluate the association of cholesterol variation with cognitive decline. We estimated the odds ratio (OR) and 95% confidence interval (CI) of the low–high and high–high groups using the low-low group as the reference; then, we also estimated the OR and 95% CI of the high–low group with the high–high group as the reference. We conducted three statistical models. The initial model was not adjusted for any covariates; the second model was adjusted for age, sex, education, marital status, smoking, drinking, exercise, BMI, diabetes, and history of disability; the last model was adjusted the same as the second model plus hypertension, heart problems (myocardial infarction, coronary heart disease, angina pectoris, heart failure, other heart diseases), stroke, and medication use (antihypertensive or antidiabetic medications). In addition, we also adjusted the number of comorbidities in the third model, which was composed of hypertension, diabetes, disability, heart problems, and stroke, because multiple morbidities are relevant to cognitive impairment.

In the sex subgroup analyses, menstrual status was adjusted in the female subgroup additionally. The subgroup analyses according to the presence or absence of cardiovascular disease was only performed in the first two statistical models. We also conducted sensitivity analyses by separating individuals with hypertension only and those with heart problems or stroke to further confirm the association between cholesterol and cognitive decline in people with different cardiovascular diseases. Null covariates were imputed by bootstrapped multiple imputation.

All statistical tests were two-sided, and the significance level was $P < 0.05$. Statistical analyses were performed using SAS software version 9.4 (SAS Institute Inc., Cary, NC).

RESULTS

Characteristics of the Participants

Among the 4,915 cognitively normal participants in 2011, 979 (19.91%) had global cognitive decline in 2015. The mean ± SD of age was 57.7 ± 8.2 years, and 49.2% of the participants were females. The participants with incident global cognitive decline were older, had lower BMI, lower education level, and higher baseline HDL-C. The proportions of individuals who were female, single and with history of disability and without drinking history were higher in the group with incident global cognitive decline. Smoking status; history of cardiovascular diseases or stroke; and baseline TC, NHDL-C, and LDL-C were not remarkably different between the participants with and without incident global cognitive decline. Females with menopause were also more likely to experience global cognitive decline (**Table 1**).

The incidence of memory function decline was lower in the low–high TC or low–high NHDL-C group, and the incidence of global cognitive decline was higher in the high–high HDL-C group. No remarkable difference in the incidence of cognitive decline was observed in the other variation groups of cholesterol (**Figure 2**).

Females were more likely to have abnormal cholesterol with larger proportions in the low–high and high–high cholesterol groups than males ($P < 0.05$). TC and NHDL-C levels were more likely to decrease in participants with at least one cardiovascular disease, and LDL-C and HDL-C were more likely to decrease in participants without any cardiovascular disease (**Supplemental Table 2**).

TABLE 1 | Baseline characteristics of participants with or without cognitive decline from 2011 to 2015.

Characteristics	Total	Persistent normal	Cognitive decline*	*P*
n (%)	4,915	3,936 (80.1)	979 (19.9)	
Age, years (mean ± SD)	57.7 ± 8.2	56.8 ± 7.8	61.3 ± 8.9	<0.001
Female, *n* (%)	2,416 (49.2)	1,874 (47.6)	542 (55.4)	<0.001
Menopause, *n* (%)#	1,676 (69.4)	1,231 (65.7)	445 (82.1)	<0.001
BMI, kg/m^2 (mean ± SD)	23.9 ± 3.7	24.1 ± 3.7	23.1 ± 3.4	<0.001
BMI, *n* (%)				<0.001
Underweight	209 (4.3)	138 (3.5)	71 (7.3)	
Normal weight	2,719 (55.3)	2,139 (54.3)	580 (59.2)	
Overweight or obesity	1,987 (40.4)	1,659 (42.2)	328 (33.5)	
Marital status, *n* (%)				<0.001
Married, living together	4,324 (88.0)	3,530 (89.7)	794 (81.1)	
Married, separated	179 (3.6)	142 (3.6)	37 (3.8)	
Single	412 (8.4)	264 (6.7)	148 (15.1)	
Education, *n* (%)				<0.001
Illiterate	811 (16.5)	410 (10.4)	401 (41.0)	
Part of primary school	878 (17.9)	656 (16.7)	222 (22.7)	
Primary or private school	1,333 (27.1)	1,100 (27.9)	233 (23.8)	
Middle school	1,274 (25.9)	1,172 (29.8)	102 (10.4)	
High school or above	619 (12.6)	598 (15.2)	21 (2.2)	
Smoking, *n* (%)				0.101
Never smoking	2,879 (58.6)	2,281 (58.0)	598 (61.1)	
Former smoking	331 (6.7)	277 (7.0)	54 (5.5)	
All the time	1,705 (34.7)	1,378 (35.0)	327 (33.4)	
Drinking, *n* (%)				0.001
Never drinking	3,276 (66.7)	2,600 (66.1)	676 (69.1)	
Former drinking	308 (6.3)	231 (5.9)	77 (7.9)	
All the time	133 (27.1)	1,105 (28.1)	226 (23.1)	
Active exercise, *n* (%)	927 (18.9)	751 (19.1)	176 (18.0)	0.430
Disability, *n* (%)	668 (13.6)	475 (12.1)	193 (19.7)	<0.001
Hypertension, *n* (%)	1,849 (37.6)	1,456 (37.0)	393 (40.1)	0.069
Diabetes, *n* (%)	705 (14.3)	567 (14.4)	138 (14.1)	0.805
Heart problems, *n* (%)	583 (11.9)	478 (12.1)	105 (10.7)	0.219
Stroke, *n* (%)	84 (1.7)	65 (1.6)	19 (1.9)	0.532
Medication use, *n* (%)	540 (11.0)	418 (10.6)	122 (12.5)	0.099
TC	193.1 ± 37.2	192.8 ± 37.2	194.2 ± 37.4	0.304
NHDL-C	142.9 ± 37.5	142.9 ± 37.5	142.7 ± 37.4	0.842
LDL-C	116.1 ± 34.6	116.2 ± 34.4	115.6 ± 35.2	0.591
HDL-C	50.2 ± 15.1	49.8 ± 15.0	51.5 ± 15.3	0.002

**Cognitive decline was determined by global cognitive function; #Proportion of menopause was calculated in females. BMI, body mass index; SD, standard deviation; TC, total cholesterol; NHDL-C, non-high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; OR, odds ratio; CI, confidence interval.*

Cholesterol Variation and Cognitive Decline in All Participants

After multivariate adjustment, the risk of global cognitive decline for the low–high TC group was borderline significant (OR [95% CI]: 0.61 [0.36–1.03]) compared with the low–low TC group. The risk of memory function decline for the high–low TC group was significantly increased (OR [95% CI]: 1.58 [1.01–2.47]) compared with the high–high TC group. The OR and 95% CI of global cognitive decline and memory function decline for the low–high NHDL-C group were 0.50 (0.26–0.95) and 0.45 (0.25–0.82), respectively, with the low–low NHDL-C group as the reference. Cognitive decline was not remarkably associated with LDL-C and HDL-C variation in all participants after adjusted covariates (**Figure 3**). In the first and second statistical models, the association of LDL-C and NHDL-C with cognitive decline was in line with that in the third model. However, the TC variation was not significantly associated with cognitive decline. The persistent high level of HDL-C was positively and significantly associated with global cognitive decline and memory function decline in the first model (**Supplemental Table 3**).

Cholesterol Variation and Cognitive Decline in the Sex Subgroups

In females, substantially lower risks of global cognitive decline (OR [95% CI]: 0.38 [0.17–0.87]) and memory function decline (OR [95% CI]: 0.44 [0.19–0.97]) were observed in the low–high NHDL-C group compared with the low–low NHDL-C group, which was used as the reference in the multivariate adjustment model; remarkably lower risk of global cognitive decline was also observed in the low–high TC group with the low–low group

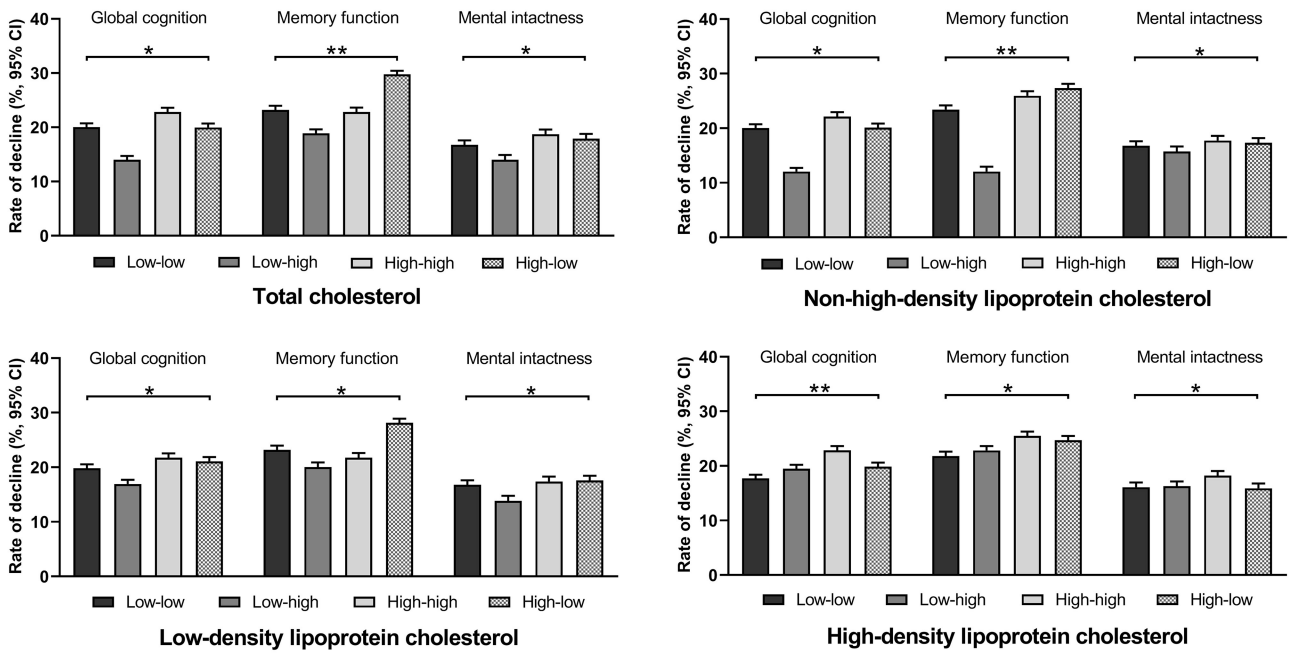

FIGURE 2 | Incidence rate of cognitive decline among cholesterol groups. *$P > 0.05$; **$P < 0.05$.

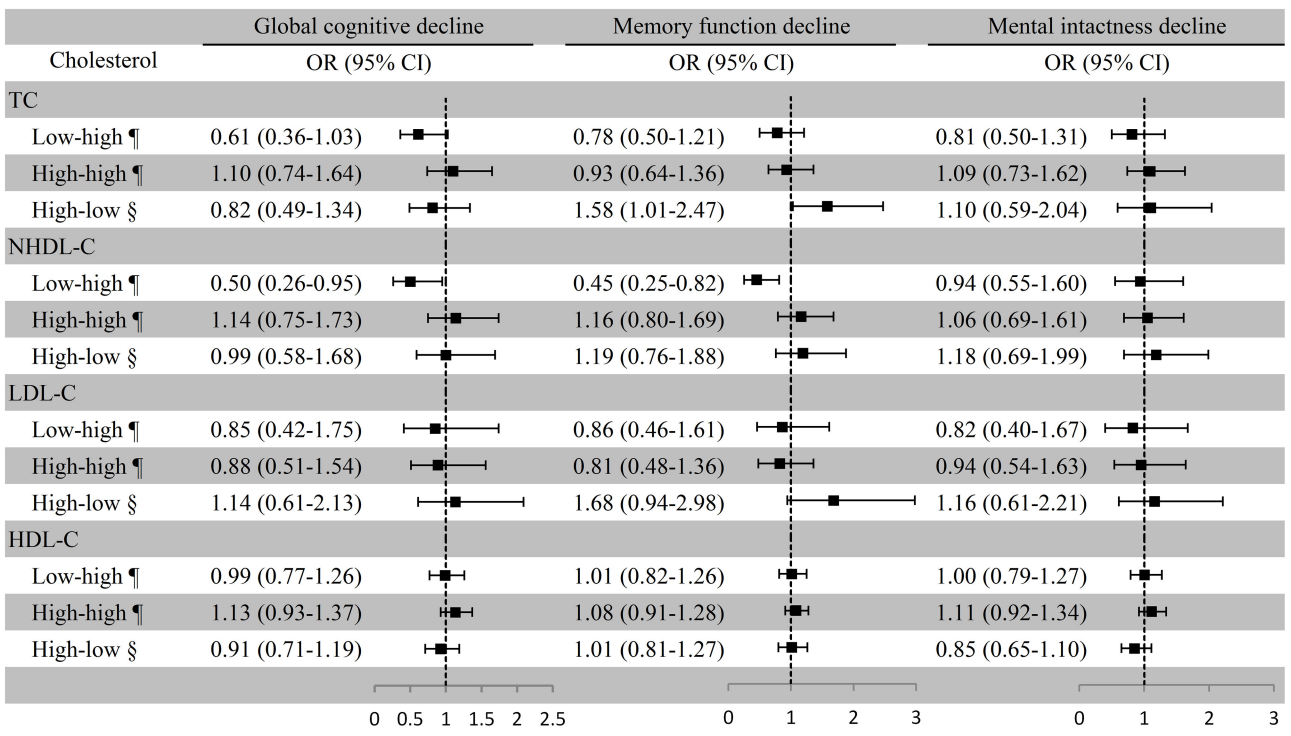

FIGURE 3 | Association between cholesterol variation and cognitive decline among all the participants. ¶Take the low–low group as a reference; §Take the high–high group as a reference. Baseline age, sex, education, marital status, smoking, drinking, BMI, exercise, physical diseases, medication use (antihypertensive or antidiabetic medications), and the number of comorbidities were adjusted. TC, total cholesterol; NHDL-C, non-high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; OR, odds ratio; CI, confidence interval.

as the reference (OR [95% CI]: 0.51 [0.26–0.95]). However, the associations of cholesterol variation with cognitive decline were not substantial in males (**Figure 4**). In the first model of the female subgroup, the increase in TC was not associated with global cognitive decline, and the persistent high level of HDL-C was positively associated with global cognitive decline and memory function decline although not significant in the third model. The association of LDL-C and NHDL-C with cognitive decline was in line with that in the third model. Consistent conclusions were found among the three models of the male subgroup (**Supplemental Table 4**).

Cholesterol Variation and Cognitive Decline in Participants With or Without Cardiovascular Disease

In participants without cardiovascular disease, remarkably lower risk of global cognitive decline (OR [95% CI]: 0.31 [0.11–0.87]) and memory function decline (OR [95% CI]: 0.34 [0.14–0.83]) were observed in the low–high NHDL-C group with the low–low group as the reference in the multivariate adjustment model, but global cognitive decline was not remarkably associated with TC variation. The associations of cholesterol variation with any cognitive decline were not substantial in participants with at least one cardiovascular disease (**Figure 5**). Similar results were also observed in the first statistical model for LDL-C and NHDL-C and cognitive decline. In addition, we observed a considerable increase in the ORs of global cognitive decline (OR [95% CI]: 1.51 [1.23–1.84]) and memory function decline (OR [95% CI]: 1.24 [1.03–1.49]) in the high–high HDL-C group with the low–low group as a reference in participants without any cardiovascular disease although it was not significant in the adjusted model (**Supplemental Table 5**).

We further stratified participants into hypertension only and with heart problems or stroke. The results in both groups were similar to those in participants with at least one cardiovascular disease (**Supplemental Table 6**).

DISCUSSION

We estimated the risk of cognitive decline based on the long-term variation patterns and relatively stable levels of cholesterol. The results show that the long-term increase in TC and NHDL-C levels were substantially associated with decreased risks of global cognitive and memory function decline. Substantial associations were mainly present in females and participants without any cardiovascular disease. The long-term decrease in cholesterol level had no protective effect on cognition. Persistent high cholesterol levels did not remarkably increase the risk of any cognitive dimensions compared with persistent low levels.

Cholesterol is a well-established risk factor for cardiovascular diseases (Brunner et al., 2019). Cardiovascular health is helpful in keeping normal cognition, and the role of cholesterol on cognition should be in line with cardiovascular disease. However, the association between cholesterol and cognitive function has been divergent in recent years. A study on a Chinese urban community indicated that higher LDL-C is associated with lower dementia risk (Zhou et al., 2018). An ideal TC level, one of seven metrics of ideal vascular health, was negatively associated with cognition in the Brazilian Longitudinal Study of Adult Health (Suemoto et al., 2021). A randomized trial in hypercholesterolemic adults also indicates that cholesterol-lowering therapy by simvastatin diminishes cognitive function slightly compared with placebo (Muldoon et al., 2004). In addition, in animal experiments, the cholesterol infusion to striatum of Huntington's disease mice prevented cognitive decline (Birolini et al., 2020). Two pieces of evidence can explain the divergent findings. First, approximately a quarter of the body's cholesterol is concentrated in the brain, and lipid composition, including lecithin, omega-3, and cholesterol, account for nearly half of the brain's weight. Although peripheral cholesterol cannot enter the central nervous system because of the blood–brain barrier, it reflects the supplement of cholesterol (Dai et al., 2021). Crucially, cholesterol is an important component of nerve cell membranes and also participates in the metabolic activities of nerve cells. Another factor is that cholesterol stores a large amount of energy, which can be sustainably provided to the brain for a long time, and the brain is the most energy-consuming organ of the body (Steiner, 2019). Therefore, the role of cholesterol in brain protection might be different from its role in cardiovascular diseases.

Previous studies clarify the associations of higher visit-to-visit cholesterol with cognitive decline (Smit et al., 2016; Grasset et al., 2020; Hua et al., 2020). In these studies, CV, VIM, and SD reflect the degree of intraindividual dispersion of cholesterol, including downward and upward fluctuations. One possible phenomenon is that the intraindividual CV, VIM, or SD of cholesterol in recommended and high ranges are comparable and, thus, collapse persistent high and low cholesterol into the same category. In our cholesterol groups, intraindividual SD and CV were higher in low–high and high–low groups and lower in low–low and high–high groups. Thus, we estimate the risk of cognitive decline from another perspective, including persistently low levels, levels that cross the diagnostic threshold, and persistently high levels. We found that variations in TC and NHDL-C levels from low to high have a remarkable protective role on cognitive function, whereas changes from high to low levels do not have the same effect.

Hua et al. (2020) find a negative association between the intraindividual SD of TC and memory function in males using the same database as our present study. Stable cholesterol is certainly favourable for cognitive ability. However, we did not observe a remarkable association between cholesterol variation and any cognitive domains in our male subgroup analyses. Hua et al. suggest the importance of maintaining a stable cholesterol level, whereas our study adds certain guiding importance on whether to use lipid-lowering intervention. This difference indicates that different grouping methods have different guiding importance for the prevention of cognitive decline.

Some studies clarify the benefit of increased TC on cognitive function. A study based on the Finland population indicates that a moderate decrease in serum TC from mid- to late life is associated with a 3.5-fold increase in the risk of cognitive impairment (Solomon et al., 2007). The same conclusion was

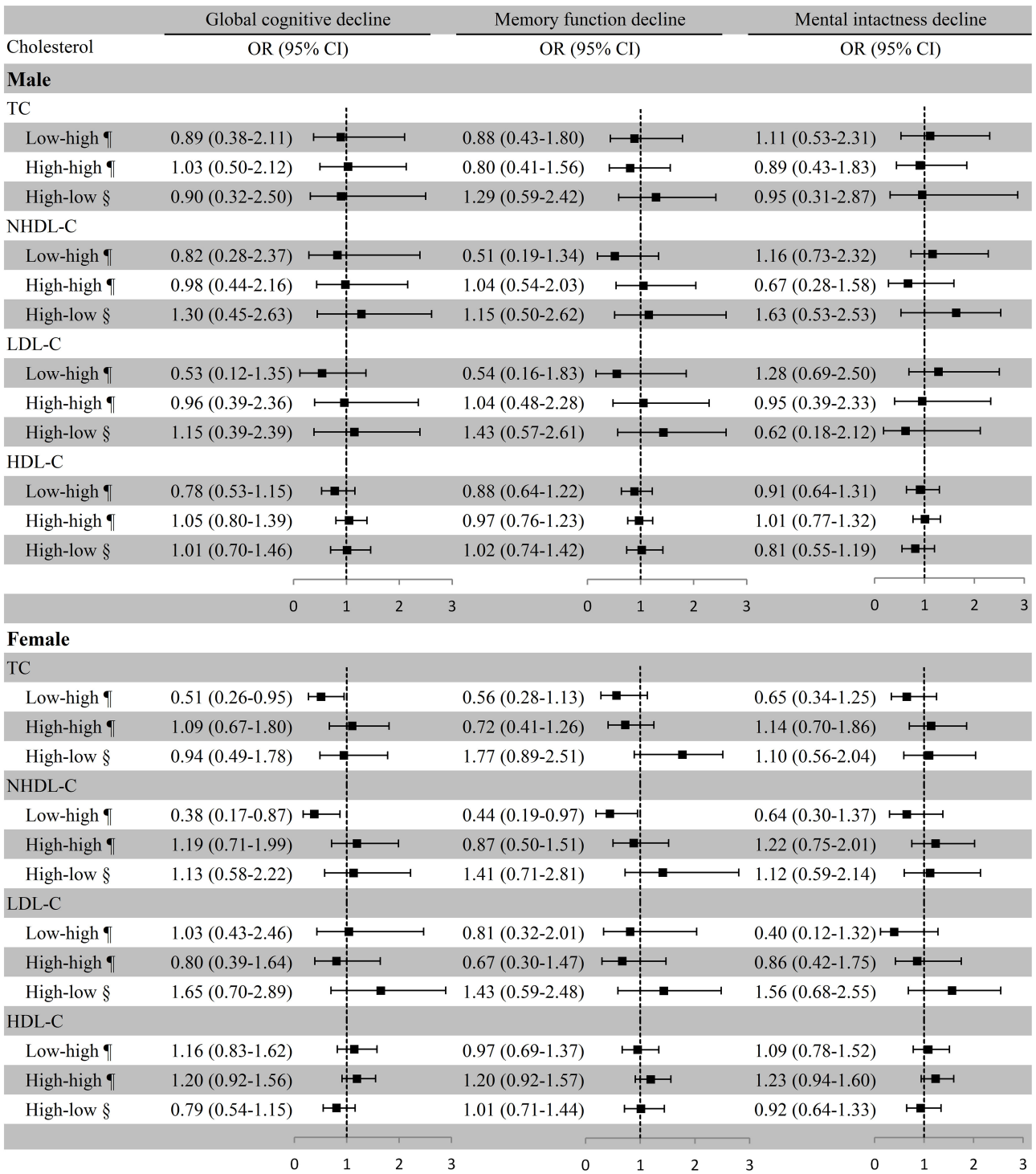

FIGURE 4 | Association between cholesterol variation and cognitive decline in sex subgroups. ¶Take the low–low group as a reference; §Take the high–high group as a reference. Baseline age, education, marital status, smoking, drinking, BMI, exercise, physical diseases, medication use (antihypertensive or antidiabetic medications), and the number of comorbidities were adjusted. Menstrual status was also adjusted in the female subgroup. TC, total cholesterol; NHDL-C, non-high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; OR, odds ratio; CI, confidence interval.

documented in Swedish females, in which the greatest decrease in TC (refers to the lowest quartile of TC difference) after 32 years of follow-up is associated with a 2.37-fold increase in the risk of dementia (Mielke et al., 2010). In a birth cohort study from Germany, TC level from baseline to endpoint decreased in participants with Alzheimer's disease but remained stable in

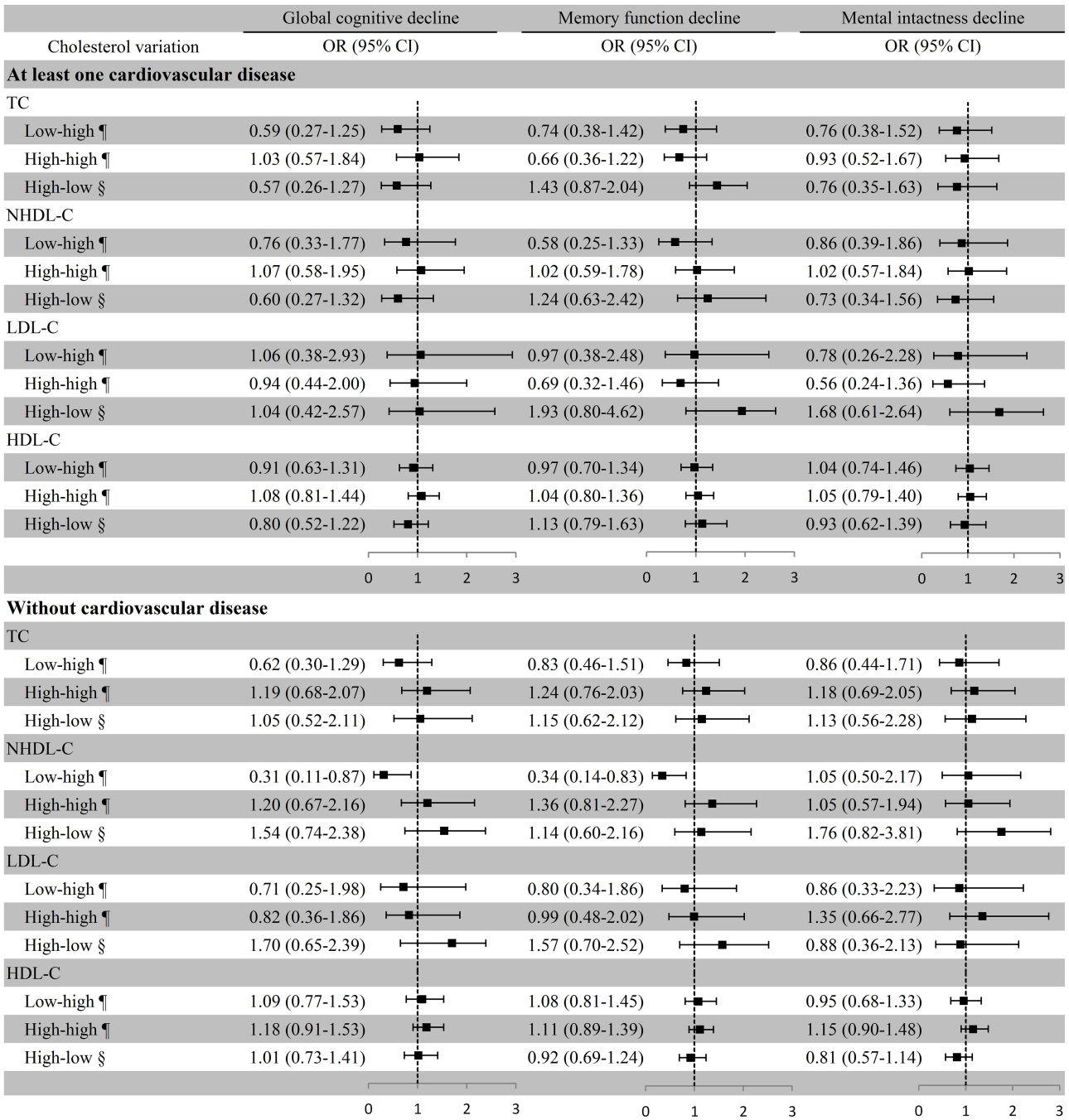

FIGURE 5 | Association between cholesterol variation and cognitive decline in participants with or without cardiovascular disease. ¶Take the low–low group as a reference; §Take the high–high group as a reference. Baseline age, education, marital status, smoking, drinking, BMI, exercise, diabetes, history of disability, medication use (antihypertensive or antidiabetic medications), and the number of comorbidities were adjusted. TC, total cholesterol; NHDL-C, non-high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; OR, odds ratio; CI, confidence interval.

those who remained healthy (Toro et al., 2014). These studies suggest that TC is protective for cognitive function, and this conclusion is consistent with our study. However, the assessment of the variation trend of cholesterol was not based on baseline cholesterol level in these studies; thus, participants with low and high baseline cholesterol levels might have the same decrease. We conducted two reference groups, the low–low and high–high groups, to address this issue. Furthermore, we demonstrated that the results for NHDL-C are still the same after we subtracted HDL-C from TC. LDL-C is the main component of NHDL-C.

However, the variation in LDL-C was not associated with cognitive function in our study. Therefore, in addition to LDL-C, the effect of other lipid compositions on cognition needs to be further determined.

Menstruation in females is of importance for cognitive decline. The decrease of estrogen levels in postmenopausal females results in low cholesterol ester transfer protein and disturbs the reverse transport of cholesterol to the liver and the metabolism of cholesterol (Guo et al., 2019). Previous studies report an increased risk of decline in psychomotor speed in females who never used hormone treatment compared with current users (Ryan et al., 2009). In our study, there were greater proportions of females in the low–high and high–high cholesterol groups, and the majority of these females (69.4%) were menopausal. We also adjusted menstrual status in the female subgroup. We still found a low risk of cognitive decline for increased TC or NHDL-C in females. Our results suggest that cholesterol may be an excellent protector of cognitive function even in postmenopausal females. The gender-specific association was documented elsewhere. Protease proprotein convertase subtilisin/kexin type 9 (PCSK9), a risk factor of cardiovascular diseases, is negatively associated with memory function in elderly females but not in males (Simeone et al., 2021). One reason that cannot be ignored is that estrogen is inversely correlated to PCSK9. However, we did not examine the association of cholesterol variation with cognitive decline in postmenopausal and premenopausal females because of the small number of premenopausal females.

Interestingly, the remarkable decrease in the risk of cognitive decline as NHDL-C levels change from low to high was only observed in participants without cardiovascular disease. Cardiovascular disease is a recognized risk factor for cognitive decline, and cholesterol promotes the progression of cardiovascular disease (Zlokovic et al., 2020). We speculated that cholesterol can play a better role in cognitive protection without the threat of cardiovascular disease. Our **Supplementary Materials** show that the levels of TC and NHDL-C are more likely to decrease in participants with at least one cardiovascular disease. Treatment for cardiovascular disease may include lipid-lowering intervention, which decreases cholesterol. This finding suggests that the pros and cons of lipid-lowering intervention should be fully weighed between cardiovascular disease and cognitive decline. The protective role of cholesterol on cognition may be covered by cardiovascular risk.

In the present study, we precisely clarify the association between cholesterol variation and cognitive decline. However, this study has several limitations. First, the blood test was only carried out in 2011 and 2015; the time of cholesterol changes is uncertain. We cannot determine the sequence of cholesterol and cognitive changes. Second, we excluded most of the participants who did not provide a blood sample in 2011 or 2015 and those who had cognitive impairment at baseline. This omission may induce selection bias. **Supplemental Table 1** shows that most of the characteristics between the excluded and included participants are remarkably different. Third, we define the lowest quartile of the difference in episodic memory or mental intactness scores from 2011 to 2015 as substantial decline, which may lead to misjudgement. Memory and mental health decline have no existing standard. Fourth, information on the use of lipid-lowering drugs was not obtained in this study. However, lipid-lowering drugs, particularly statins, have controversial effects on cognition. The combined effect of cholesterol and lipid-lowering drugs on cognition needs to be further studied. Last, the existing conditions that were not officially diagnosed also resulted in information bias. In the community, not everyone could see a doctor in the time when they were sick, leading to patients not knowing that they were sick and, thus, being regarded as healthy people in the survey.

CONCLUSIONS

In conclusion, the longitudinal increase in TC or NHDL-C is associated with better cognitive function among females or participants without cardiovascular disease. This study adds further evidence that lipid regulation strategies based on gender and cardiovascular disease diagnosis should be considered in the primary prevention of cognitive impairment.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analysed in this study. This data can be found at: http://charls.pku.edu.cn/en.

ETHICS STATEMENT

The survey was approved by the Institutional Review Board of Peking University, China (IRB00001052-11015). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

HL and LZ wrote the article. HL and RZ performed the data analysis. XW and JZ drafted and critically revised the manuscript. SG provided clinical guidance. MZ and DH reviewed language and made substantial interpretation. KW, ZH, and ZY organized database. XW contributed to the study concept and design and reviewed the article. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnagi.2021.691423/full#supplementary-material

Supplementary Figure 1 | Intraindividual SD and CV in cholesterol variation group. SD, standard deviation; CV, coefficient of variation.

Supplemental Table 1 | Characteristics between included and excluded participants.

Supplemental Table 2 | Variation profile of cholesterol in subgroups.

Supplemental Table 3 | The OR (95% CI) between cholesterol variation and cognitive decline amongst all the participants.

Supplemental Table 4 | The OR (95% CI) between cholesterol variation and cognitive decline in sex subgroups.

Supplemental Table 5 | The OR (95% CI) between cholesterol variation and cognitive decline in participants with or without cardiovascular disease.

Supplemental Table 6 | The OR (95% CI) between cholesterol variation and cognitive decline in participants just with hypertension and with heart problems or stroke.

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frontiers in Aging Neuroscience

REVIEW
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Cholesterol and Alzheimer's Disease; From Risk Genes to Pathological Effects

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While the central nervous system compromises 2% of our body weight, it harbors up to 25% of the body's cholesterol. Cholesterol levels in the brain are tightly regulated for physiological brain function, but mounting evidence indicates that excessive cholesterol accumulates in Alzheimer's disease (AD), where it may drive AD-associated pathological changes. This seems especially relevant for late-onset AD, as several of the major genetic risk factors are functionally associated with cholesterol metabolism. In this review we discuss the different systems that maintain brain cholesterol metabolism in the healthy brain, and how dysregulation of these processes can lead, or contribute to, Alzheimer's disease. We will also discuss how AD-risk genes might impact cholesterol metabolism and downstream AD pathology. Finally, we will address the major outstanding questions in the field and how recent technical advances in CRISPR/Cas9-gene editing and induced pluripotent stem cell (iPSC)-technology can aid to study these problems.

Keywords: cholesterol, Alzheimer, ApoE, gliosis, iPSC models

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INTRODUCTION

Dementia affects over 46 million people worldwide, a number that is expected to double within the next 20 years due to our increased life expectancy (Prince et al., 2015). Alzheimer's Disease (AD) is the most common type of dementia and no successful treatment that can cure AD, or halt its progression, is available today. At the pathological level, AD is characterized by the accumulation of extracellular amyloid beta (Aβ) plaques, and intracellular neurofibrillary tangles (NFT) consisting of hyperphosphorylated Tau species (Scheltens et al., 2016). AD can develop early (<65 years) referred to as early-onset AD (EOAD), which is in part explained by autosomal dominant inheritance of coding mutations in the amyloid precursor protein (APP) or presenilin genes (*PSEN1* and *PSEN2*), in that case called familial AD (FAD). The FAD related mutations directly affect Aβ production and their identification therefore contributed to formation of the amyloid cascade hypothesis that postulates a linear relation between development of Aβ plaques and NFT in AD (Pimenova et al., 2018). Whilst EOAD only represents around 5% of all AD cases, the vast majority of AD patients suffer from late-onset AD (LOAD), for which aging is the biggest risk factor in addition to genetic and lifestyle factors (Scheltens et al., 2016). Multiple studies on the lifestyle and genetic interactions with AD have connected altered circulating cholesterol metabolism and hypercholesterolemia with aging and AD pathogenesis (**Box 1**).

BOX 1 | Hypercholesterolemia, high fat diet and AD.

With the identification of lipoprotein ApoE4 as the biggest risk factor for LOAD in the early nineties, the interest for lipids and cholesterol metabolism in AD rapidly developed (Corder et al., 1993; Saunders et al., 1993; Strittmatter et al., 1993). Expression of the ApoE4 allelic variant of ApoE had previously been shown to increase plasma low-density lipoprotein (LDL) levels and increase the risk for atherosclerosis. Moreover, carriers of the ApoE4 gene were overrepresented in hyperlipidemic and cardiovascular patients (Huang, 2010; Mahley, 2016). At the epidemiological level, retrospective studies have shown that obesity, type 2 diabetes, cardiovascular disease and hypercholesterolemia at middle-age increase the risk for dementia at older age in humans (Pappolla et al., 2003; Whitmer et al., 2005; Stampfer, 2006; Solomon et al., 2009; Pedditizi et al., 2016; Anstey et al., 2017; Ma et al., 2020; Tini et al., 2020; Barbiellini Amidei et al., 2021). In line, increased plasma and CSF levels of the cholesterol metabolite 24-hydroxycholesterol (24S-OHC) that is selectively generated in neurons, have been linked to early AD development (Lütjohann et al., 2000; Papassotiropoulos et al., 2002; Schönknecht et al., 2002; Li et al., 2018). Cholesterol has also been shown to accumulate in mature Aβ-plaques in AD patients and APP(SW) mice (Mori et al., 2001) and cholesterol levels in the brain positively correlate with the severity of dementia in AD patients (Cutler et al., 2004). In line, lower AD incidence was associated with statin use in retrospective studies (Jick et al., 2000; Wolozin et al., 2000; Cramer et al., 2008; Haag et al., 2009; Li et al., 2010; Lin et al., 2015; Zissimopoulos et al., 2017; Zhang et al., 2018). The protective effect of statin usage was present independent of ApoE status (Haag et al., 2009; Li et al., 2010). A subset of these studies showed that the protective effect of statin usage was no longer present in participants that fell within the oldest age categories (>80). This could be due to a survival bias, where participants that survive till old age have fewer additional medical conditions, that would have increased their risk for AD development. Alternatively, this could point to a beneficial effect of statin usage only when taken at a timepoint before pathological hallmarks of AD would typically develop in the brain. A link between high circulating cholesterol levels and AD was also corroborated in AD mouse models where a hypercholesterolemic diet increased Aβ-plaque load (Refolo et al., 2000). In addition, high cholesterol diet in mice induced Tau hyperphosphorylation, which was amplified by loss of ApoE expression (Rahman et al., 2005; Glöckner et al., 2011). Glial activation, contributing to gliosis as seen in AD, has also been reported in mice on a high cholesterol diet (Crisby et al., 2004). Metabolic changes accompanied by AD phenotypes in the brain, where also described in rabbits on a high cholesterol diet (Jin et al., 2018). Finally, a high fat/high cholesterol diet in young (4-month old) versus aged (14-month old) rats negatively affected memory performance at both ages, while also increasing hippocampal pTau levels at old age, indicating the detrimental combination of disturbed circulating cholesterol homeostasis and aging (Ledreux et al., 2016).

In the brain, already a century ago, in addition to plaques and tangles, Dr. Alzheimer described as a third characteristic of AD: the extensive accumulation of 'adipose saccules' (Alzheimer, 1907). These 'adipose saccules' were likely what we now refer to as lipid droplets, and are major storage organelles of intracellular lipids such as cholesterol and fatty acids (Farmer et al., 2020). While mostly ignored since their first discovery, these 'adipose saccules' in the brain have gained renewed interest in light of the findings on cholesterol metabolism and AD in the last two decades.

In this review, we will discuss the basic regulation of cholesterol homeostasis at the cellular level, and how crosstalk between different brain-cell types regulates "healthy" cholesterol homeostasis in the human brain. We will then discuss how brain cholesterol metabolism is affected by aging and how neuronal cholesterol can contribute to downstream AD pathologies such as Amyloid- and Tau accumulation. Next, we will examine the contribution of human-specific AD risk polymorphisms to cholesterol dyshomeostasis and gliosis in different brain cell types, an area of research that greatly benefits from the development of CRISPR gene-editing and iPSC techniques. Lastly, we will formulate some of the major questions still outstanding in the field, and how they could be addressed to develop disease-modifying interventions for AD based on our knowledge of cholesterol metabolism in AD.

INTRACELLULAR CHOLESTEROL METABOLISM; THE BASICS

The largely hydrophobic molecule cholesterol localizes primarily in cell membranes where it regulates membrane fluidity and can interact with neighboring lipids and proteins to regulate membrane trafficking or signal transduction (Luo et al., 2019). Cholesterol levels in cells are balanced by *de novo* biosynthesis, uptake, storage and export [extensively reviewed by Luo et al. (2019)]. In short: *De novo* synthesis of cholesterol starts when sterol regulatory element binding protein 2 (SREBP2), the key regulator of cholesterol synthesis, transfers from the endoplasmic reticulum (ER)-membrane to the Golgi where it is processed in order to enter the nucleus and induce transcription of its numerous target genes involved in cholesterol synthesis (**Figure 1**). Together around 30 consecutive reactions ensure cholesterol synthesis in the ER starting from acetyl-CoA. HMG-CoA reductase (HMGCR) and squalene monooxygenase (SM), both SREBP2 targets, are rate limiting enzymes in this process. Cholesterol is transported from the ER to the plasma membrane without passing through the Golgi (Dai et al., 2021). As an alternative to synthesis, cells can acquire cholesterol trough uptake. When not incorporated in the lipid bilayer of a membrane, most cholesterol is protein bound in apolipoprotein particles that facilitate transport (Zhang and Liu, 2015). Uptake of these cholesterol containing particles depends on Low-density lipoprotein receptors (LDLRs) on the plasma membrane. Binding of lipoprotein particles to the LDLR causes incorporation of LDL into clathrin-coated vesicles that enter the endocytic pathway (Goldstein and Brown, 2009). LDLR is either recycled via endosomal recycling or directed to lysosomes for degradation (Rudenko et al., 2002; Bartuzi et al., 2016; Fedoseienko et al., 2018). When LDLs arrive in lysosomes, cholesterol is freed by hydrolysis of CEs present in the LDLs. NPC2, NPC1 and lysosome-associated glycoprotein LAMP2 control delivery of the newly formed cholesterol to the ER (Kwon et al., 2009). Excess cholesterol can be stored in lipid droplets as CEs and converted back to cholesterol by acidic lipases in the lysosome when needed (Ikonen, 2008). As an alternative to intracellular storage, excess cholesterol can also be exported as part of LDL- or High-density lipoprotein (HDL) particles in a process named reverse cholesterol transport. This is mediated through ATP-binding cassette (ABC) transporters, like ABC subfamily A member 1 (ABCA1) and ABC subfamily G member 1 (ABCG1), which are widely expressed in the body and coordinately

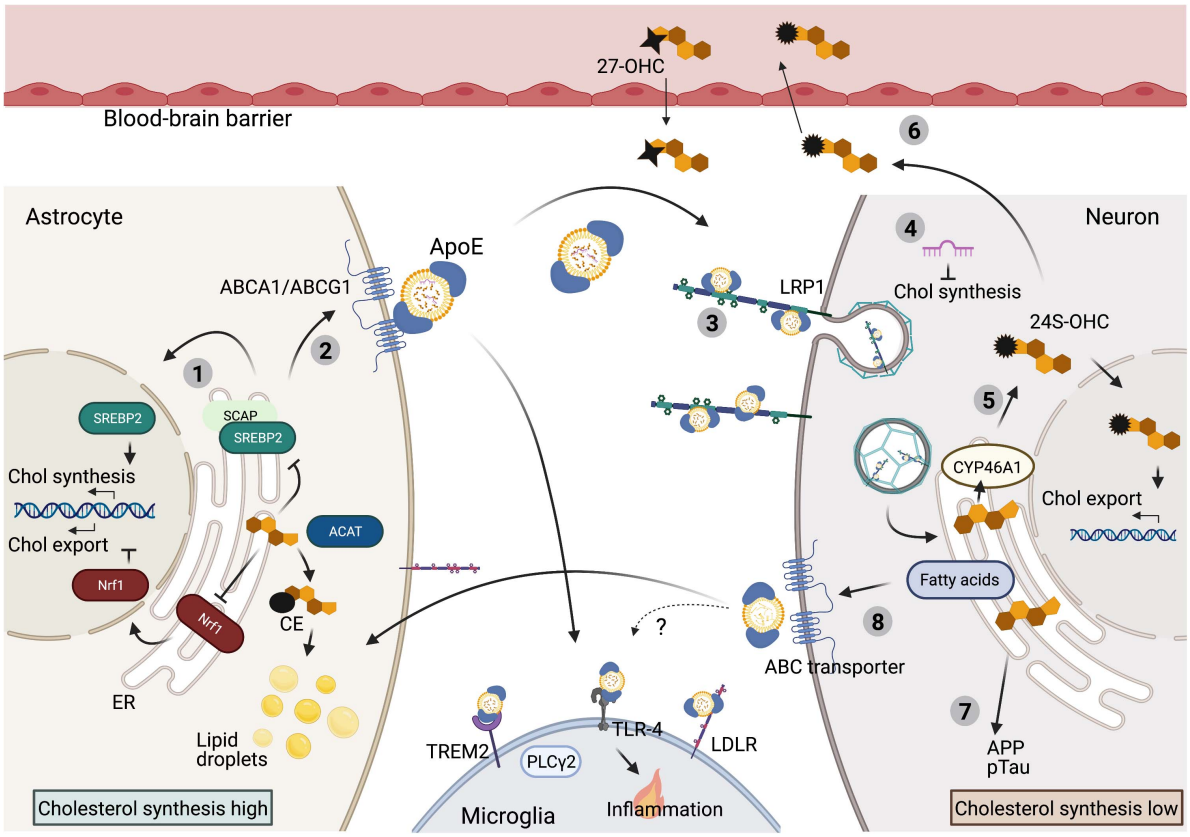

FIGURE 1 | Cholesterol metabolism in the brain. Overview of cholesterol metabolism in the mature brain. (1) In the brain cholesterol is predominantly synthesized in astrocytes. Cholesterol synthesis is under tight control of ER-cholesterol level. High ER-cholesterol concentrations prevent SREBP2 processing and thereby suppress cholesterol synthesis. High ER-cholesterol levels also inhibit Nrf1 processing to induce cholesterol export. In addition, excess ER cholesterol is converted to CE for storage in lipid droplets. (2) Cholesterol and cholesterol precursors are exported via ABC transporters and transported from astrocytes to neurons and microglia via ApoE bound lipoprotein-particles. (3) These lipoprotein particles can bind to lipoprotein receptors (LRP1 on neurons, TREM2, TLR-4 and LDLR on microglia) to be internalized. (4) Neuronal cholesterol synthesis is inhibited by ApoE dependent delivery of astrocytic derived microRNAs that target cholesterol synthesis genes in neurons. (5) Specifically in neurons, excess cholesterol is converted to 24S-OHC, which activates a transcriptional program to promote cholesterol export. (6) 24S-OHC itself can be secreted from the brain via crossing of the BBB, while 27OHC can enter the brain from the periphery. (7) High cholesterol load in neurons can contribute to amyloidogenic APP processing and pTau accumulation. (8) Astrocytes can also prevent toxic overload of (peroxidized) fatty acids in neurons via ApoE-dependent lipid-particle transport from neurons to astrocytes, but whether cholesterol is also transported into this direction remains unknown (BioRender, 2021).

regulate cholesterol export from the cell (**Figure 1**) (Luo et al., 2019). Although the exact mechanism is still under debate, cholesterol effluxed by ABCA1 appears to be loaded on lipid-free Apolipoprotein A-I (ApoA-I), which can subsequently acquire additional cholesterol from ABCG1 (Gelissen et al., 2006).

While representing only 1% of cellular cholesterol, cholesterol levels in the ER play a central role in the regulation of all aspects of cholesterol metabolism described above. When cholesterol levels are low, SREBP2 interacts with SCAP in the ER membrane which promotes SREBP2 trafficking to the Golgi, it's processing and transcription of cholesterol-synthetic genes (Sakai et al., 1997, 1998; Brown et al., 2018). The uptake of cholesterol is also directly regulated through this process, as LDLR is a transcriptional target of SREBP2 (Luo et al., 2019). In this way, low ER cholesterol drives increased synthesis and uptake of cholesterol in order to balance cellular cholesterol levels (**Figure 1**). Reversely, too high levels of cholesterol can be toxic to cells. Therefore, when a surplus of cholesterol accumulates in the plasma membrane, cholesterol is transported back to the ER where it (i) inhibits SREBP2 activation and (ii) can be esterified by acyl-coA: cholesterol acyltransferase (ACAT1) to from CE for storage in lipid droplets (Chang et al., 1997; Zhang and Liu, 2017). Furthermore, when cholesterol levels in the ER are high, cholesterol is converted to oxysterols (Olsen et al., 2012). As the major sensor for cholesterol overload in a cell oxysterols prevent SREBP2 activation and directly activate the Liver × receptor (LXR), which promotes cholesterol efflux by transcription of ABC transporters (Radhakrishnan et al., 2007; Olsen et al., 2012). In addition to SREBP2, the transcription factor Nuclear factor erythroid 2-related factor 1 (NFE2L1 aka Nrf1) also senses ER cholesterol levels. When ER cholesterol levels are low, Nrf1 is cleaved and the transcription-part domain enters the nucleus where it inhibits LXR dependent transcription and thus prevents cholesterol export. When ER cholesterol levels rise, cholesterol

binding to Nrf1 prevents its translocation to the nucleus, which causes de-repression of the LXR locus and promotes cholesterol efflux (**Figure 1**) (Widenmaier et al., 2017). Together, in a Yin-Yang manner, SREBP2 and Nrf1 sense ER cholesterol levels to maintain cellular cholesterol homeostasis (**Figure 1**).

It is important to note, that many basic rules underlying intracellular cholesterol metabolism have been uncovered so far, as discussed above. However, most of this knowledge is acquired from experiments in dividing fibroblast culture systems. Cholesterol metabolism in the central nervous system (CNS) and, particularly in neurons, is under added pressure due to the postmitotic nature of these cells, their long-life span, large size and highly specialized metabolic demand which requests specific mechanisms to maintain lifelong cholesterol homeostasis in the brain.

CHOLESTEROL METABOLISM IN THE BRAIN; DIFFERENT NEEDS FOR DIFFERENT CELLS

Due to the blood-brain barrier (BBB) cholesterol metabolism in the CNS is largely separated from the periphery and it is generally understood that little diet-derived cholesterol enters the brain (Björkhem and Meaney, 2004). Therefore the brain is largely dependent on its own cholesterol synthesis and separate metabolism regulated by a complex interplay between different highly specialized cell types, each with their own demand for cholesterol (Dietschy and Turley, 2004). In adults, biosynthesis of cholesterol is thought to almost exclusively take place in astrocytes, from where cholesterol is transported to neurons via ApoE lipoproteins (**Figure 1**) (Pfrieger and Ungerer, 2011). Although cell type specific cholesterol synthesis rates have not been determined *in vivo*, cholesterol synthesis rates in cultured rat astrocytes are double as high as in cultured neurons (Nieweg et al., 2009). Moreover, conditional depletion of cholesterol synthesis in neuronal cells in mice did not result in neurodegeneration or synapse loss, indicating that mature neurons can acquire sufficient cholesterol levels supplied by surrounding glia (Fünfschilling et al., 2007). Accordingly, neuronal synaptogenesis has been shown to depend on ApoE dependent cholesterol transport from astrocytes to neurons (Mauch et al., 2001; Pfrieger, 2003). Of interest is the recent finding that astrocytes might also suppress cholesterol synthesis in neurons, as astrocytic ApoE was shown to deliver microRNAs to neurons that target and suppress expression of cholesterol biosynthesis genes (Li et al., 2021). Astrocyte-derived lipoproteins carry cholesterol and phospholipids as well as cholesterol precursors, presumably used by neurons for processing, but contain little CE or triglycerides making them substantially different from plasma lipoproteins (Pfrieger and Ungerer, 2011). Instead of ApoA-I, ApoE is the main apolipoprotein responsible for lipid transport in the CNS. ApoE is highly expressed in astrocytes where it is lipidated and exported via ABC transporters like ABCA1 and ABCG1 (**Figure 1**) (Koldamova et al., 2003; Xu et al., 2006). Which ABC transporters are responsible for cholesterol efflux in the CNS seems to be cell-type dependent. Blocking ABCA1 or ABCG1 mediated transport in primary rat astrocytes reduced ApoE mediated cholesterol export, but had no effect on cholesterol efflux from primary cultured neurons. In contrast, knock down of ABCG4 selectively affected cholesterol export in primary cultured neurons (Chen et al., 2013). Neurons can take up astrocyte-derived HDL-like lipoprotein particles containing ApoE through receptors of the LDLR family (**Figure 1**), of which LRP1 is highest expressed in neurons (Vance and Hayashi, 2010). Similar to neurons, cholesterol biosynthesis levels are relatively low in microglia, which also mainly depend on astrocytes for cholesterol production (Zhang et al., 2014; Loving and Bruce, 2020). On the microglial cell surface, ApoE lipoprotein particles can interact with Triggering Receptor Expressed on Myeloid Cells 2 (TREM2), Toll Like Receptor 4 (TLR-4) and the LDLR to internalize lipoprotein particles into the microglia (**Figure 1**) (Loving and Bruce, 2020).

As mentioned above, regulation of cholesterol metabolism is particularly important for neurons. To further fine-tune cholesterol metabolism, neurons contain another cholesterol-regulating enzyme; cholesterol 24-hydroxylase (CYP46A1), which is CNS specific and under healthy conditions only expressed by neurons (Brown et al., 2004; Ramirez et al., 2008). CYP46A1 converts excess cholesterol to 24S-hydroxycholesterol (24S-OHC) (Lund et al., 2003; Ramirez et al., 2008; Zhang and Liu, 2015; van der Kant et al., 2019), which can be released by neurons and crosses the BBB through diffusion, forming a major export pathway for excess cholesterol from the brain (**Figure 1**) (Lütjohann et al., 1996; Lund et al., 1999, 2003; Xie et al., 2003). Due to its neuron specific origin, 24S-OHC levels in the blood also provide a direct measure of cholesterol turnover levels in the brain (Sodero, 2020). Besides being an export product, as other oxysterols, 24S-OHC can promote ApoE-mediated cholesterol export by activating liver X receptor (LXR) (**Figure 1**) (Abildayeva et al., 2006; Matsuda et al., 2013). Additional oxysterols that are produced in the brain include 27-OHC, which is generated by the enzyme CYP27A1 and can be further processed by CYP7B to form 7α-hydroxy-3-oxo-4-cholestenoic acid (7-OH-4-C). 7-OH-4-C can cross the BBB to be eliminated by the liver. CYP27A1 is expressed in multiple brain cell types, yet 27-OHC levels in the brain are only a fraction of the far more abundant 24S-OHC (Brown et al., 2004; Heverin et al., 2004; Gilardi et al., 2009). In fact most 27-OHC is not produced in the brain but enters the brain via the BBB originating outside the CNS (Gamba et al., 2015).

While neurons depend on the astrocyte-to-neuron lipid shuttle for supply of cholesterol and cholesterol precursors, recent studies have shown that lipids under certain circumstances can also be transported from neurons to astrocytes. For example, neuronal lipids can become peroxidized when they encounter oxidative stress, potentially generated due to hyperactivity or as an incidental of aging. Neurons are not well equipped to deal with these toxic lipids, and peroxidized lipids in neurons are therefore transported to astrocytes in an ApoE-dependent manner (**Figure 1**) (Liu et al., 2015, 2017; Moulton et al., 2021; Qi et al., 2021; Ioannou et al., 2019). Astrocytes store these lipids while also upregulating expression of genes responsible for oxidative energy metabolism to process these peroxidized lipids,

thereby protecting neuronal integrity (Ioannou et al., 2019). While this directional of lipid-transport is now well established for fatty acids, it is unknown how this neuron-to-astrocyte lipid shuttle affects cholesterol transport and overall cholesterol metabolism in the healthy brain.

BRAIN CHOLESTEROL METABOLISM; CHANGES FROM DEVELOPMENT TO AGING

The processes that maintain cholesterol homeostasis in the healthy brain are not static, but change during early human development and later again in the aging brain. Cholesterol synthesis rates are at the highest-level during brain development to support the generation of an extensive neuronal network (Waelsch et al., 1940; Quan et al., 2003; Pfrieger and Ungerer, 2011). Therefore, all brain cell types, including both neurons and glial cells are thought to contribute to cholesterol biosynthesis during development (Pfrieger and Ungerer, 2011; Genaro-Mattos et al., 2019). Once an adult, brain cholesterol synthesis rate declines and astrocytic cholesterol production ensures sufficient levels to support neuronal plasticity and glial performance (Andersson et al., 1990; Lütjohann et al., 1996; Dietschy and Turley, 2004; Zhang and Liu, 2015). A further decline in cholesterol synthesis upon aging is suggested by detection of lower cholesterol precursor levels in post mortem hippocampal tissue from middle-aged and elderly (>38 years) donors compared to young (<38 years) donors (Thelen et al., 2006). Yet, absolute cholesterol levels were stable in aged human hippocampal tissue, while a decrease is observed in white and gray matter regions (Söderberg et al., 1990; Thelen et al., 2006). Hippocampal 24S-OHC levels showed a downward trend in middle-aged and elderly (>38 years) donors, which also suggests a decrease in cholesterol metabolism and cholesterol turnover (Thelen et al., 2006). Possibly, lower cholesterol turnover helps to keep cholesterol levels relatively stable in the aging brain when cholesterol synthesis decreases, but less cholesterol turnover might also contribute to reduced neuronal plasticity associated with aging (Thelen et al., 2006). In addition, the BBB, which normally separates CNS and peripheral cholesterol, has been shown to lose integrity during aging (Montagne et al., 2015). This might affect brain cholesterol levels especially in the hippocampus where BBB break-down has been reported to occur first (Montagne et al., 2015; Segarra et al., 2021). Indeed in mice, BBB breakdown results in entry of peripheral cholesterol into the brain, and reversely BBB breakdown also led to increased release of 24S-OHC from the brain into the circulation (Saeed et al., 2014). Brain cholesterol synthesis was increased upon BBB disruption in mice, which might be induced to compensate for the lowered 24S-OHC level (Saeed et al., 2014). What happens to intracellular levels of cholesterol in neurons, astrocytes and microglia during the aging process or downstream of BBB breakdown is not well known.

Age-associated neurodegeneration itself also has a major impact on brain cholesterol metabolism. For example, dying neurons generate high levels of cholesterol-rich debris, in part due to the dismantling of myelin sheets formed by oligodendrocytes. This debris is subsequently phagocytosed by microglia (Callaghan et al., 2014). Recently, Cantuti-Castelvetri et al. (2018) showed that in the aged brain, phagocytes (mainly representing microglia) had lost the ability to process excess amounts of cholesterol, which depended on ApoE and led to accumulation of cholesterol into crystals in the phagocytic cells. The intracellular accumulation of cholesterol in microglia induced an inflammatory response and prevented successful re-myelination. Re-myelination could be restored by stimulation of reverse cholesterol transport or inhibition of the inflammatory response, indicating that aging can affect the cholesterol efflux capacity of immune cells in the brain, which perturbs timely reversal of immune responses needed for proper re-myelination (Cantuti-Castelvetri et al., 2018). Increased presence of lipid droplets, which are storage sites for neutral lipids like glycerolipids and CEs has also been observed in aged microglia of both mouse and human brains (Shimabukuro et al., 2016; Farmer et al., 2020; Marschallinger et al., 2020). While the lipid composition in these droplets has not been well characterized in humans, lipidomic analysis was performed on lipid-droplet containing microglia from aged mouse hippocampus. These lipid droplets contained predominantly glycerolipids, like triacylglycerols (TAGs), diacylglycerols (DAGs), and monoacylglycerols (MAGs), but little CE, indicating that cholesterol is not a major contributor to this age-related phenotype at least in mice (Marschallinger et al., 2020). Therefore, these so-called Lipid-droplet-accumulating microglia (LDAM) seem to be distinct from the microglia in aged mice that accumulated cholesterol after de-myelination (Cantuti-Castelvetri et al., 2018; Marschallinger et al., 2020). In contrast to the general aging process, in AD intracellular cholesterol accumulation has been broadly reported for a number of cell types, as discussed below.

CHOLESTEROL AND AD, A DUAL DRIVER OF Aβ AND TAU PATHOLOGY IN NEURONS

Cholesterol, APP Processing and Aβ Generation in Neurons

The relationship between cholesterol metabolism, APP processing and Aβ production has been characterized in much detail. Aβ is generated when the amyloid precursor protein (APP) is sequentially processed by β-secretase (BACE1) and γ-secretase. Alternatively, APP can be cleaved by α-secretases, a pathway known as the non-amyloidogenic pathway. APP is normally present in the bilayer membrane of the cell and concentrated in neuronal synapses (Zheng and Koo, 2011). Exogenous addition of cholesterol to human brain tissue lysates promoted BACE1 and γ-secretase activity (Xiong et al., 2008) and treatment of primary mouse neuronal cultures with excess cholesterol was sufficient to increase $A\beta_{42}$ secretion (Marquer et al., 2014). In addition, exogenous cholesterol addition in APP transfected HEK293 cells reduced APP processing via the non-amyloidogenic pathway

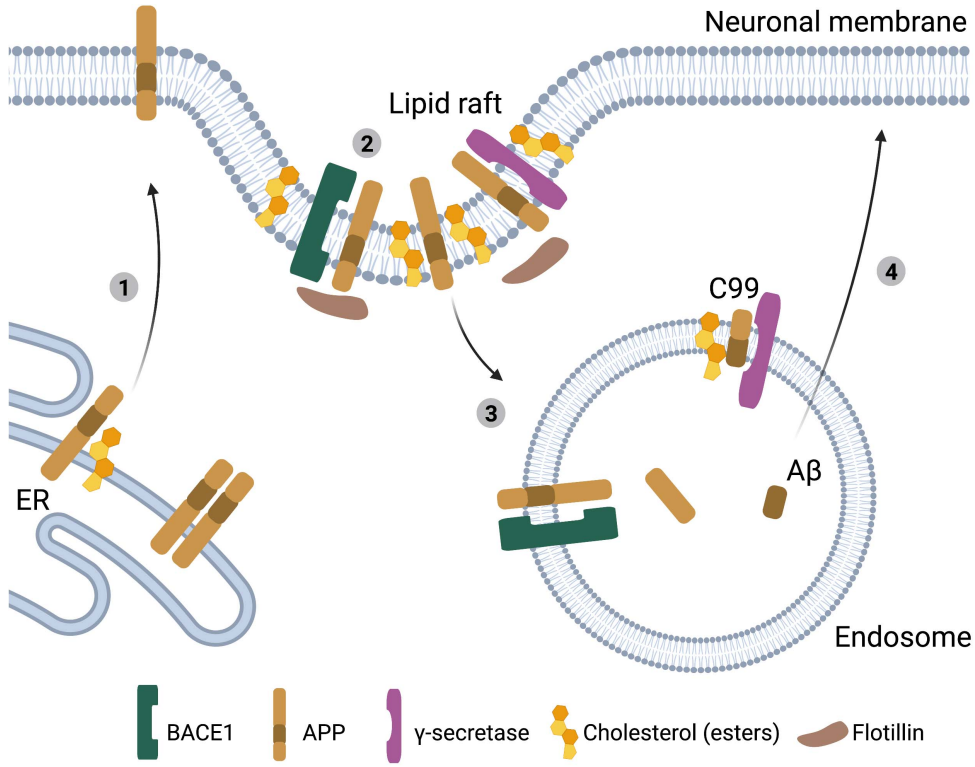

FIGURE 2 | Cholesterol, APP processing and Aβ generation in neurons. Schematic representation of the interactions between cholesterol and APP processing in neurons. (1) High ER-cholesterol levels prevent APP dimerization and promote APP transport to the plasma membrane. (2) Cholesterol or cholesterol esters in the plasma membrane stimulate the clustering of APP, BACE1 and γ-secretases in lipid rafts. (3) These lipid rafts undergo clathrin-mediated endocytosis resulting in amyloidogenic processing of APP to Aβ. (4) Aβ peptides are subsequently secreted from the neuron (BioRender, 2021).

by α-secretase (Bodovitz and Klein, 1996). Accordingly, lower cholesterol levels have been shown to inhibit APP processing by BACE1 and γ-secretase, while promoting processing of APP via the non-amyloidogenic pathway (Simons et al., 1998; Kojro et al., 2001; Refolo et al., 2001; Schneider et al., 2006; Grimm et al., 2008). These effects of cholesterol on APP processing could be mediated by effects of cholesterol on membrane composition. As a transmembrane protein APP can localize in lipid rafts, which are small sterol- and sphingolipid enriched domains that facilitate protein and lipid interactions and play a role in cellular signaling and membrane transport (Hicks et al., 2012). Multiple studies together uncovered that an increase in cholesterol levels promotes APP and BACE1 colocalization in lipid rafts which promotes clathrin-mediated endocytosis and APP processing via the amyloidogenic pathway (**Figure 2**) (Wahrle et al., 2002; Cordy et al., 2003; Ehehalt et al., 2003; Osenkowski et al., 2008; Cossec et al., 2010; Marquer et al., 2011). This is supported by a recent study in human iPSC-derived neurons, where lowering cholesterol levels reduced the interaction between full-length APP (flAPP) and BACE1, potentially explaining why APP processing is inhibited and flAPP levels are increased upon statin treatment (Langness et al., 2021). In addition, a cholesterol dependent interaction between flotillin and APP in lipid rafts might further promote endocytosis (Chen et al., 2006; Schneider et al., 2008; Cho et al., 2020). Importantly the cholesterol effect is APP specific, as higher membrane cholesterol levels did not affect endocytosis of other membrane proteins like transferrin (Cossec et al., 2010). Also, high membrane cholesterol levels only promote endocytosis and amyloidogenic processing of APP that is localized in lipid rafts, while APP located in the membrane outside of lipid rafts is unaffected (Cho et al., 2020). When analyzing lipid raft composition in postmortem AD vs. control brain tissue, Fabelo et al. (2014) actually detected lower cholesterol, but higher CE presence in lipid rafts of AD subjects, indicating that plasma membrane CE might also contribute to regulation of APP processing. Increased levels of CE, have also been observed both in human postmortem AD brain tissue and in mouse models of AD (Chan et al., 2012; Tajima et al., 2013; Yang et al., 2014).

Cholesterol can also bind directly to APP in the transmembrane C-terminal domain (C99) (Beel et al., 2008; Barrett et al., 2012; Song et al., 2013; Nierzwicki and Czub, 2015). In iPSC derived neurons mutating the cholesterol-binding domain in APP results in reduced APP processing and Aβ production (van der Kant et al., 2019). In addition, inhibition of Aβ secretion by cholesterol-lowering statin treatment depended on the cholesterol binding domain in APP (van der Kant et al., 2019). Export of APP from the ER and subsequent APP processing to Aβ is also cholesterol and/or CE dependent (Puglielli et al., 2001; Hutter-Paier et al., 2004;

Huttunen et al., 2009; van der Kant et al., 2019), and regulated by the cholesterol-binding domain in APP (Langness et al., 2021). APP dimerization inhibits exit of flAPP from the ER, and subsequent processing to Aβ (Kaden et al., 2008; Eggert et al., 2009, 2018; Decock et al., 2015; Langness et al., 2021). Interestingly, residues required for binding of APP to cholesterol overlap with residues required for APP dimerization (Song et al., 2013), and thus binding of cholesterol to APP might prevent APP dimerization thereby enhancing ER-export of monomeric APP (Nierzwicki and Czub, 2015; Langness et al., 2021). Whether the cholesterol-binding domain of APP also directly affects APP recruitment into lipid rafts, thereby providing another level of cholesterol-dependent regulation of APP-processing is currently unknown. Also, whether ER cholesterol, or ER CE's are the main driver of these processes needs to be further established. It is, however, interesting to note, that like SREBP processing, the regulation of APP processing is very much dependent on intracellular cholesterol levels, raising the possibility that APP is a regulator of intracellular cholesterol homeostasis, which is also supported by a number of publications (**Box 2**). Overall, there is evidently a strong correlation between cholesterol levels and Aβ generation, with increased levels of cholesterol and/or CE in neurons driving Aβ generation (**Figure 2**).

Cholesterol and Tau

In addition to an established connection between cholesterol and APP processing, cholesterol metabolism was also recently found to directly regulate phosphorylated Tau (pTau) levels in iPSC-derived neurons. As identified by an unbiased high-throughput drug screen, drugs that reduced CE levels in iPSC-derived neurons from familial AD (FAD) patients, also decreased pTau levels (van der Kant et al., 2019). This reduction of pTau was mediated by an increase in proteasomal degradation of pTau, and independent on the effect of CE on APP processing and Aβ (van der Kant et al., 2019). Interestingly, genetically lowering cholesterol esterification in triple-transgenic AD mice (3xTg-AD) mice also lowered pathological Tau accumulation (Shibuya et al., 2015). In addition, *in vivo*, genetic inhibition of ApoE-mediated cholesterol transport from astrocytes to neurons also reduced neuronal pTau levels in mice (Wang et al., 2020). While the exact mechanism underlying cholesterol-dependent regulation of Tau needs to be further established, these findings do further implicate cholesterol as a central player in AD pathogenesis upstream of Aβ and Tau pathology (Kant et al., 2019).

BOX 2 | Tables turned; APP as a regulator of cellular cholesterol metabolism. While cholesterol levels are known to regulate APP processing (**Figure 2**), accumulating data indicate that APP and its cleavage products can also regulate cholesterol metabolism in turn. In primary cultures of rat cortical neurons, higher expression of full length APP decreased HMGCR-mediated cholesterol synthesis, while lowering APP levels increased cholesterol biosynthesis (Pierrot et al., 2013). Similarly, deletion of APP caused increased SREBP2 target gene expression in human iPSC derived astrocytes (Fong et al., 2018). In addition, the C99 APP-fragment has been demonstrated to cluster cholesterol in the ER membrane thereby lowering *de novo* cholesterol synthesis (Montesinos et al., 2020), while the APP intracellular domain (AICD), a cytosolic fragment generated from C99, can directly bind to, and suppress, the LRP1 promoter thereby potentially lowering LRP1 dependent uptake of ApoE delivered cholesterol into neurons (Liu et al., 2007). In line with the position of the cholesterol binding domain in APP (Barrett et al., 2012), $A\beta_{40}$ and $A\beta_{42}$ peptides have been shown to bind extracellular cholesterol, thereby competing with ApoE or LDL driven cholesterol import, and reducing ApoE-dependent cholesterol delivery (Yao and Papadopoulos, 2002). In astrocytes, exogenous Aβ stimulated cholesterol transport from plasma membrane to the Golgi, thereby lowering plasma membrane cholesterol levels (Igbavboa et al., 2009). Finally, $A\beta_{42}$ has been shown to inhibit astrocytic ABCA1 expression (Canepa et al., 2011), which would reduce cholesterol secretion and transport to neurons. Together these results show that APP processing and cleavage fragments can directly affect brain cholesterol homeostasis. This raises the interesting question whether FAD-associated mutations that affect APP-processing also alter brain cholesterol metabolism, which could then further contribute to AD pathology in a cholesterol-dependent manner. Indeed, accumulation of CE has been demonstrated in multiple mouse models of FAD (Chan et al., 2012; Tajima et al., 2013; Yang et al., 2014), indicating that altered brain cholesterol metabolism could hurry pathogenesis also in FAD.

DYSREGULATION OF BRAIN CHOLESTEROL IN AD; IT IS IN THE GENES

The last decade has seen the discovery of numerous genetic risk factors for LOAD by genome-wide-association studies (GWAS) on LOAD patients vs. healthy controls (Lambert et al., 2013; Jansen et al., 2019; Kunkle et al., 2019; Bellenguez et al., 2020). A high number of the LOAD risk genes have roles in lipid homeostasis, which is best defined for ApoE.

ApoE

Three common allelic ApoE genetic variants exist in the human population; ApoE2, ApoE3 and ApoE4. ApoE3 is the most common isoform present homozygous in over 60% of the population and is considered the reference allele for LOAD risk (Jeong et al., 2019). ApoE4 is a strong risk factor for LOAD: carriers of one ApoE4 allele have a 3 to 4-fold increased risk for LOAD, while homozygous ApoE4 carriers have an approximate 14-fold increased risk of developing LOAD compared to ApoE3 carriers (Liu et al., 2013). It has to be noted, however, that the penetrance of the ApoE4-risk allele varies in different ethnicities, possibly due to differences in ApoE expression levels (Griswold et al., 2021). In contrast to ApoE4, expression of the ApoE2 allele confers a decreased risk for LOAD and hence is considered protective (Corder et al., 1994; Reiman et al., 2020). Despite their strong effects on LOAD risk, the three ApoE isoforms only differ from each other by two amino acids (Chen et al., 2020). ApoE3 contains a Cys112 and Arg158, of which Cys112 is changed to Arg112 in ApoE4 and Arg158 is changed to Cys158 in the ApoE2 variant (**Figure 3**). As an apolipoprotein ApoE interacts with lipoproteins to execute its function as a cholesterol and lipid carrier. Via a receptor binding domain ApoE can interact with lipoprotein receptors to be internalized and deliver the cargo of lipids to cells. As described above, this ApoE-dependent route is crucial for transport of cholesterol and cholesterol precursors from astrocytes to neurons in the mature brain.

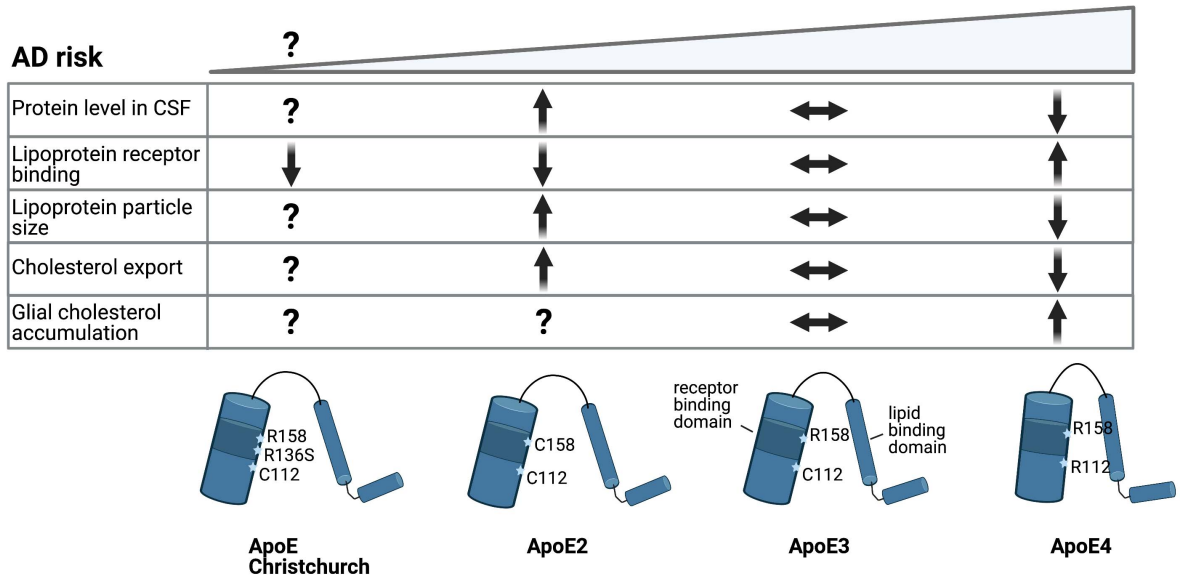

FIGURE 3 | Alzheimer's disease risk variants in ApoE affect cholesterol metabolism. Schematic representation of ApoE allelic variants and their consequences. Single amino acid changes in ApoE confer different risk for LOAD. Conformational change of the protein structure by expression of the different ApoE alleles changes LDL receptor binding and lipid particle binding capacity, which might affect brain cholesterol metabolism (BioRender, 2021).

Strikingly enough, while peripheral ApoE4 is a major risk factor for hypercholesterolemia (**Box 1**), it remains largely unknown how ApoE isoforms affect cholesterol metabolism in the brain. Astrocytes are the highest ApoE-expressing cell type in the brain (Huang et al., 2004), and transcriptomic analysis of human iPSC-derived neurons, astrocytes and microglia, revealed that ApoE4 driven changes in gene expression were most dramatic in astrocytes (Julia et al., 2019). Compared to ApoE3 astrocytes, ApoE4 astrocytes expressed higher levels of genes with a role in cholesterol biosynthesis and displayed cholesterol accumulation in lysosomes, while CE levels were not increased (Lin et al., 2018; Julia et al., 2019). The dysregulation in lipid metabolic genes in ApoE4 astrocytes was also confirmed in human control and AD brain samples (Julia et al., 2019). Also, an increased number of smaller lipid droplets has been detected in ApoE4 astrocytes compared to ApoE3 astrocytes derived from human ApoE-replacement mice (Farmer et al., 2019).

How ApoE4 affects cholesterol metabolism in other brain cell types like neurons and microglia is even less clear. ApoE4 expressing human iPSC-derived astrocytes showed reduced support of neuronal survival in an iPSC-derived neuron-astrocyte co-culture compared to ApoE3 expressing astrocytes (Zhao et al., 2017). One way by which different ApoE polymorphisms could affect total brain lipid metabolism is by altering the export of ApoE-lipoprotein particles (**Figure 3**). In human CSF and upon overexpression in mice, ApoE2 has been shown to generate bigger HDL particles compared to ApoE3, while ApoE3 in turn is associated with bigger HDL particles then ApoE4, suggesting less sterol transport by ApoE4 (Hu et al., 2015; Heinsinger et al., 2016). Accordingly, an isoform dependent effect on cholesterol efflux, ApoE2 > ApoE3 > ApoE4, was detected previously in primary rat or mouse astrocytes and neurons (Michikawa et al., 2000; Rawat et al., 2019). No isoform dependent changes in binding of ApoE to ABCA1 were found that could explain reduced cholesterol efflux from ApoE4 expressing astrocytes, although ApoE4 has been suggested to affect ABCA1 membrane trafficking (Krimbou et al., 2004; Rawat et al., 2019). In addition, ABCA1 has recently been identified as a LOAD risk gene itself further implicating this pathway in LOAD pathogenesis (Bellenguez et al., 2020). In addition to export, ApoE genotype might also differentially affect the internalization of ApoE-lipoprotein particles. Lipidation of the ApoE protein triggers a conformational change that increases its binding affinity for the LDL receptors. Lipidated ApoE4 shows the strongest binding affinity for LDLR, while the binding of lipidated ApoE2 to LDLR is reduced compared to ApoE3 (**Figure 3**) (Chen et al., 2020). How these differences in receptor binding affinity affect uptake of ApoE-lipoprotein particles in specific brain cell types remains to be determined. The protein levels of ApoE also differ between isoforms, where ApoE2 levels are highest and ApoE4 levels are lowest in CSF and plasma (Castellano et al., 2011; Cruchaga et al., 2012). This might be a result of their different receptor affinities, as LDLR loss caused an increase in ApoE3 and ApoE4 levels, but not ApoE2 levels (Fryer et al., 2005). Importantly, the recently described Christchurch mutation in *APOE* (R136S) results in strongly reduced LDLR binding of ApoE, similar to ApoE2 (**Figure 3**). Presence of this mutation has been reported in an individual who had no signs of cognitive decline or Tau-pathology until advanced age despite carrying a PSEN1 mutation that causes autosomal-dominant AD (Arboleda-Velasquez et al., 2019). These results suggest a strong protective effect of the Christchurch ApoE variant, although this conclusion awaits further confirmation. Together, these results suggest strong ApoE genotype dependent effects

on cholesterol metabolism in astrocytes. Whether, and how, this affects the proper astrocyte-dependent support of neuronal function and/or downstream AD pathology in neurons and glia remains to be determined.

Besides ApoE, other AD risk genes also have a predicted role in lipid metabolism. This is established for AD-genes that act in similar processes as ApoE and ABCA1, such as CLU and ABCA7 (discussed below), but also increasingly recognized for genes which are highly, or exclusively, expressed in microglia (discussed in the next section).

CLU and ABCA7

Like ApoE, clusterin (CLU) is a component of lipoproteins. The *CLU* gene producing clusterin (also referred to as ApoJ) is expressed in astrocytes and has a wide range of biological functions including cholesterol and lipid transport. In periphery, clusterin can form HDL particles that are transported to the liver (Baralla et al., 2015; Foster et al., 2019). Also, clusterin is expressed upon damage in arteries and could remove cholesterol from macrophage-foam cells that promote formation of atherosclerotic lesions (Gelissen et al., 1998; Foster et al., 2019). Clusterin plasma levels have been shown to correspond to severity of AD in patients (Thambisetty et al., 2010; Jongbloed et al., 2015), yet the specific role in brain cholesterol homeostasis is unknown.

Another AD risk gene, ABCA7 shares 54% homology with ABCA1, the protein known to load cholesterol onto ApoE particles (Kaminski et al., 2000). However, what role ABCA7 has in intracellular cholesterol- and lipid transport remains unclear. ABCA7 is highly expressed in the brain, predominantly in neurons and microglia (Zhang et al., 2014). Reduced ABCA7 levels are observed in AD brain (Lyssenko and Praticò, 2020) and hippocampus of mice on a high fat diet (Zou et al., 2020). Contrary to ABCA1, transcription of ABCA7 is downregulated when cholesterol levels are high in the cell (Iwamoto et al., 2006). Recent studies on detergent purified ABCA7 showed that removal of cholesterol led to increased ATPase activity in ABCA7 (Le et al., 2021). In addition, ATPase activity was stimulated by interaction with apolipoproteins, ApoA-I and ApoE, where ATPase activity was hardly stimulated in presence of the ApoE4 compared to the ApoE3 variant. Interestingly, also the ApoE2 variant had a lower effect in stimulating ATPase activity of ABCA7 compared to ApoE3 (Le et al., 2021). The physiological consequences of these ApoE genotype dependent effects on ABCA7 and cholesterol metabolism remain to be determined. *In vitro* analysis in baby hamster kidney (BHK) cells transfected with either ABCA1-GFP or ABCA7-GFP revealed that free cholesterol efflux through ABCA7 was much lower compared to ABCA1 and was unaffected by ApoE genotype, while cholesterol efflux by ABCA1 was greatly reduced in presence of ApoE4 (Tomioka et al., 2017). Interestingly, recent data on the ABCA7 homolog in Drosophila suggests that ABCA7 might play a role in the neuron-to-glial transport of lipids to protect neuronal functionality and viability from toxic accumulation of peroxidized lipids, for instance generated by oxidative stress (Moulton et al., 2021). These results open the door for future studies on the role of ABCA7 in brain cholesterol metabolism and AD development.

CHOLESTEROL AS A DRIVER OF (MICRO)GLIAL DYSFUNCTION AND GLIOSIS

TREM2, PLCγ2, and Microglial ApoE

Multiple LOAD risk genes such as ApoE, TREM2 and PLCγ2 are highly -or exclusively- expressed in (micro)glia and have been shown to regulate lipid metabolism and microgliosis. Increased expression of genes associated with lipid metabolism are found in microglia during development, damage or disease (Efthymiou and Goate, 2017; Keren-Shaul et al., 2017; Hammond et al., 2019; McQuade and Blurton-Jones, 2019). A good example is triggering receptor expressed on myeloid cells 2 (TREM2), which in brain is primarily expressed in microglia. Carriers of the R47H or R62H variant in this gene have an up to 4-fold increased risk for LOAD (Ulrich and Holtzman, 2016). TREM2 has multiple ligands including the apolipoproteins ApoE and clusterin, and binding of TREM2 to these proteins is enhanced by their lipidation (Atagi et al., 2015; Bailey et al., 2015; Yeh et al., 2016). Binding of TREM2 to lipoprotein particles is reduced by the TREM2 R47H variant, which could lead to altered cholesterol load in microglia and also affects phagocytosis of lipoprotein bound Aβ by microglia (Yeh et al., 2016). Both astrocytes and microglia can phagocytose Aβ, thereby contributing to Aβ clearance from the brain (Tarasoff-Conway et al., 2015; Ries and Sastre, 2016). Single-cell RNA-sequence (RNA-seq) data from AD mouse models revealed so-called disease associated microglia (DAM) as a reactive microglial population that is generated when AD pathology is present in the brain (Keren-Shaul et al., 2017). The transition to a DAM-phenotype was dependent on TREM2 (Zhou et al., 2020). DAM have reduced expression of several homeostatic microglial genes accompanied by a significant increase in expression of lipid metabolism and phagocytosis genes, including ApoE. These activated DAM microglia localize predominantly around amyloid plaques where they form a neuroprotective barrier, prevent propagation of Tau pathology in mice and are believed to play a role in Aβ clearance (Condello et al., 2015; Yuan et al., 2016; Keren-Shaul et al., 2017; Leyns et al., 2019). Of note, although increased ApoE levels were detected by recent (single-cell and single-nuclear) RNAseq studies in microglia from human AD postmortem brain tissue, a subpopulation with DAM signature was not identified (Grubman et al., 2019; Olah et al., 2020; Srinivasan et al., 2020; Zhou et al., 2020). While this may indicate differences between AD pathogenesis in mouse models and in humans, it could also be a consequence of technical limitations, e.g., too few single microglial cells analyzed or the low sensitivity of single-nuclear RNAseq to detect microglial activation genes (Del-Aguila et al., 2019; Mathys et al., 2019; Thrupp et al., 2020). As a consequence of TREM2 loss, microglia from *TREM2*$^{-/-}$ mice on a demyelinating cuprizone (CPZ) diet failed to upregulate DAM-genes needed for cholesterol transport and lipid metabolism like ApoE, and could not clear myelin

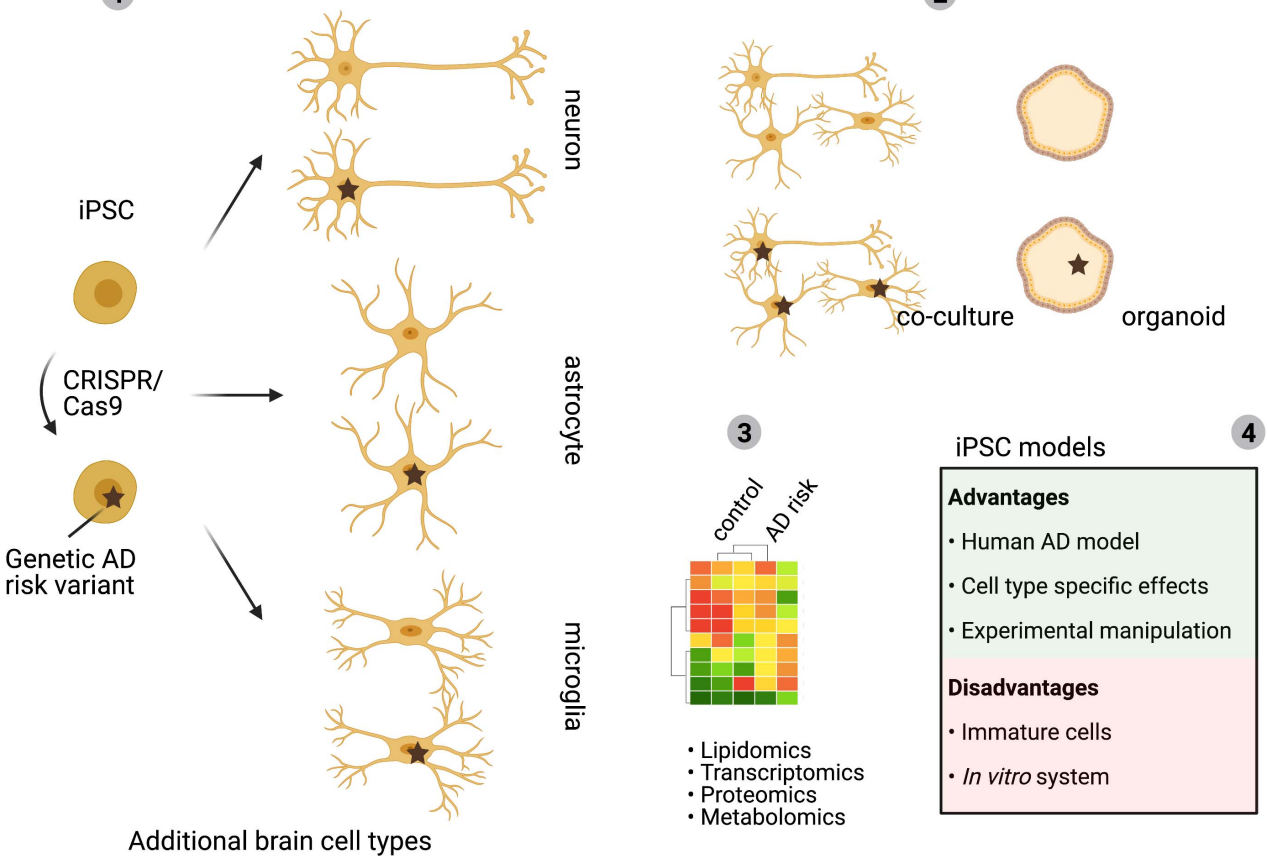

FIGURE 4 | Human iPSC models to study cell type specific effects. Multiple LOAD-risk variants are found in genes with an expected role in lipid metabolism and changes in cholesterol metabolism are linked to AD development. Yet, how individual LOAD-risk variants affect brain cholesterol metabolism and AD pathology is largely unclear. As described in this review, the complex organization of brain cholesterol metabolism depends on cell intrinsic metabolism as well as on the transport of cholesterol between the different brain cell types. Development of human iPSC derived cell models allows for separation of these processes and gives the possibility to introduce risk variants in each selected brain cell type (1). For example, analysis of cell type specific effects of LOAD-risk gene expression will uncover in which cell type a LOAD-risk variant has the biggest impact. Co-culture models of different brain cell types can subsequently be used to identify how LOAD-risk variants affect lipid transport, cholesterol metabolism between different brain cell types and how this affects downstream AD pathology (2). iPSC-derived brain cell models allow for -omics approaches to determine complex cell type dependent effects (3). Advantages and disadvantages of iPSC-models are discussed in (4). Mechanistic insights acquired by iPSC studies can contribute to the identification of novel (cell type) specific targets for future therapy development (BioRender, 2021).

derived cholesterol, leading to microglial CE accumulation. A similar accumulation of CE was observed in human TREM2 knock out iPSC-derived microglia-like cells (iMG), when these were treated with exogenous myelin (Nugent et al., 2020). The role of TREM2 in lipid metabolism is (at least in part) mediated by the enzyme phospholipase C γ2 (PLCγ2), for which a LOAD-protective variant has been identified (P522R). PLCγ2 is an intracellular enzyme, which is also specifically expressed in microglia. The protective P522R variant is associated with a gain of function and hence loss of TREM2 or PLCγ2 are both expected to negatively affect LOAD risk (Magno et al., 2019). Indeed, TREM2 or PLCγ2 deficient iMG showed a similar defect in upregulation of genes needed for lipid metabolism. Accordingly, both TREM2- and PLCγ2-deficient iPSC derived microglia fail to clear cholesterol after phagocytosis of myelin debris (Andreone et al., 2020; Nugent et al., 2020). Analysis of the lipidome after myelin treatment revealed accumulation of free cholesterol, CE, myelin-derived ceramides [Cer, hexosylceramides (HexCer), sulfatides and diacylglycerols (DAGs) and triacylglycerols (TAGs) in the TREM2- and PLCγ2-deficient iMG compared to WT iMG (Andreone et al., 2020)]. Importantly, Andreone et al. (2020) show that expression of the LOAD-protective variant P522R reduced CE accumulation to a greater extent than WT PLCγ2, further indicating that TREM2 and PLCγ2 work together in microglial lipid metabolism and demonstrating the relevance of this pathway for LOAD development.

Accumulation of CE was also observed in sorted microglia from *ApoE*$^{-/-}$ mice, indicating that ApoE dependent transport prevents cholesterol overload in microglia (Nugent et al., 2020). ApoE levels are increased in AD-brain microglia and both intracellular and extracellular clearance of Aβ is greatly facilitated by lipidated ApoE particles (Jiang et al., 2008). Lipidated ApoE

can directly interact with Aβ to support phagocytosis. In addition, independent of a direct ApoE-Aβ interaction, depletion of cellular cholesterol from microglia via ApoE-containing HDL particles promoted Aβ degradation in primary mouse microglial cultures (Lee et al., 2012). How the TREM2-PLCγ2-ApoE axis contributes to LOAD development remains an important question for future studies. Whether the effects of the LOAD risk genes on AD pathology are directly coupled to the function of these genes in (micro)glial cholesterol metabolism is currently unknown, but of great interest to the field.

From Cholesterol Dysregulation to (Micro)Gliosis

Glial LOAD risk genes that affect (micro)glial cholesterol metabolism could impact AD pathology via their effect on gliosis, represented by accumulation of reactive astrocytes and immune activated microglia (Shi and Holtzman, 2018). Gliosis is detected early in AD development, and seen as a major pathological hallmark (Heneka et al., 2005; Carter et al., 2012). While considered primary a neuroprotective response, gliosis is also thought to contribute to progressive AD development (Heneka et al., 2015). Changes in cholesterol metabolism have been linked to gliosis by various studies. For example, a high cholesterol diet induces astrocytic activation and increased expression of ApoE in mice (Chen et al., 2016). In line, exogenous cholesterol addition in rat astrocytes triggered astrocyte activation, indicated by upregulation of glial fibrillary acidic protein (GFAP) (Avila-Muñoz and Arias, 2015). Finally, exogenous addition of a mixture of oxysterols, representing oxysterols that are produced when cholesterol accumulates in the AD brain, promote upregulation of reactive astrocyte markers, which contributed to synaptotoxicity (Staurenghi et al., 2021). In microglia, high cholesterol can affect immune function, as particularly studied in respect of cholesterol-rich myelin debris, which promotes inflammatory activation of microglia (Cantuti-Castelvetri et al., 2018). Myelin debris treatment in bone-marrow derived macrophages from mice caused NLRP3 inflammasome activation, possibly due to lysosomal rupture after formation of cholesterol crystals (Cantuti-Castelvetri et al., 2018). Mechanistic understanding of altered immune activation in microglia upon high cholesterol load requires further studying. In macrophages, changes in membrane cholesterol load have also been shown to affect lipid raft composition and TLR mediated signaling, where high cholesterol levels cause hyperresponsiveness to LPS treatment (Fessler and Parks, 2011). In addition, high cholesterol levels could drive CD36-dependend inflammatory signaling via inhibition of Nrf1 in the ER (Widenmaier et al., 2017). As described above reversed cholesterol transport is needed to revert the pro-inflammatory state in microglia and promote re-myelination (Cantuti-Castelvetri et al., 2018). Microglia increase post-squalene sterol synthesis in response to cholesterol overload to activate LXR dependent transcription and promote cholesterol export (Berghoff et al., 2021). Increased secretion of pro-inflammatory cytokines IL-1β and IL-18 upon myelin treatment combined with TLR activation in TREM2 KO iMG suggests that increased sterol levels might indeed contribute to excessive inflammatory responses by microglia, which might be further enhanced in presence of LOAD risk genes (Andreone et al., 2020). Moreover, Lin et al. (2018) recently suggested that ApoE4 expression in microglia might be sufficient to convert them into an immune-active state. In line, ApoE4 expressing primary mouse microglia respond stronger to immune activation compared to ApoE2- or ApoE3 expressing microglia (Wong et al., 2020). Whether these processes are mediated by altered cholesterol metabolism in glia downstream of ApoE remains to be established.

CONCLUSION

Cholesterol is a central player in AD affecting Amyloid, Tau and gliosis. In addition, LOAD genetic risk factors point to a strong effect of lipid metabolism in AD development. Yet, mechanistic understanding of the pathways by which dysregulation of cholesterol metabolism contributes to AD development remains largely lacking. A number of major outstanding questions are; (1). How is cholesterol metabolism affected in each specific brain cell type in AD patients (2). How do LOAD-risk genes affect cholesterol metabolism in specific brain cell types and the transport of lipids between these cells (3). How do changes in brain cholesterol metabolism contribute to AD pathology (Amyloid, Tau, and gliosis) and finally (4) is cholesterol itself or one of its derivatives most toxic in this context?

Progress in technology development has delivered new tools to address these questions in human cells and tissue. Techniques that will help to answer the outstanding questions include the generation of human brain cell types from induced pluripotent stem cells and introduction of specific LOAD-risk mutations by CRISPR/Cas9 gene-editing (**Figure 4**). This approach allows the mapping of cell type specific effects of LOAD-risk variants on cholesterol metabolism combined with the possibility to mechanistically study AD pathology for a certain genetic background. Extension of this approach to co-culture or 3D organoid models of different iPSC derived brain cell types gives the opportunity to further study complex interplay between different cell types of the brain (**Figure 4**). Finally, single-nuclear- or single-cell-RNA sequencing of postmortem brain tissue, or 3D iPSC derived brain cell models, with distinct LOAD-risk genotypes will result in comprehensive data on cell type specific effects on gene expression. In addition, subcellular populations that might impact cholesterol metabolism or AD pathology can be identified by this approach. A better mechanistic understanding of the cholesterol-dependent pathways that drive (early) AD development will also uncover novel (cell type) specific targets for rational drug discovery. An example of such a drug discovery effort for brain-lipid targeting drugs is the discovery that Efavirenz, an FDA approved HIV-drug, activates the neuronal specific enzyme CYP46A1 to promote conversion of excess cholesterol to 24S-OHC that can be secreted from the brain via the BBB. Efavirenz lowered pTau levels in iPSC-derived neurons from AD patients and improved behavior in 5xFAD mice

(Petrov and Pikuleva, 2019; Petrov et al., 2019; van der Kant et al., 2019). A phase I clinical trial with intermediate-to-high doses of Efavirenz has started in patients with MCI in the United States (Nugent et al., 2020) and a similar trial with low-dose Efavirenz is planned to start in the Netherlands.

The approaches described above, and the rapidly increasing knowledge on brain lipid metabolism, will contribute to tackling the outstanding questions in this field and will undoubtedly provide much needed new insights on AD etiology and the role of cholesterol metabolism in this process. Such knowledge will likely be fundamental to develop targeted therapies to prevent, delay or cure AD in the future.

AUTHOR CONTRIBUTIONS

FF and RK provided the original conception and design of the manuscript and revised the manuscript. FF researched data and wrote the manuscript to which RK provided feedback. Both authors contributed to the article and approved the submitted version.

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REVIEW
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Cholesterol Hydroxylating Cytochrome P450 46A1: From Mechanisms of Action to Clinical Applications

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Cholesterol, an essential component of the brain, and its local metabolism are involved in many neurodegenerative diseases. The blood-brain barrier is impermeable to cholesterol; hence, cholesterol homeostasis in the central nervous system represents a balance between *in situ* biosynthesis and elimination. Cytochrome P450 46A1 (CYP46A1), a central nervous system-specific enzyme, converts cholesterol to 24-hydroxycholesterol, which can freely cross the blood-brain barrier and be degraded in the liver. By the dual action of initiating cholesterol efflux and activating the cholesterol synthesis pathway, CYP46A1 is the key enzyme that ensures brain cholesterol turnover. In humans and mouse models, CYP46A1 activity is altered in Alzheimer's and Huntington's diseases, spinocerebellar ataxias, glioblastoma, and autism spectrum disorders. In mouse models, modulations of CYP46A1 activity mitigate the manifestations of Alzheimer's, Huntington's, Nieman-Pick type C, and Machao-Joseph (spinocerebellar ataxia type 3) diseases as well as amyotrophic lateral sclerosis, epilepsy, glioblastoma, and prion infection. Animal studies revealed that the CYP46A1 activity effects are not limited to cholesterol maintenance but also involve critical cellular pathways, like gene transcription, endocytosis, misfolded protein clearance, vesicular transport, and synaptic transmission. How CYP46A1 can exert central control of such essential brain functions is a pressing question under investigation. The potential therapeutic role of CYP46A1, demonstrated in numerous models of brain disorders, is currently being evaluated in early clinical trials. This review summarizes the past 70 years of research that has led to the identification of CYP46A1 and brain cholesterol homeostasis as powerful therapeutic targets for severe pathologies of the CNS.

Keywords: CYP46A1, cholesterol, 24-hydroxycholesterol, brain, phosphorylation, plasma membranes, lipid rafts, neurodegenerative diseases

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Abbreviations: 24HC, 24-hydroxycholesterol; AAV, adeno-associated virus; AD, Alzheimer's disease; Aβ, amyloid β; ALS, amyotrophic lateral sclerosis; APP, amyloid precursor protein; CSF, cerebrospinal fluid; EFV, efavirenz; ER, endoplasmic reticulum; Glu, glutamate; HD, Huntington's disease; LTD, long-term depression; LTP, long-term potentiation; MJD; Machao-Joseph disease, MWM, Morris water maze; NMDAR, N-methyl-D-aspartate receptor; polyQ, polyglutamine; Sca, spinocerebellar ataxia; shRNA, short hairpin RNA.

INTRODUCTION

Cholesterol is an essential component of the brain constituting 22 and 27.5% of dry weight in human gray and white matter, respectively (Quarles et al., 1999). In gray matter, cholesterol is mainly found in cellular membranes of glial cells and neurons (∼30% of total brain cholesterol as determined in mice). In white matter, cholesterol is at an abundance in myelin sheaths (∼70% of total brain cholesterol as determined in mice) (Dietschy and Turley, 2001, 2004), which represent greatly extended and modified plasma membranes of oligodendrocytes wrapped around axons in a spiral fashion (Quarles et al., 1999). As a membrane constituent, cholesterol influences biophysical (e.g., ordering, fluidity, and permeability) and biological (e.g., function of membrane-bound proteins and lipid rafts) properties of the membranes (Yeagle, 1991; Simons and Ehehalt, 2002), and thereby is involved in important brain processes such as transmission of electrical impulses along axons, synaptogenesis, and synaptic function (Mauch et al., 2001; Pfrieger, 2003; Segatto et al., 2014; Egawa et al., 2016). In addition, cholesterol is used in the brain for production of neurosteroids and oxysterols, the biologically active compounds involved in different brain regulatory and signaling pathways (Mellon and Griffin, 2002; Moutinho et al., 2016; Sun et al., 2016b; Björkhem and Diczfalusy, 2020).

The majority of cerebral cholesterol is synthesized locally, as the blood-brain barrier prevents cholesterol exchange with the systemic circulation (Dietschy and Turley, 2001). Similarly, cerebral cholesterol removal is also mainly enzymatic with the major route being 24-hydroxylation catalyzed by cytochrome P450 46A1 or CYP46A1 (Bjorkhem et al., 1998; Lund et al., 2003). Studies in humans and mouse models detected changes in CYP46A1 expression or activity in a number of neurodegenerative diseases [Alzheimer's (AD), Huntington's (HD), Nieman-Pick type C, spinocerebellar ataxias (Sca), multiple sclerosis (MS), and amyotrophic lateral sclerosis (ALS)] as well as other brain disorders (epilepsy, autism, Rett syndrome, glioblastoma, and prion infection) (**Figure 1**; Loftus et al., 1997; Boussicault et al., 2016; Lopez et al., 2017; Grayaa et al., 2018; Lütjohann et al., 2018; Vejux et al., 2018; Han et al., 2019; Huang et al., 2019; Nobrega et al., 2019; Sodero, 2020; Steriade et al., 2020; Ali et al., 2021). Hence, CYP46A1 has emerged as a therapeutic target for these diseases because of its key role in cerebral cholesterol elimination.

This review will first summarize the early seminal research, which created conceptual and pre-clinical bases for the on-going clinical pharmacological and gene therapy strategies developed to use CYP46A1 as a therapeutic target to treat neurodegenerative diseases. Then, the current clinical trials evaluating CYP46A1 modulations will be described, after which a discussion of the most recent studies aimed at understanding how this single enzyme, CYP46A1, can represent a common therapeutic target for various brain diseases. Finally, a list of pressing questions will be offered from the viewpoints of a biochemist (IAP), who pioneered the investigation of CYP46A1 pharmacological modulations and is now identifying brain processes affected by CYP46A1 activity, and a gene therapy neuroscientist (NC), aiming to ultimately bring this therapeutic target to patients affected with severe neurodegenerative diseases. More comprehensive reviews on CYP46A1, oxysterols, and the links between CYP46A1, cholesterol homeostasis in the brain, and neurological disorders can be found elsewhere (Russell et al., 2009; Moutinho et al., 2016; Bjorkhem et al., 2019; Loera-Valencia et al., 2019; Petrov and Pikuleva, 2019; Choi and Finlay, 2020; Griffiths and Wang, 2020; Sodero, 2020).

24-HYDROXYCHOLESTEROL AND CYP46A1

24-Hydroxycholesterol (24HC) was first detected in the brain of different mammalian species almost 70 years ago and was called cerebrosterol because of its abundance in this organ (Di Frisco et al., 1953; Ercoli et al., 1953). Then, about 50 years ago, the enzymatic origin of 24HC was established–from brain cholesterol and the reaction catalyzed by an endoplasmic reticulum (ER) mixed function oxidase or a P450 enzyme (Dhar et al., 1973; Lin and Smith, 1974). Later, the monooxygenase mechanism of the 24HC formation was also shown in a different study (Bjorkhem et al., 1997). The investigations in 1996–1998 revealed that the brain contains ∼80% of 24HC in the human body, and that there is a continuous flux of 24HC from the brain in the systemic circulation. Most of the circulating 24HC was found to be of cerebral origin and accounted for at least 75% of cholesterol removal and turnover in human brain; the liver represented the major site for subsequent 24HC biotransformations (Bjorkhem et al., 1998). Plasma 24HC levels were determined to markedly depend on age and be ∼5 times lower in the sixth decade of human life than in the first decade of life (Lutjohann et al., 1996).

cDNAs for mouse and human cholesterol 24-hydroxylases were cloned in 1999 and demonstrated to convert cholesterol to 24HC when transfected into cultured cells (Lund et al., 1999). In both species, RNA and protein expression were predominantly confined to the brain, where the signals were detected in some, but not all, neurons of the cortex, hippocampus, dentate gyrus, and thalamus. The amino acid sequences of mouse and human cholesterol 24-hydroxylases shared a high 95% identity and suggested protein localization to the ER. The identified enzymes were the members of a new cytochrome P450 family, 46, subsequently designated as cytochromes P450 46A1 or CYP46A1. Ontogeny measurements in mice and humans documented a marked discrepancy between the 24HC levels in the serum and brain. In mice, serum 24HC levels declined with age after postnatal days 12–15, whereas the brain 24HC levels increased with age and were closely matched by an increase in the CYP46A1 expression. In human brain (the frontal lobe), CYP46A1 expression was also low at young age (before 1 year) and much higher between the ages of 1.5 and 72 years (Lund et al., 1999). CYP46A1 was found to be ectopically expressed in brain astrocytes in AD and other cell types in mouse models of MS and traumatic brain injury (Bogdanovic et al., 2001; Brown et al., 2004; Teunissen et al., 2007; Cartagena et al., 2008). Still, CYP46A1 selectivity in neuronal expression and ER localization remained valid for normal conditions (Ramirez et al., 2008).

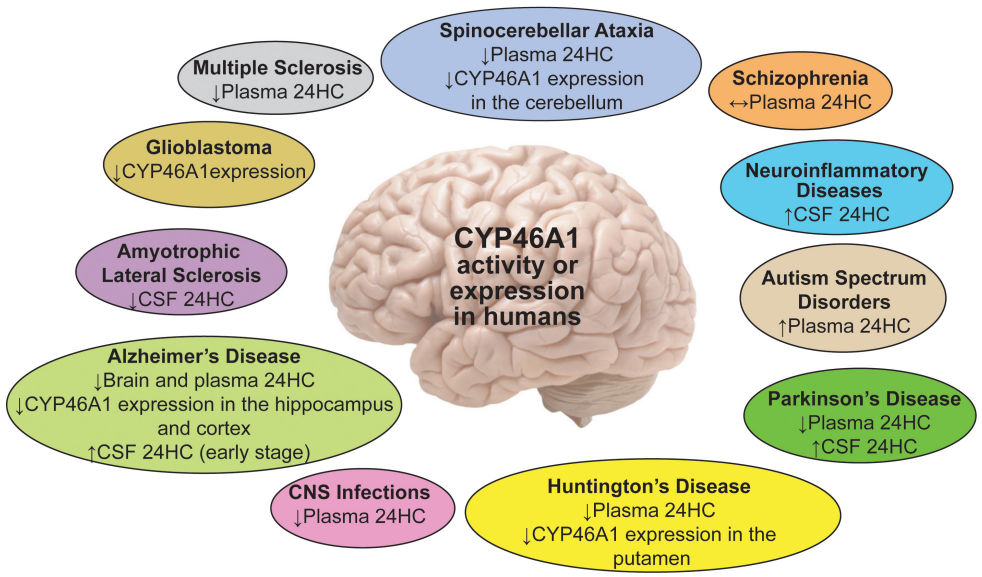

FIGURE 1 | A summary of the 24HC or CYP46A1 measurements in human plasma, CSF and brain regions in different brain disorders. Upwards (↑), downwards (↓), and left-right (↔) arrows indicate increase, decrease, and no change.

Recently, the spatial 24HC distribution in mouse brain was determined and found to reflect local *Cyp46a1* expression (Yutuc et al., 2020).

Cyp46a1$^{-/-}$ mice were generated in 2003 and revealed to have a tight coupling between cholesterol elimination and biosynthesis from the brain as their brain cholesterol levels were unchanged because of a compensatory decrease in the brain cholesterol biosynthesis (Lund et al., 2003). The CYP46A1 contribution to cholesterol removal and turnover in mouse brain was determined and was shown to be at least 40–50% (Lund et al., 2003). Detailed studies of CYP46A1 enzymatic properties were initiated in 2003 when human CYP46A1 was heterologously expressed in *E. coli* and purified. In the reconstituted system *in vitro*, purified CYP46A1 converted cholesterol to 24HC and further hydroxylated 24HC to 24, 25- and 24, 27-dihydroxycholesterols. The rate of the CYP46A1-mediated cholesterol hydroxylation was slow when compared with other cholesterol hydroxylases (CYP7A1, CYP11A1, and CYP27A1) (Mast et al., 2003), but consistent with slow cholesterol turnover in human (∼9.1 years) and mouse (∼0.7 year) brains (Bjorkhem et al., 1998; Dietschy and Turley, 2001). Besides cholesterol, other compounds (some marketed drugs and neurosteroids) were metabolized by CYP46A1 *in vitro*, thus suggesting that the P450 active site is plastic and can accommodate molecules of different sizes, shapes, and polarities. Later, this plasticity of the enzyme active site was confirmed by crystal structures of CYP46A1 with and without the substrate cholesterol sulfate and in complex with seven different pharmaceuticals: the antidepressants tranylcypromine and fluvoxamine; the antifungals voriconazole, clotrimazole, and posaconazole; the anticonvulsant thioperamide; and the anticancer drug bicalutamide (Mast et al., 2008, 2010, 2012, 2013a,b). The biochemical and biophysical characterizations of purified CYP46A1 finalized the 24HC-CYP46A1 link and created the basis for subsequent P450 investigations as a pharmacologic target.

24HC IN PLASMA AND CEREBROSPINAL FLUID (CSF) AS A POTENTIAL BIOMARKER IN NEUROLOGICAL DISEASES

The potential diagnostic power of plasma 24HC for onset and progression of several neurodegenerative diseases has been the subject of multiple studies with fascinating, yet sometimes inconsistent, results (**Figure 1**). These inconsistencies were likely due to multiple factors: heterogeneity of patient populations, disease stage, brain atrophy associated with advanced disease, specific kinetics of disease evolution, complex interactions involving brain metabolic balance, function of the blood-brain barrier, and inflammatory components. Indeed, at an early disease stage, cell loss from myelin breakdown and neurodegeneration may lead to a release of both cholesterol and 24HC, thus increasing cholesterol availability to CYP46A1. Accordingly, brain cholesterol turnover and plasma 24HC levels may be increased. Conversely, at an advanced disease stage, there is significant brain atrophy and a reduced number of the CYP46A-containing cells along with a decreased neuronal expression of CYP46A1 (see below). Hence, at this stage, brain cholesterol turnover and plasma 24HC levels may decrease. During intermediate stages, opposite effects on the brain cholesterol turnover may overlap and compensate each other, with no change in the plasma 24HC levels (Leoni and Caccia, 2013a; Björkhem and Diczfalusy, 2020).

A summary of the reproducible findings is presented in **Figure 1** with the most compelling data coming from the studies in patients with AD and HD. More recently, investigations of PD, ALS, and MS as well brain trauma, neonatal hypoxia, and glioblastoma have reinforced the interest for 24HC as a biomarker of disease progression and highlighted the role of CYP46A1 in this process or even disease genesis.

Plasma 24HC in Neurological Diseases

In AD, an increase, decrease or no change in plasma 24HC levels were documented in affected patients (Bretillon et al., 2000; Lutjohann et al., 2000; Papassotiropoulos et al., 2002; Schonknecht et al., 2002; Heverin et al., 2004; Kolsch et al., 2004; Zuliani et al., 2011; Popp et al., 2012; Leoni and Caccia, 2013a; Li et al., 2018). This inconsistency was explained by the disease stage, as different criteria were used for the disease stage determination and patient enrollment. Also, multiple other factors were found to affect plasma 24HC levels (Leoni and Caccia, 2013a; Björkhem and Diczfalusy, 2020). These factors included inflammation, dysfunction of the blood-brain barrier, ectopic CYP46A1 expression in astrocytes, and medications that altered the whole body and brain cholesterol metabolism. These factors further limited the diagnostic power of 24HC for AD (Leoni and Caccia, 2013a). Recently, a systematic oxysterol profiling in the brain of patients with different stages of AD provided important insight into oxysterol changes during the disease progression (Testa et al., 2016). In the early stages of AD, the levels of 24HC in the frontal and occipital cortex were only slightly below those of controls, while at the later AD stages, the 24HC levels were markedly and significantly decreased (40%), thus raising a possibility that this concentration change may play an important role in the end-phase of the disease.

In HD, decreases in plasma 24HC levels were reported at all stages, but were normal in premanifest patients. This result was explained to reflect disease burden, loss of neurons, and degree of structural atrophy (Leoni et al., 2008; Leoni and Caccia, 2013b; Leoni et al., 2013a). Also, the finding of the same reduction in plasma 24HC across different HD stages, despite a progressive decrease in the caudate volume (Leoni et al., 2008), suggested that perhaps there was an uncoupling of the 24HC production in the HD-affected brains and in the cholesterol biosynthesis, which was also reduced. As a result, cholesterol content in the caudate in the HD brain was elevated (Del Toro et al., 2010), and more cholesterol became available to CYP46A1 for the 24HC production to compensate for the caudate volume decrease.

In MS, a recent study showed that serum 24HC levels were lower in primary progressive and older relapsing remitting patients. Furthermore, the serum levels of cholesterol precursors (lathosterol, lanosterol, desmosterol) were decreased in all clinical subtypes (Teunissen et al., 2003; van de Kraats et al., 2014; Mukhopadhyay et al., 2017).

In PD, initial studies investigating plasma 24HC levels showed inconsistent results (Lee et al., 2009; Bjorkhem et al., 2013; Huang et al., 2019). In the cohorts of up to 25 disease-affected participants, both lower and unchanged plasma oxysterol levels were found relative to the controls (Lee et al., 2009; Bjorkhem et al., 2013; Di Natale et al., 2018). However, in a recent investigation with a sample size of 35 PD patients and a controlled analysis for potential confounders, a robust evidence was provided that higher levels of 24HC are inversely associated with the risk of the disease (Huang et al., 2019).

Severe CNS infections were also associated with a reduction in plasma 24HC levels (Bretillon et al., 2000; Leoni and Caccia, 2013b). Conversely, children with autism spectrum disorders were shown to have higher plasma 24HC levels, which inversely correlated with age (Grayaa et al., 2018). No changes in the plasma 24HC levels were found in schizophrenia (Chiappelli et al., 2020).

CSF 24HC in Neurological Diseases

Normally, >99% of brain 24HC is directly fluxed into the systemic circulation, and only <1% is first secreted into the CSF to enter then the systemic circulation (Leoni et al., 2004). Hence, not only the levels of plasma 24HC but also the levels of CSF 24HC could be sensitive to intracerebral changes in cholesterol homeostasis. The latter changes could be a result of cholesterol release from dying cells with subsequent conversion to 24HC by CYP46A1 and/or due to altered CYP46A1 expression or activity. In addition, the levels of plasma and CSF could be affected by dysfunction of the blood-brain and blood-CSF barriers, respectively (Leoni et al., 2003). Indeed, the CSF levels of 24HC were shown to be increased in several conditions.

In AD, the 24HC levels in the CSF appeared to have a better diagnostic power than plasma levels and were even suggested to be a marker of brain health (Leoni et al., 2013b). In most studies, the CSF 24HC was increased in AD (Papassotiropoulos et al., 2002; Schonknecht et al., 2002; Leoni et al., 2006, 2013b; Bjorkhem et al., 2019), possibly due to a sterol release as a result of neurodegeneration (Björkhem and Diczfalusy, 2020) and a lack of confounding contribution of hepatic clearance.

In MS, patients treated with natalizumab, a drug which reduces inflammation and degeneration of the CNS, showed a decrease in the CSF 24HC levels, which was interpreted as an indicator of a reduced neurodegeneration (Novakova et al., 2015).

In PD, an increase in the 24HC levels in the CSF was detected, and 10% of patients even had a correlation between the CSF 24HC and disease duration. Therefore, CSF 24HC was proposed to be of value for following the disease progression (Bjorkhem et al., 2013, Björkhem et al., 2018).

Increased 24HC levels were also detected in several neuroinflammatory diseases (neuroborreliosis, viral meningitis, and Gullian-Barre syndrome) (Leoni et al., 2003, 2004; Bjorkhem et al., 2013, Björkhem et al., 2018).

Thus, a number of brain disorders appeared to have changes in CYP46A1 activity, and these disorders were not only limited to neurodegenerative diseases. Even some conflicting results still need to be clarified, the importance of 24HC quantifications is that they pointed to the diseases which could benefit from CYP46A1 activity modulation and suggested that more neurological conditions should be evaluated for changes in the plasma or CSF 24HC levels.

CYP46A1 Protein Levels Are Modified in Brain Regions Affected by Neurological Disorders

Not only CYP46A1 activity and concentrations of 24HC vary in the plasma and CSF depending on a specific disease and its stage of progression, but also CYP46A1 protein levels are altered in the brain. In patients with HD as well as Machado-Joseph disease (MJD or Sca3), the levels of CYP46A1 were reduced in the affected brain regions, the striatum in HD and the cerebellum in MJD patients (Boussicault et al., 2016; Nobrega et al., 2019). Interestingly, additional CYP46A1 expression in non-neuronal cells (astrocytes) was documented in the AD- and HD-affected brains, perhaps to compensate for CYP46A1 loss in neurons due to neurodegeneration (Bogdanovic et al., 2001; Brown et al., 2004).

CYP46A1 IS A RELEVANT THERAPEUTIC TARGET IN ANIMAL MODELS

CYP46A1 Regulation

CYP46A1 does not appear to be regulated at transcriptional levels as shown by experiments on cells transfected with the *CYP46A1* reporter constructs. The *CYP46A1* transcription was found to be insensitive to the major regulatory axes, and only oxidative stress significantly increased the gene transcription (Ohyama et al., 2006). In addition, the specificity (Sp) transcription factors were shown to contribute to the control of the basal *CYP46A1* transcription (Milagre et al., 2008, 2012; Moutinho et al., 2016). Epigenetic mechanisms were shown to alter the *CYP46A1* expression *in vivo* and *in vitro* (Shafaati et al., 2009; Milagre et al., 2010; Nunes et al., 2010).

Role of CYP46A1 in Neurotransmission and Stress Response

The role of CYP46A1 as an important neuronal stress response factor and the effects of 24HC on neuronal survival were demonstrated (Sodero et al., 2012). Current studies also strongly support the neurotransmission-CYP46A1 activity link (Sodero et al., 2011, 2012; Mast et al., 2017a). *In vitro*, CYP46A1 activity is increased by L-Glu, and 24HC is a potent positive allosteric modulator of the GluN2B subunit of N-methyl-D-aspartate receptors (NMDARs), whose activation is a key mediator of the long-term potentiation (LTP) induction (Paul et al., 2013; Linsenbardt et al., 2014; Emnett et al., 2015; Sun et al., 2016b; Wei et al., 2019). Conversely, *Cyp46a1*$^{-/-}$ mice were shown to have reduced activity and function of these receptors, which govern experience-dependent synaptic plasticity (Sun et al., 2016a). Collectively, these results suggest a reciprocal relationship between CYP46A1 activity and neurotransmission: excitatory neurotransmission increases CYP46A1 activity, and increased CYP46A1 activity enhances neurotransmission. This relationship could have important implications for brain plasticity and, interestingly, for both conditions of NMDAR hypofunction and hyperfunction. CYP46A1 activation could be beneficial for AD, HD and other polyglutamine diseases (Scas), in which either brain levels of CYP46A1 or 24HC are decreased (Boussicault et al., 2016; Testa et al., 2016; Nobrega et al., 2019) and, hence, can affect the NMDAR function (Zhou and Sheng, 2013). Conversely, CYP46A1 inhibition could be of therapeutic value in pathological conditions that promote excessive glutamatergic excitation such as those occurring in schizophrenia, epilepsy, and hyperalgesia (Coyle, 2006; Xia et al., 2010; Sun et al., 2016b; Lu et al., 2020). Thus, depending on the context, both upregulation/mimicry of CYP46A1 activity along with 24HC signaling and downregulation/antagonism may have therapeutic potential.

Increased CYP46A1 activity in response to an excitotoxic stress raises the key question of the cause or the consequence of this response. In mice with excessive stimulation of glutamate receptors after a single injection of kainic acid, a loss of membrane cholesterol due to 24-hydroxylation was demonstrated. This cholesterol loss was shown to be a normal response to high excitatory neurotransmission in neurons, and it was suggested to modulate the magnitude of the depolarization-evoked calcium response (Sodero et al., 2012). Consistent with this cause–effect relationship, knockdown of *CYP46A1* prevented Glu-mediated cholesterol loss in cultured neurons (Sodero et al., 2012). In rats exposed for 11 weeks to subneurotoxic doses of L-Glu, the regulation of cholesterol metabolism and transport by chronic Glu exposure was confirmed (Zhang et al., 2020). The brain ratio of 24HC to cholesterol and CYP46A1 expression was increased, and Glu induced an elevation of CYP46A1 and ApoE. These modulations could either be a toxic event or a compensatory change during the chronic excitotoxicity (Zhang et al., 2020). The data obtained are consistent with recent observations in a neonatal model of hypoxic ischemia in mice, in which increased production of 24HC in the injured hemisphere and simultaneous metabolite excretion into the bloodstream were demonstrated (Lu et al., 2021). Enhanced brain cholesterol turnover and upregulation of CYP46A1 were observed. However, further investigations are needed to demonstrate that this is a protective stress response.

CYP46a1$^{-/-}$ and *CYP46A1*^{Tg} Mice

Cyp46a1 knockout did not impair mouse survival, potentially due to the compensatory mechanisms that were in place during development. However, the characterization of *Cyp46a1*$^{-/-}$ mice revealed that animals of both sexes had severe cognitive deficits, thus linking CYP46A1, cholesterol turnover in the brain with higher order brain functions (Kotti et al., 2006). Decreased cholesterol biosynthesis in the brain, and thereby decreased protein prenylation by geranylgeraniol (a cholesterol biosynthetic intermediate), were shown to contribute to this link *via* the impairment of the hippocampal LTP (Kotti et al., 2008).

CYP46A1 transgenic mice were generated as well and demonstrated increased cholesterol elimination from the brain. While no apparent changes were present in young animals, 15-month- old female mice demonstrated improved spatial memory in the Morris water maze (MWM) test and showed increased expression of some of the synaptic proteins, including one of the NMDAR subunits (Maioli et al., 2013).

Altogether, the two *in vivo* models suggested that CYP46A1 could play a key role in memory processes and synaptic plasticity, and that modulating CYP46A1 activity could have therapeutic implications. So far, two major approaches have been tested *in vivo* to investigate CYP46A1 activity modulation—genetic targeting of *CYP46A1* expression and direct CYP46A1 activity modifications by enzyme inhibitors or activators. Both were evaluated in mouse models of different brain diseases (**Figure 2**).

GENETIC TARGETING OF CYP46A1 EXPRESSION

CYP46A1 Inhibition Using a Short Hairpin RNA Is Detrimental in Mouse Brain

Decreases of CYP46A1 levels in patients with severe neurodegenerative diseases, like AD, HD, and Scas, and the phenotypic impairment observed in *Cyp46a1*$^{-/-}$ mice, prompted studies of potential detrimental consequences of CYP46A1 inhibition in adult mice. This was achieved by injecting an adeno-associated virus (AAV) vector carrying a short hairpin RNA (shRNA) expression cassette specific to the *Cyp46a1* gene.

Hippocampal injection of the AAV-shRNA *Cyp46a1* to wild type mice led to cognitive deficits, hippocampal atrophy, enhanced amyloid β (Aβ) production, abnormal tau phosphorylation, and stress of the ER, all symptoms strongly resembling AD phenotype (Djelti et al., 2015). Injection of the AAV-sh*Cyp46a1* in the APP23 model of AD exacerbated their AD phenotype by increasing the Aβ content, tau phosphorylation, and neuronal loss leading to epileptic seizures (Chali et al., 2015; Djelti et al., 2015). This phenotype was associated with major lipid abnormalities and modifications of the lysosomal compartment. Specifically, accumulation of sphingolipids and increased expression of the enzymes involved in phosphatidylcholine and sphingolipid metabolism after the AAV-sh*CYP46A1* injection were associated with alterations in the lysosomal cargo, accumulation of phagolysosomes, and impairment of endosome-lysosome trafficking (Ayciriex et al., 2017).

In the striatum of wild type mice, the knockdown of the *Cyp46a1* mimicked HD phenotype with spontaneous medium spiny striatal neuron degeneration, motor deficits (Boussicault et al., 2016), and accumulation of endosomes and lysosomes (Nobrega et al., 2019).

Restoring of CYP46A1 Is Therapeutic in AD, HD, Scas, and ALS Mouse Models

Mouse models of amyloid and tau pathologies, HD, and MJP (Sca3) were shown to have a decrease in the levels of *Cyp46a1* mRNA and protein, thus confirming a decrease in CYP46A1 expression in patients with AD, HD, and Sca3 (Hudry et al., 2010; Burlot et al., 2015; Boussicault et al., 2016; Kacher et al., 2019; Nobrega et al., 2019). The injections of the AAV-*CYP46A1* to the brain of these models were used to increase the P450 expression and positively affect the disease manifestations (Hudry et al., 2010; Burlot et al., 2015; Boussicault et al., 2016; Kacher et al., 2019; Nobrega et al., 2019).

In the AD models of amyloidogenesis (APP23 and APP/PS mice), neuronal overexpression of CYP46A1 before or after the onset of amyloid plaques decreased the brain Aβ deposits and improved animal performance in the MWM test of the investigated APP23 model. This was further confirmed in the very severe APP/PS1 knock-in model in which AAV-CYP46A1 not only decreased amyloid burden but also improved spine density and LTP (Alves et al., 2018). Cerebral delivery of *CYP46A1* was shown to reduce the cleavage of amyloid precursor protein (APP), possibly due to a decreased content of cholesterol and presenilin 1 in lipid rafts; the latter being an important component of the γ-secretase complex, which yields the Aβ peptides (Hudry et al., 2010).

Notably, the therapeutic benefit of CYP46A1 expression was not only observed on the amyloid component of AD but also on the tauopathy. In a model of the AD-like tau pathology (THY-Tau22 mice), the CYP46A1 gene therapy completely restored impaired cholesterol metabolism, rescued cognitive deficits, and mitigated impairments in long-term depression (LTD) as well as spine defects that characterize this model. Tau hyperphosphorylation and the associated gliosis were not reduced, but the persistence of glial cells could be in line with the role of glial cells in the clearance of misfolded protein accumulation in AD (Burlot et al., 2015).

Defects of brain cholesterol metabolism are particularly well documented in HD and are associated with increased content of membrane cholesterol and a decreased production of cholesterol due to a reduction in cholesterol biosynthesis (Karasinska and Hayden, 2011; Alves et al., 2018). In R6/2 and zQ175 knock-in mice, *CYP46A1* delivery into the striatum rescued the cholesterol synthesis pathway, improved motor function, and decreased neuronal atrophy. Importantly, the AAV-*CYP46A1* administration significantly decreased the number and area of the polyglutamine (polyQ)-mutant huntingtin aggregates in both models to 40–50% (Boussicault et al., 2016; Kacher et al., 2019). These studies allowed major insights into the mechanisms of therapeutic effects of CYP46A1 *in vivo* as the striatal transcriptomic profile of zQ175 mice was improved by the *CYP46A1* injections. CYP46A1 increased expression of genes implicated in lipid metabolism, synaptic transmission, autophagy, innate immunity, and DNA repair. Experimental evidence was presented that these modifications were translated into improvements of synaptic activity and connectivity along with the activation of the proteasome and autophagy machineries, which participate in the clearance of the mutant polyQ-huntingtin aggregates (Kacher et al., 2019). In particular, it was shown that the maturation of autophagosomes was improved. It was also further confirmed that CYP46A1 expression is protective against NMDAR-mediated excitotoxicity in two different HD neuronal cell models and helps to reduce neuronal cholesterol content in lipid raft extracts (Boussicault et al., 2018).

MJD (Sca3), a disease characterized by the accumulation of polyQ-ataxin-3, is the most prevalent form of ataxia worldwide.

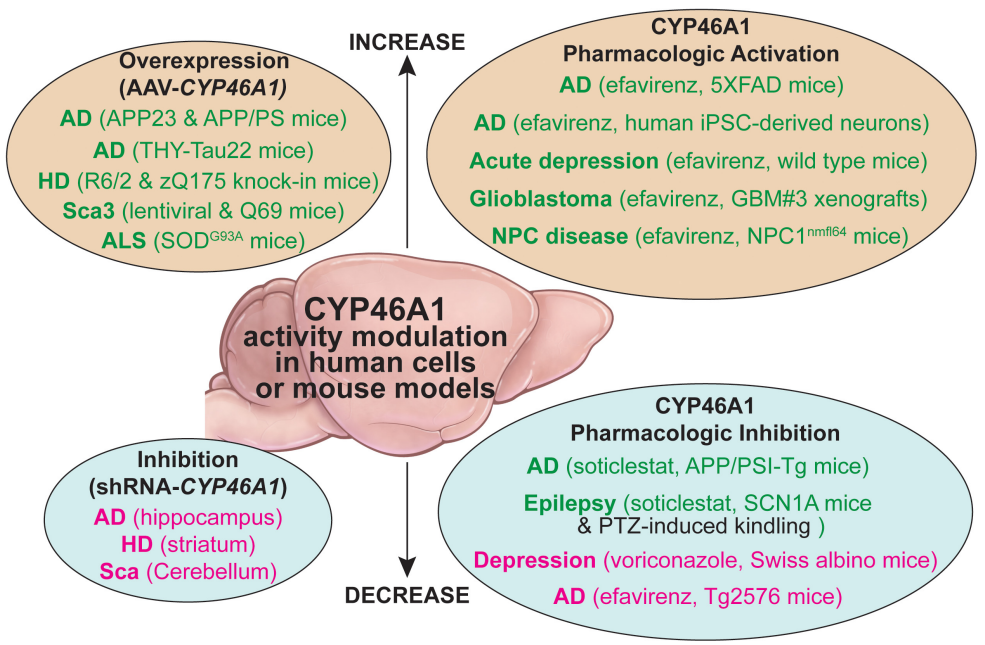

FIGURE 2 | A summary of the CYP46A1 activity modulation by genetic (the AAV-based gene delivery or shRNA inhibition of gene transcription) and pharmacologic means in human cells and mouse models of different brain disorders. AD, Alzheimer's disease; ALS, amyotrophic lateral sclerosis; HD, Huntington's disease; NPC, Nieman-Pick type C disease; iPSC, human induced pluripotent stem cells; PTZ, pentylenetetrazol; Sca3, spinocerebellar ataxia 3. The beneficial and detrimental effects of the CYP6A1 activity modulation are indicated in green and magenta fonts, respectively, black font indicates no effects on the studied process.

The CYP46A1 protein level is decreased in the cerebellum of MJD patients (Nobrega et al., 2019). To investigate potential therapeutic consequences of restoring CYP46A1 expression, two models (lentiviral and Q69) of Sca3 were tested as decreased expression of CYP46A1 was confirmed in the brain of Sca3 mice. In the lentiviral-based model, the striatal administration of the AAV-*CYP46A* reduced the accumulation of the mutant ataxin-3 aggregates, a hallmark of Sca3, and preserved neuronal markers (Nobrega et al., 2019). In the severe transgenic Sca3 Q69 model, cerebellar delivery of *CYP46A1* was strongly neuroprotective in adult mice through the significant decrease of the ataxin-3 aggregation, the alleviation of motor impairments, and the improvement of the Sca3-associated neuropathology (preservation of Purkinje cells and of the cerebellum volume). *CYP46A1* activated autophagic clearance of ataxin-3 (Nobrega et al., 2019).

The role of the cholesterol pathway and CYP46A1 activity were recently investigated in ALS. Cholesterol metabolism seems to play a major role in neuromuscular junctions, synaptic vesicle cycle, neurotransmitter release, and synaptic integrity (Krivoi and Petrov, 2019). The effect of exogenous 24HC on neuromuscular transmission in the diaphragm of SOD^{G93A} mice, a model of ALS, was evaluated (Mukhutdinova et al., 2018). 24HC was found to suppress the exocytotic release of neurotransmitter in response to intense activity *via* the NO/lipid raft-dependent pathway. Also, 24HC increased the staining of the neuromuscular junction membranes with CTxB (a lipid raft marker), indicating an increase in membrane ordering that likely attenuated NO production. Moreover, treatment with the raft-disrupting agents (methyl-β-cyclodextrin or sphingomyelinase) markedly suppressed the effect of 24HC on both lipid raft integrity and activity-induced NO production. These results were consistent with increased sphingomyelinase gene transcription observed after CYP46A1 inhibition in the brain of normal mice (Ayciriex et al., 2017). Given that the disturbance of cholesterol-rich microdomains at the neuromuscular junctions might occur as a result of muscle disease and affect synaptic transmission (Gil et al., 2006; Petrov et al., 2017), the potential membrane ordering effect of 24HC might protect neuromuscular junctions (Mukhutdinova et al., 2018).

Beneficial role of CYP46A1 in ALS was confirmed in $SOD1^{G93A}$ mice. A single intravenous administration of AAV-*CYP46A1* at the presymptomatic or symptomatic stage improved the severe phenotype characterized by a rapid decrease in muscular strength and motor dysfunction leading to progressive paralysis at 3 months. A significant and prolonged motor rescue of animals treated at the preventive or symptomatic stages was observed and improved survival. Clinical improvement was associated with preservations of motoneurons in the spinal cord, the muscular fiber structure, and neuromuscular junctions (Piguet et al., 2019; Wurtz et al., 2020).

Collectively, the AAV-*CYP46A1* delivery to animal models of AD, HD, Sca3, and ALS enabled major insights into the role of the CYP46A1 defect in neurodegenerative diseases and CYP46A1 mechanisms of therapeutic action. These extensive studies provided strong support for CYP46A1 as a therapeutic target and suggested that a one-time *CYP46A1* delivery approach could

be translatable to human patients. Feasibility and safety proof-of-concept experiments in non-human primates are ongoing in preparation for the submission of a first clinical application in human patients (Piguet et al., 2020).

PHARMACOLOGIC TARGETING OF CYP46A1 ACTIVITY

Screening of the marketed drugs for the effect on activity of purified CYP46A1 *in vitro* led to the identification of both CYP46A1 inhibitors and activators (Mast et al., 2008, 2012) and gave impetus to *in vivo* testing of the identified modulators. Results from these studies have shed light on the CYP46A1 activity effects in different pathophysiological conditions.

Consequences of Pharmacological Inhibition of CYP46A1

The antifungal medicine voriconazole, a CYP46A1 inhibitor, was the first enzyme modulator that was tested *in vivo* on wild type mice. Intraperitoneal voriconazole injections (once a day for 5 consecutive days) of a clinically relevant drug dose (60 mg/kg of body weight) were used and resulted in reduced brain 24HC levels. This reduction led in turn to a compensatory decrease in the brain cholesterol biosynthesis; hence, the brain cholesterol levels remained unchanged (Shafaati et al., 2010). Thus, it was revealed that *in vivo*, pharmacologic inhibition of CYP46A1 could be achieved within a relatively short time and does not affect the tight coupling between the brain cholesterol elimination and brain cholesterol biosynthesis. Voriconazole was then evaluated on a mouse model of depression and recently on hippocampal slices of rats (Patel et al., 2017; Popiolek et al., 2020). In mice with depression, the CYP46A1-inhibiting dose of voriconazole (75 mg/kg of body weight per day) had a pro-depressive effect and decreased the brain serotonin levels (Patel et al., 2017). In hippocampal slices of rats, voriconazole inhibited LTD at a 3 μM concentration (Popiolek et al., 2020).

Due to an interest in CYP46A1 inhibitors, new compounds have been developed for commercialization, and several of them were assessed *in vivo*. Two new CYP46A1 inhibitors were evaluated at different doses and post-treatment times on wild type mice, and one of them was shown to decrease the brain 24HC levels as early as 4 h post-treatment. In hippocampal slices, these inhibitors ablated LTD at 1 or 10 μM concentrations, a comparable effect to that of voriconazole (3 μM) (Popiolek et al., 2020).

In a different investigation, a novel CYP46A1 inhibitor called soticlestat (also known as TAK-935 and OV935) was administered to the AD model of amyloidogenesis (APP/PS1-Tg mice) and evaluated for pharmacokinetics, pharmacodynamics, and functional effects. In only 8 h (the first measured time point), soticlestat inhibited CYP46A1 after a single oral dose of 10 mg/kg of body weight, whereas the three daily administrations reduced the brain 24HC levels to a steady state (Nishi et al., 2020). An 8-week treatment of young APP/PS1-Tg mice with soticlestat (10 mg/kg of body weight) starting at the age of 7 weeks increased animal survival from 16 to 28 in the groups of 30 animals. A 2-week treatment of APP/PS1-Tg mice with the same dose of soticlestat followed by a 100 mM KCl hippocampal perfusion to induce the hyperexcitability phenotype suppressed the elevation of extracellular Glu and reduced seizure-like behaviors. In another experiment, a 2-week treatment of 3-month old APP/PS1-Tg mice did not alter the hippocampal levels of the Aβ42 peptide and did not have notable effects on motor coordination or spontaneous locomotor activity (Nishi et al., 2020). Besides APP/PS1-Tg mice, soticlestat was assessed on mouse models of epilepsy (*SCN1A* mice and pentylenetetrazol-induced kindling) and was shown to decrease seizure burden in the former but not the latter (Bialer et al., 2018), although full papers describing these findings remain to be published. Based on these animal studies, soticlestat was advanced to a clinical trial, which will be presented in the next section.

The ^{11}C-labeled form of soticlestat was synthesized for use in positron emission topography (Chen et al., 2020). Yet, the drug characterization in this study yielded different results when compared to those of the developers of this compound (Nishi et al., 2020). ^{11}C-soticlestat was found to have low uptake by the brain and a marginal CYP46A1 specificity (Chen et al., 2020). Furthermore, experiments with purified recombinant human CYP46A1 and subsequent 24HC quantifications by isotope-dilution gas chromatography-mass spectrometry, a highly accurate method, did not confirm tight soticlestat binding to P450 as indicated by a high K_i value of 7.3 μM (Chen et al., 2020). This is in contrast to the reported 4.5 nM IC_{50} determined in cultured cells transfected with *CYP46A1* using 24HC detection by thin layer chromatography (Nishi et al., 2020). Apparently, additional soticlestat characterizations are needed to ascertain its binding specificity for CYP46A1.

CYP46A1 Activation

Pharmaceuticals that activate CYP46A1 *in vitro* were found during screening of the FDA-approved drugs for CYP46A1 inhibition (Mast et al., 2008, 2012). This was particularly exciting because not every enzyme could be activated, and only very few drugs on the market act as enzyme activators rather than inhibitors (Blum et al., 2011; Haberle, 2011; Matschinsky et al., 2011). A CYP46A1 activator and anti-HIV drug efavirenz (EFV) was chosen for subsequent studies in mice, which proved to be a challenge as many drug doses and treatment durations had to be tested to identify the treatment paradigm that activated CYP46A1 *in vivo* (Mast et al., 2014). Ultimately, the CYP46A1 activating dose was identified and appeared to be very small (0.1 mg/kg of body weight per day delivered in drinking water), ∼86-times lower than that given to HIV-positive subjects to keep their viral load low (∼8.6 mg/kg of body weight per day or 600 mg/day). Increasing the EFV daily dose to > 0.2 mg/kg of body weight inhibited CYP46A1 and brain cholesterol 24-hydroxylation, thus indicating a narrow therapeutic window for CYP46A1 activation by EFV. Mouse treatment by EFV had to last at least 4 weeks for the brain sterol levels to reach a new steady state, at which point the content of 24HC as well as lathosterol and desmosterol [cholesterol precursors and markers of cholesterol biosynthesis in the neurons and astrocytes, respectively (Nieweg et al., 2009)] were increased but

the cholesterol levels remained unchanged. This new steady state indicated a coupling between increased cholesterol elimination and biosynthesis in the brain to keep the brain cholesterol at normal levels (Mast et al., 2014). Mechanistically, EFV was found to be the CYP46A1 allosteric activator, which bound only to the allosteric site when used at small concentrations and to both allosteric and active sites when used at high concentrations (Mast et al., 2014; Anderson et al., 2016).

The CYP46A1 activating EFV dose of 0.1 mg/kg of body weight per day was next tested on the AD model of rapid amyloidogenesis (5XFAD mice) in two treatment paradigms. In the first, mice were started on EFV at 1 month of age before the Aβ plaque appearance and continued for 8 months until 9 months of age (Mast et al., 2017c). In the second treatment paradigm, the drug administration began at 3 months of age, after the amyloid plaques developed, and continued for 6 months until 9 months of age (Petrov et al., 2019a). In both paradigms, CYP46A1 was activated, the brain cholesterol turnover was enhanced, and mouse performance in the MWM test was improved (Mast et al., 2017c; Petrov et al., 2019a). The common general effects included changes in gene expression and protein phosphorylation (also observed in *Cyp46a1*$^{-/-}$ mice), which encompassed targets from different pathways and processes (e.g., APP processing, inflammation, immune response, autophagy, ubiquitin-proteasome systems, hypoxia, apoptosis, glucocorticoid-related stress, synaptic function, neurite growth and Ca^{2+}-, small GTPase, and catenin signaling) (Mast et al., 2017b, 2020a; Petrov et al., 2019b). Changes in the Aβ levels, astrocyte and microglia activation, and expression of essential synaptic proteins were treatment paradigm-specific with both age (or initial Aβ load) and treatment duration appearing to determine, in part, the outcome of treatment (Mast et al., 2017c; Petrov et al., 2019a). Notably, when EFV was tested in a different study using the AD model of cerebral amyloidosis (Tg2576 mice) and the CYP46A1-inhibiting dose (15 mg/kg of body weight for 10 days), the drug increased the brain Aβ load (Brown et al., 2014).

In a human model of AD (induced pluripotent stem cell-derived neurons and astrocytes), EFV treatment (10 μM) reduced aberrant accumulation of phosphorylated tau in the neurons, which was independent of APP and Aβ. Importantly, EFV was well tolerated by astrocytes and was found to reduce the amount of esterified cholesterol in neurons, thereby leading to an increase in proteasomal activity and proteasomal degradation of phosphorylated tau (van der Kant et al., 2019a,b).

Besides models of AD, EFV was tested on mouse models of acute depression, glioblastoma, Nieman-Pick type C disease, and prion-infected mice (Patel et al., 2017; Han et al., 2019; Mitroi et al., 2019; Ali et al., 2021). These treatments demonstrated that therapeutic benefits of CYP46A1 activation may not be limited to AD. In stressed mice, EFV showed anti-depressive effects and increased the brain serotonin levels (Patel et al., 2017). In mice bearing intracranial tumors (the LN229 and GBM#P3 orthotopic xenografts), EFV treatment prolonged animal survival and inhibited tumor growth in the GBM#P3 model. Mechanistically, EFV administration was shown to affect the tumor cholesterol homeostasis by regulating the activity of the two important transcription factors–liver X receptors (LXRs), for which 24HC is a ligand (Janowski et al., 1996), and sterol regulatory element-binding protein 1 (SREBP1), a sensor of cellular cholesterol and oxysterol levels (Goldstein et al., 2006). In addition, the treatment increased the levels of cleaved poly (ADP-ribose) polymerase (PARP, an apoptosis marker) and decreased the levels of proliferating cell nuclear antigen (PCNA, a proliferation marker) as well as SRY (sex determining region Y)-box transcription factor 2 (SOX2, a stemness marker) (Han et al., 2019). In a mouse model of Nieman-Pick type C disease (*NPC1*^{nmfl64} mice), EFV treatment extended the mouse life span by 30%, normalized synaptic levels of cholesterol and LTP, and improved performance in behavioral tests as well as the lysosomal phenotype (Mitroi et al., 2019). In prion-infected mice, EFV administration significantly mitigated the propagation of the infectious isoform of the cellular prion protein while preserving physiological cellular prion protein and lipid raft integrity. Also, drug treatment significantly prolonged the lifespan of animals (Ali et al., 2021).

To discriminate between CYP46A1-dependent and independent effects of EFV treatment, *Cyp46a1*$^{-/-}$5XFAD mice were recently generated and treated along with 5XFAD animals with EFV (0.1 mg/kg body weight per day) for 6 months, from 3 to 9 months of age (Mast et al., 2020a). A comparison of these two lines vs. their corresponding controls indicated that the CYP46A1-dependent EFV effects include changes in the levels of brain sterols, steroid hormones, proteins such as glial fibrillary acidic protein (GFAP, a marker of astrocyte activation), ionized calcium binding adaptor molecule 1 (Iba1, a marker of microglia activation), Munc13-1, post synaptic density-95 (PSD-95), gephyrin, synaptophysin, and synapsin-1, as well as genes involved in neuroprotection, neurogenesis, synaptic function, inflammation, oxidative stress, and apoptosis. The CYP46A1-independent EFV effects included the transcription of genes from cholinergic, monoaminergic, and peptidergic neurotransmission, the homeostasis of sulfated steroids, steroidogenesis, and vitamin D_3 activation. Apparently, even at a small dose, EFV acted as a transcriptional regulator, yet this regulation did not appear to lead to functional effects. This study further confirmed that CYP46A1 is a key enzyme for cholesterol homeostasis in the brain, and that the therapeutic EFV effects on 5XFAD mice are likely mostly realized *via* CYP46A1 activation (Mast et al., 2020a).

Of pertinence to the CYP46A1-independent EFV effects is a study showing increased somatic *APP* recombination in neurons from individuals with AD as compared to neurons from individuals who lacked the disease (Lee et al., 2018). This recombination was shown to depend, among other processes, on reverse transcriptase activity and was linked to AD, which is known to be rare in HIV-positive individuals 65 years of age and older (Turner et al., 2016). This work supported testing of the anti-HIV reverse transcriptase inhibitors in AD patients (Lee et al., 2018) and suggested that a potential CYP46A1-independent EFV effect could be of benefit for treatment of AD.

The search for CYP46A1 activators continues. The goal is to identify compounds that have both a broader therapeutic window and a higher potency for CYP46A1 activation than EFV.

These properties should enhance the potentially beneficial effects of CYP46A1 activation and lower the possibility of CYP46A1 inhibition at high EFV doses (Mast et al., 2020b). Eight EFV-related compounds were evaluated *in vitro* on purified CYP46A1, and one (8,14-dihydroxyEFV) activated CYP46A1 1.5-times stronger than EFV. Importantly, 8,14-dihydroxyEFV did not inhibit CYP46A1 at the range of the concentrations (60–100 μM) that were already inhibiting for EFV. Remarkably, 8,14-dihydroxyEFV is an initial product of EFV clearance by the liver, thus suggesting that not only EFV but also its metabolites could activate CYP46A1 *in vivo*. Furthermore, 8,14-dihydroxyEFV was used as a racemic mixture but not as a pure *S* enantiomer, like EFV, and evidence was obtained that CYP46A1 could be activated by both, *S* and *R* EFV metabolites. This finding pointed to replacing *S*-EFV with racemic EFV as an approach to avoid possible EFV toxicity at high doses (Mast et al., 2020b). Experiments on 5XFAD mice are in progress to evaluate the effects of racemic 8,14-dihydroxyEFV.

CYP46A1 AS A THERAPEUTIC TARGET IN HUMAN DISEASES

Preclinical proofs of concept have provided strong support for CYP46A1 being a viable therapeutic target and justified the initiation of first clinical trials. Currently, one trial (NCT03650452[1]) has already been completed on July 20, 2020, and another (NCT03706885, see text footnote 1) is still on-going.

The NCT03650452 clinical trial (called ELEKTRA) was a phase 2 study to evaluate the efficacy, safety, and tolerability of soticlestat as a CYP46A1 inhibitor in pediatric patients with Dravet syndrome and Lennox Gastaut syndrome. In these diseases with excessive glutamatergic excitation, CYP46A1 inhibition was hypothesized to negatively modulate NMDARs and be of therapeutic value. One hundred forty-one participants were enrolled and 126 completed the study, which had two arms, experimental and placebo. Both arms had 2 periods: the screening to establish the baseline seizure frequency; and the treatment period, which consisted of an 8-week dose optimization phase followed by a 12-week maintenance period. The primary endpoint was% change from baseline in frequency of all seizures per 28 days in the treatment vs. placebo arms during the maintenance period. No results have yet been published. A press release from Takeda Pharmaceutical Company Limited and Ovid Therapeutics[2][3] stated that the study achieved its primary endpoint. For the 12-week maintenance period, the median changes from the baseline in seizure frequency in the combined Dravet and Lennox Gastaut syndrome cohorts were −27.8 and + 3.1% in the treatment and placebo arms, respectively (P = 0.0007). For the full 20-week treatment period, the corresponding numbers were −29.8 and 0.0% (P = 0.0024). When the data for the latter were broken down by the cohorts, the changes for the Dravet syndrome cohort (convulsive seizure frequency) were −33.8 and + 7.0% (P = 0.0007) and for the Lennox Gastaut syndrome cohort (drop seizure frequency) were −20.6 and −6.0% (P = 0.1279). There was a reduction of plasma 24HC levels with soticlestat treatment vs. placebo. All patients who completed the ELEKTRA study elected to enroll into the ENDYMION open-label extension study. The primary objectives of the ENDYMION are to assess the long-term safety and tolerability of soticlestat over a 4 year treatment in patients with rare epilepsies and to evaluate the effect of soticlestat on seizure frequency over time (see text footnote 3). Thus, the ELEKTRA trial supports that CYP46A1 inhibition could reduce seizure frequency in children with certain epileptic disorders.

While inhibition of CYP46A1 was proposed to be beneficial in very specific diseases with excessive glutamatergic excitation, a large number of neurodegenerative conditions are associated with glutamatergic hypofunction. In such situations converging proofs-of-concept mentioned above were made in animal models that set the basis for therapeutic intervention aiming to activate or deliver CYP46A1 to the affected brain regions. Reproducible demonstrations in mouse models of AD of beneficial effects of the CYP46A1 expression increase or CYP46A1 pharmacologic activation justified the safety and tolerability phase 1 study of EFV (Sustiva) in patients with mild cognitive impairment due to AD (NCT03706885 trial called EPAD). At a 600 mg per day dose given to HIV-positive subjects, EFV can have some adverse effects (Apostolova et al., 2015, 2017) and likely inhibits CYP46A1. Therefore, lower EFV doses are investigated for CYP46A1 activation and safety in the geriatric population. Specifically, the study has three arms, two treatment and one placebo, with participants in the treatment arms receiving 50 and 200 mg of EFV daily for 20 weeks. The trial's primary endpoint is to identify EFV doses that engage CYP46A1, as assessed by changes in the plasma 24HC levels. In addition, participants are genotyped for the APOE isoform status (E2, E3, or E4) and the presence of SNPs rs754203 and rs3745274 in *CYP46A1* and *CYP2B6*, respectively. This is done because the APOE status and *CYP46A1* SNP may affect CYP46A1 activity, whereas the *CYP2B6* SNP can increase the plasma EFV concentrations because CYP2B6 is the major enzyme metabolizing EFV (Ward et al., 2003). The trial is planned to be completed in summer of 2021 and will provide insight into whether the investigated EFV doses activate CYP46A1 in human brain or if EFV doses should be lowered to accomplish this goal.

HOW ALTERED CYP46A1 ACTIVITY CAN AFFECT MULTIPLE CELLULAR PROCESSES IN VARIOUS BRAIN DISEASES

The primary and proven role of CYP46A1 is to catalyze cholesterol 24-hydroxylation, the rate-limiting step in cholesterol removal from the brain and the reaction that controls the brain cholesterol turnover (Lutjohann et al., 1996; Bjorkhem et al., 1998; Lund et al., 1999, 2003). Yet, numerous studies of the biological effects of CYP46A1 activity modulation

[1] ClinicalTrials.gov

[2] https://ovidrx.com/science/clinical-studies/

[3] https://www.businesswire.com/news/home/20200825005303/en/

suggest that the roles of CYP46A1 in the brain are much broader than originally thought and extend beyond those in cholesterol elimination. Collectively, CYP46A1 activity modulation was shown to alter processes such as synaptic plasticity; gene expression; protein phosphorylation; apoptosis; inflammation; immune response; proteasome, ubiquitin, and autophagy systems; endosomes, lysosomes, and ER; as well as signaling *via* different receptors. In addition, CYP46A1 was demonstrated to be a major neuronal stress response factor in conditions like aging, excitatory neurotransmission, and accumulation of reactive oxygen species by regulating the TrkB/PI3K/Akt pro-survival pathway (Sodero et al., 2011).

Altogether these results raise the question of general unifying mechanisms for the multiple CYP46A1 activity effects. In addressing this question, the four types (individual or combined) of the CYP46A1 activity effects should be considered: (1) on total brain cholesterol levels and cholesterol distribution in the membrane compartments; (2) on total brain levels of 24HC; (3) on levels of the precursors in the mevalonate pathway (e.g., desmosterol and geranylgeraniol) that play key roles in neuronal function (Moutinho et al., 2017); and (4) on the rate of the brain cholesterol turnover (reviewed in Petrov and Pikuleva, 2019).

Herein we will focus on how, by both activating the cholesterol synthesis (the mevalonate pathway) and ensuring the cholesterol turnover (or sterol flux), CYP46A1 may exert broad effects on neuronal functions and survival (**Figure 3**).

The Role of the Mevalonate Pathway

Cholesterol biosynthesis, called the mevalonate pathway, leads to the production of the key compounds, besides cholesterol, that are involved in diverse cellular processes such as transcription (isopentenyl tRNAs), protein N-glycosylation (dolichol phosphate), protein prenylation (farnesylation and geranylgeranylation), and mitochondrial electron transport (ubiquinone) (**Figure 3**). Although cholesterol biosynthesis mostly occurs in astrocytes in adults, neurons keep the mevalonate pathway active, even with sustained cholesterol supply from the aforementioned astrocytes, to ensure the production of these essential compounds (Moutinho et al., 2017). Isoprenoids from the mevalonate pathway were shown to act as anchors for membrane association after being covalently bound to proteins like most of the small guanosine triphosphate-binding proteins, which are critical to neuronal cell function. The mevalonate pathway also influences cell size, growth, and proteostasis by regulating basal autophagic flux through geranylgeranylation of the small GTPase RAB11 (Moutinho et al., 2017). Accordingly, the control of autophagy by the mevalonate pathway has potential implications for inflammation and neurodegenerative diseases (Miettinen and Bjorklund, 2016). This role on autophagy has a major impact on a number of neurodegenerative diseases in which the accumulation of misfolded proteins is critical in disease phenotype.

The Sterol Flux Hypothesis

The sterol flux hypothesis (**Figure 3**) was put forward and tested based on the characterizations of EFV-treated vs. control 5XFAD mice and *Cyp46a1*$^{-/-}$ vs. wild type mice, which had improved and impaired performance, respectively, in behavioral tests and changes in the brain phosphoproteome (Mast et al., 2017b,c; Petrov and Pikuleva, 2019; Petrov et al., 2019b). These animals also had an increase (EFV-treated 5XFAD mice) and decrease (*Cyp46a1*$^{-/-}$) in their brain cholesterol turnover (Mast et al., 2017c; Petrov and Pikuleva, 2019), and hence, increased and decreased, respectively, sterol fluxes through the plasma membranes. Accordingly, it was hypothesized that it could be the sterol flux rates that mainly mediate the CYP46A1 activity effects as they alter the properties of the plasma membranes and/or membrane lipid rafts that serve as the major platforms for signal transduction and targeted protein trafficking (Petrov and Pikuleva, 2019; Petrov et al., 2019b, 2020). The latter would be consistent with the modifications of the lipid raft cholesterol content after CYP46A1 modulation, which was demonstrated earlier in several models (Hudry et al., 2010; Djelti et al., 2015; Boussicault et al., 2018). Accordingly, the membrane and/or lipid raft effects of the CYP46A1 activity modulation could then trigger changes in various brain processes including synaptic transmission and protein phosphorylation. The former is a membrane-dependent process (Jahn and Sudhof, 1994), and the latter relies on the activity of protein kinases and phosphatases, many of which reside in, or are associated with, the membranes and lipid rafts (Antal and Newton, 2013; Prakash, 2020). These sterol flux effects could be similar to those of the altered membrane cholesterol content as they are known to affect the physico-chemical properties of the membranes, the formation of lipid rafts, and the conformation and membrane distribution of integral membrane proteins (Simons and Ehehalt, 2002; Pike, 2003; Yang et al., 2016).

To test the sterol flux hypothesis, synaptosomal factions from the brain of EFV-treated and control 5XFAD mice, *Cyp46a1*$^{-/-}$ and wild type mice as well as B6SJL mice, the background strain for 5XFAD animals, were prepared and characterized (Petrov et al., 2020). In EFV-treated *vs* control 5XFAD mice, these fractions had increases in cholesterol accessibility, membrane ordering, resistance to osmotic stress, thickness of the synaptic membranes, total Glu content, and ability to release Glu in response to mild stimulation. Conversely, synaptosomal fractions from *Cyp46a1*$^{-/-}$ mice had opposite changes in all these properties consistent with the opposite change in the sterol flux rate as compared to EFV-treated 5XFAD mice. Furthermore, incubations of synaptosomal fractions with the inhibitors of glycogen synthase kinase 3, cyclin-dependent kinase 5, protein phosphatase 1/2A, and protein phosphatase 2B revealed that increased sterol flux in EFV-treated *vs* control 5XFAD mice affected the ability of all four enzymes to modulate Glu release. In contrast, in *Cyp46a1*$^{-/-}$ vs. wild type mice, decreased sterol flux altered the ability of only cyclin-dependent kinase 5 and protein phosphatase 2B to regulate the glutamate release (Petrov et al., 2020). Thus, evidence was obtained in support of the cytochrome P450 46A1-mediated sterol flux as an important contributor to the fundamental properties of the membranes and thereby protein phosphorylation and synaptic transmission. An explanation was provided for how one enzyme, CYP46A1, can affect multiple pathways and processes in the brain and serve as a common potential target for various brain disorders.

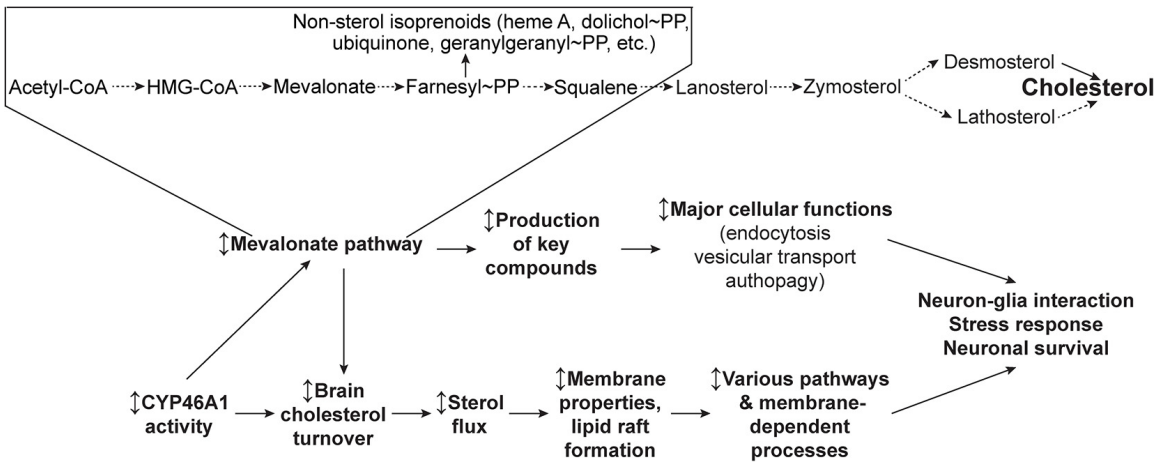

FIGURE 3 | Schematic representation of the two general CYP46A1 mechanisms of action—*via* the mevalonate pathway and sterol flux. Modulation (the ↕ up-down arrow) of CYP46A1 activity affects the mevalonate pathway (or the rate of cholesterol biosynthesis), a critical metabolic circuitry which plays key roles in multiple cellular processes by synthesizing the non-sterol isoprenoids and sterol cholesterol precursors. Modulation of CYP46A1 activity also alters the brain cholesterol turnover and sterol flux through the plasma membranes. The latter in turn affects membrane properties and thereby membrane-dependent events. See main text for details. Dashed arrows indicate multiple steps.

Additional testing is required to further prove or disprove the sterol flux hypothesis.

PRESSING QUESTIONS AND HOW TO ADDRESS THEM

Complementary expertise and efforts of many laboratories throughout the world contributed to our current knowledge of CYP46A1 and suggested that this P450 is of translational value. Hence, the future directions in the CYP46A1 research should include those related to the CYP46A1 mechanism of action as well as its therapeutic applications as exemplified by the questions below.

1. How can CYP46A1 affect multiple brain processes and be associated with different brain diseases? Most of the CYP46A1 activity effects could be explained by the sterol flux hypothesis and the production of the intermediates in the mevalonate pathway. Nevertheless, other unifying hypotheses are needed to fully understand the brain significance of CYP46A1. These hypotheses should certainly consider the biological activity of 24HC, a signaling molecule, which binds to different receptors and other important proteins. In parallel, testing of the sterol flux hypothesis and the role of the biosynthetic cholesterol precursors should continue to identify more processes and proteins in the brain as well as in the plasma membranes and/or lipid rafts that depend on cholesterol input and turnover.
2. What is the CYP46A1 role *in vivo* in non-neuronal cells? What is the reason for ectopic CYP46A1 expression in brain astrocytes in AD (Bogdanovic et al., 2001; Brown et al., 2004), in microglia in a mouse model of traumatic brain injury (Cartagena et al., 2008), and in macrophages in a mouse model of MS (Teunissen et al., 2007)? Could CYP46A1 be expressed in the plasma membranes upon excessive stimulation of glutamate receptors in mice (Sodero et al., 2012) and could it be catalytically active if its activity requires a redox partner (cytochrome P450 oxidoreductase) and a source of reducing equivalents (NADPH)? Specific experiments should be designed and conducted to address these important questions and further advance our knowledge of cellular and subcellular CYP46A1 effects.
3. Why are the CYP46A1 levels decreased in certain diseases? Is increased CYP46A1 expression, as evidenced at an early stage of hypoxia, a protective mechanism or a cause of tissue damage (Lu et al., 2018)? Impaired transcription is a common feature of many neurodegenerative diseases, as misfolded protein accumulation in AD, HD, PD, or ALS severely affects the transcription of multiple genes (Yang and Hu, 2016). Because CYP46A1 is a key enzyme of cholesterol balance and an essential neuronal stress response factor, the defect of its gene transcription could lead to severe dysfunction as observed in HD or Sca3 and after *in vivo* inhibition in different brain regions through shRNAs. Increased CYP46A1 expression at an early stage of hypoxia could thus be viewed as a protective mechanism that could be enhanced to potentially improve or accelerate recovery.
4. How to use CYP46A1 delivery/activation as a therapeutic strategy in humans? What are the CYP46A1 activating doses of EFV in humans? In diseases with decreased CYP46A1 protein levels, the beneficial effects of EFV might be limited. Combining *CYP46A1* delivery and EFV activation could therefore represent a relevant option.
5. Does only EFV activate CYP46A1 *in vivo*, or do some of its metabolites also act as CYP46A1 activators, potentially

better than EFV? Clinical trials are required to address these questions, and EFV pills need to be manufactured with a smaller drug content than that which is currently available (50, 200, and 600 mg) to test a much broader range of EFV doses.

AUTHOR CONTRIBUTIONS

IP and NC wrote the manuscript and are accountable for the content of the work. Both authors contributed to the article and approved the submitted version.

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The Cerebrospinal Fluid Profile of Cholesterol Metabolites in Parkinson's Disease and Their Association With Disease State and Clinical Features

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Disordered cholesterol metabolism is linked to neurodegeneration. In this study we investigated the profile of cholesterol metabolites found in the cerebrospinal fluid (CSF) of Parkinson's disease (PD) patients. When adjustments were made for confounding variables of age and sex, 7α,(25R)26-dihydroxycholesterol and a second oxysterol 7α,x,y-trihydroxycholest-4-en-3-one (7α,x,y-triHCO), whose exact structure is unknown, were found to be significantly elevated in PD CSF. The likely location of the additional hydroxy groups on the second oxysterol are on the sterol side-chain. We found that CSF 7α-hydroxycholesterol levels correlated positively with depression in PD patients, while two presumptively identified cholestenoic acids correlated negatively with depression.

Keywords: sterol, oxysterol, dihydroxycholesterol, bile acid biosynthesis, mass spectrometry

INTRODUCTION

Parkinson's disease (PD) is a chronic neurodegenerative disorder of the central nervous system (CNS) that presents with motor deficits, but which also has many non-motor features, including cognitive and neuropsychiatric problems. In PD, the core motor features result mainly from a loss of dopaminergic neurons in the substantia nigra of the midbrain and their projection to the striatum, but more widespread pathology in subcortical and cortical regions, and even outside the CNS, underlies many of the non-motor features.

About 25% of total body cholesterol is found in the brain (Dietschy and Turley, 2004), and dysregulated cholesterol metabolism is linked to PD as it is to a number of other neurodegenerative conditions (Leoni et al., 2004; Leoni and Caccia, 2011; Bjorkhem et al., 2013, 2018).

Cholesterol will not pass the blood brain barrier (BBB), and cannot be imported from the circulation, so essentially all brain cholesterol is synthesised *in situ*. Excess cholesterol is removed from the brain by the neuron-specific cytochrome P450 (CYP) 46A1- catalyzed metabolism to 24S-hydroxycholesterol (24S-HC, see **Figure 1** for structure), which by virtue of its side-chain hydroxy group can cross the BBB and enter the circulation (Lutjohann et al., 1996). While 24S-HC exits the brain, (25R)26-hydroxycholesterol (26-HC), also known by the non-systematic name 27-hydroxycholesterol (Fakheri and Javitt, 2012), enters the brain from the circulation (Heverin et al., 2005), and is metabolised by CYP7B1, CYP27A1 and hydroxysteroid dehydrogenase (HSD) 3B7 to 7α-hydroxy-3-oxocholest-4-en-(25R)26-oic acid [7αH,3O-CA(25R), **Figure 1**] which is exported from the brain to the circulation and is also found in cerebrospinal fluid (CSF) (Meaney et al., 2007; Ogundare et al., 2010). Plasma and CSF levels of 24S-HC have been suggested as biomarkers for neurodegenerative disorders (Leoni et al., 2004), and while the prevailing evidence suggests that 24S-HC in plasma does not provide a diagnostic marker for PD (Bjorkhem et al., 2013, 2018), some data suggests that there may be a statistically significant elevation of 24S-HC in the CSF of PD patients (Bjorkhem et al., 2018).

Currently, oxysterols in the circulation and in CSF are almost exclusively analysed by mass spectrometry (MS) either in combination with gas chromatography (GC) (i.e., GC-MS) or with liquid chromatography (LC) (i.e., LC-MS) (Leoni et al., 2004; Griffiths et al., 2013). Most studies of oxysterols in CSF are not performed on the "free" non-esterified molecules which are exported from brain but on a combination of esterified and non-esterified molecules (Leoni et al., 2004; Bjorkhem et al., 2018). This is for practical reasons as the non-esterified molecules make up only a small proportion of the total as they become esterified by lecithin–cholesterol acyltransferase (LCAT) in lipoprotein particles within the CSF. However, there is value in analysing the non-esterified molecules alone as these are the precise forms exported from brain.

In the current study, we analysed "free" non-esterified oxysterols (including cholestenoic acids) in the CSF of PD patients and healthy controls with an aim of identifying metabolites or pathways linked to PD. To achieve the necessary sensitivity, we adopted a two-step derivatisation approach named "enzyme-assisted derivatisation for sterol analysis" (EADSA) in combination with LC-MS (**Figure 2**; Crick et al., 2015, 2017). Although we did not find a statistical increase in 24S-HC in CSF from PD patients compared to controls, we did find an increase in 7α,(25R)26-dihydroxycholesterol (7α,26-diHC), an intermediate in the pathway from 26-HC to 7αH,3O-CA(25R) (**Figure 1**). In addition, we found a positive correlation between the CSF concentration of 7α-hydroxycholesterol (7α-HC) and scores on the Beck Depression Inventory (BDI), which is a rating scale commonly used to assess depression in PD. Interestingly there were negative correlations between the presumptively identified cholestenoic acids, 7α-hydroxy-3,24-*bis*oxocholest-4-en-26-oic acid (7αH-3,24-diO-CA) and 7α,12α-dihydroxy-3-oxocholeste-4-en-26-oic acid (7α,12α-diH,3O-CA), and scores on the BDI but not other clinical measures. This work highlights the potential clinical significance of the bile acid biosynthesis pathway in PD and defines a methodology that can be used to measure the pathway intermediates within a clinical laboratory setting.

MATERIALS AND METHODS

Subjects and Sample Collection

This work was designed in two studies: Study 1 primarily focused on oxysterol and cholestenoic acid identification while Study 2 focused on their quantitation and relationship with a range of PD relevant clinical measures. All patients were recruited from the Parkinson's Disease Research Clinic at the John van Geest Centre for Brain Repair in Cambridge. The study was approved by the Cambridgeshire 2 Research Ethics Committee (Ref. 08/H0308/331) and written informed consent was obtained from all participants. Controls for Study 2 were carers of patients with PD with no known neurological disease, or patients attending Addenbrooke's Hospital NHS Neurology clinics for a lumbar puncture to investigate other symptoms (such as headache), but with no known neurodegenerative disease.

Lumbar punctures were performed using an aseptic technique as per standard clinical guidelines. 2–5 mL of CSF was collected. The CSF was centrifuged at 2,000–3,000 g for 15 min and the supernatant was stored at −80°C prior to analysis.

Standard demographic data was collected along with assessments of disease severity including the Movement Disorder Society-Unified Parkinson's Disease Rating Scale (MDS-UPDRS); neuropsychological assessments including the Addenbrooke's Cognitive Examination Revised (ACE-R) and semantic fluency and assessment of depression using the BDI.

LC-MS

The LC-MS method is described in Crick et al. (2015, 2017); it incorporated EADSA (**Figure 2**) to enhance sensitivity and specificity, reversed-phase chromatography to separate diastereoisomers, accurate mass measurement (<5 ppm) at high-resolution (30,000 in Study 1, 120,000 in Study 2, both at *m/z* 400) and multistage fragmentation (MS^n) for structure determination. Quantification was performed against added isotope-labelled standards. In Study 1 quantification was against [25,26,26,26,27,27,27-$^2H_7$]24R/S-hydroxycholesterol ([$^2H_7$]24R/S-HC) which has been shown to be an adequate surrogate for side-chain oxysterols and cholestenoic acids (Crick et al., 2015). For Study 2, the additional standard [26,26,26,27,27,27-$^2H_6$]7α,25-dihydroxycholesterol ([$^2H_6$]7α,25-diHC) was included to allow quantification of 7α,25-dihydroxycholesterol (7α,25-diHC) and 7α,26-diHC and their 3-oxo analogues (Crick et al., 2017).

Patient Data and Statistical Analysis

Study 1

This study was designed to allow for the identification of oxysterols including cholestenoic acids in CSF from PD patients. CSF from 18 PD patients was analysed and compared to a historical data set (Crick et al., 2017) of 18 control CSF samples from people without neurodegenerative conditions. Statistical

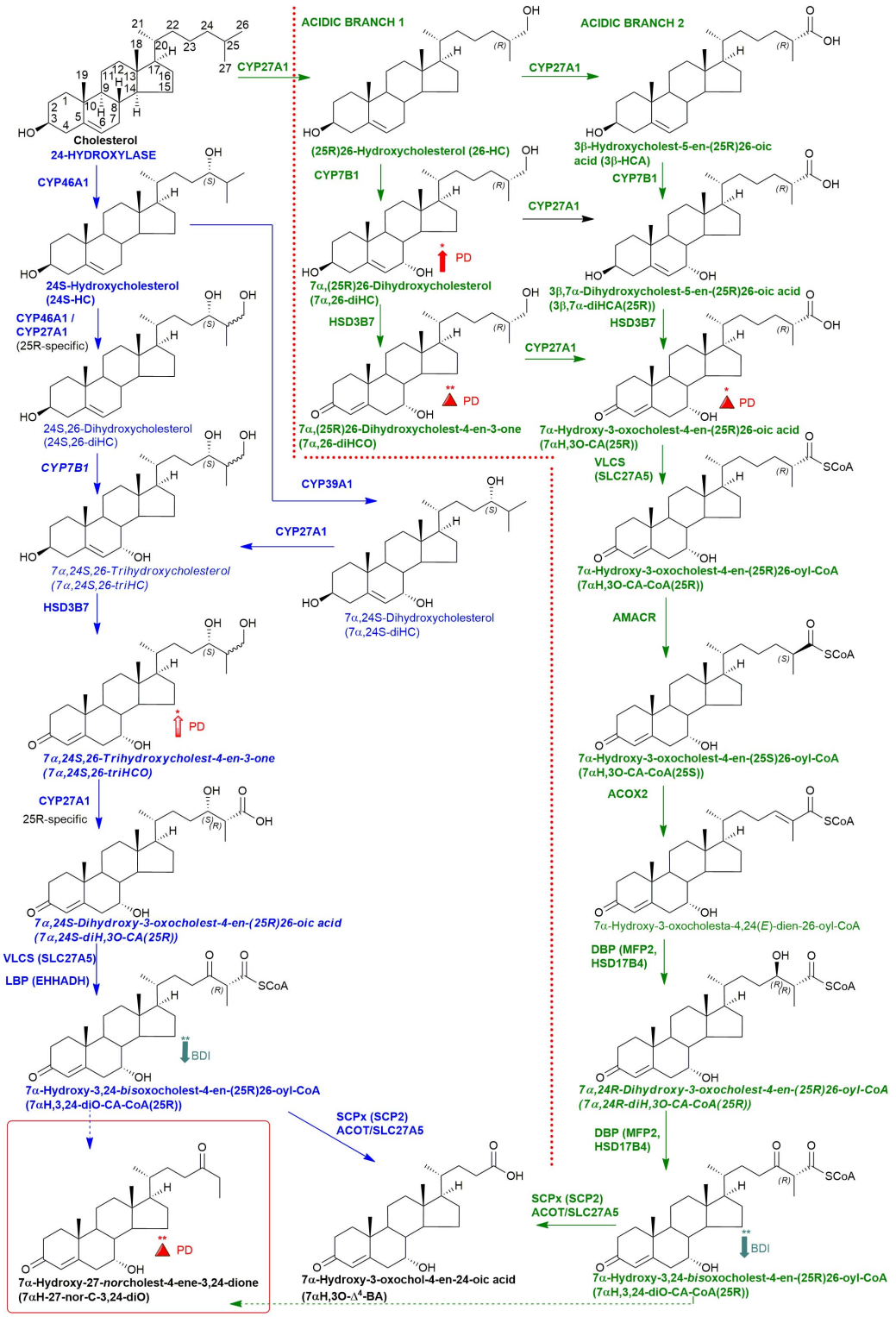

FIGURE 1 | Abbreviated versions of the cerebral 24-hydroxylase (left) and acidic (right) pathways of bile acid biosynthesis. Enzymes, metabolites, and reactions of the 24-hydroxylase pathway are indicated in blue, those of the acidic pathway are in green. Enzymes/genes expressed in the brain, and metabolites observed, in CSF are in bold. CoA intermediates are observed as the unconjugated acids in CSF. *Italics* indicate that the named structure is one of a number of possibilities. The broken arrows indicate a reaction leading to elimination of C-27. Thick coloured arrows pointing upwards or downwards indicate significant positive or negative correlations even when the confounding variables are considered. Red triangles indicate significance ignoring confounding variables, in at least one of the two studies. The full stereochemistry and numbering system for cholesterol is indicated. Abbreviated structures are shown for other sterols ignoring ring-stereochemistry.

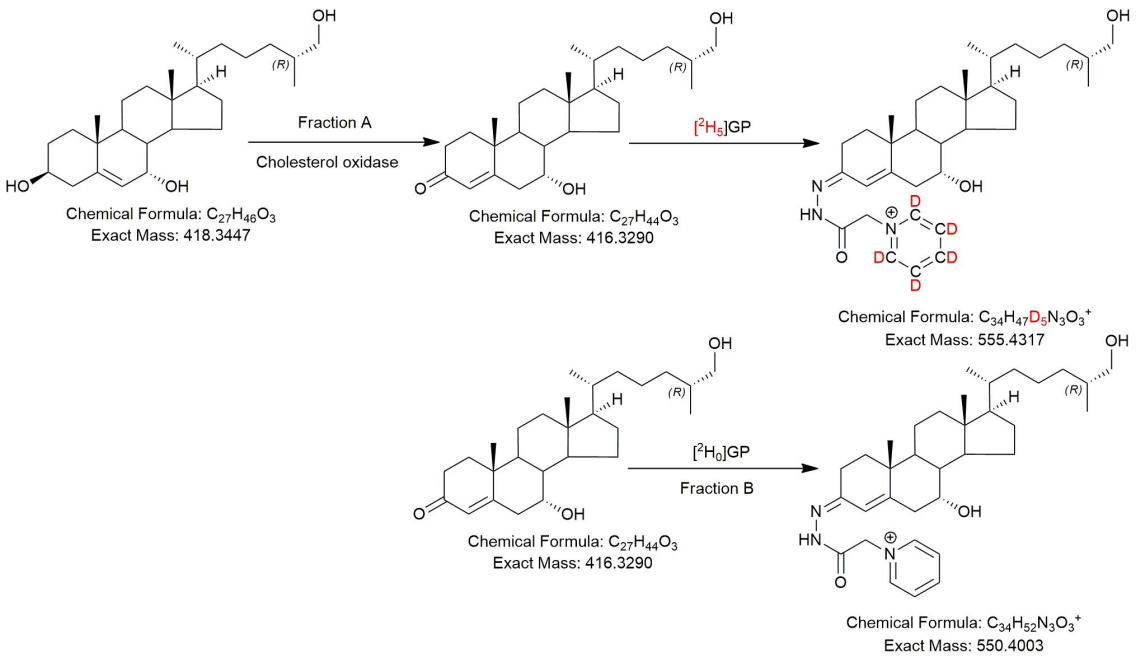

FIGURE 2 | The EADSA derivatisation method. Samples are split into two equal fractions A and B. A-fractions are treated with cholesterol oxidase enzyme and $[^2H_5]$GP, B-fractions are treated with $[^2H_0]$GP in the absence of cholesterol oxidase. LC-MS(MS^3) analysis of fraction-A reveals compounds with a 3β-hydroxy-5-ene structure plus those with a native 3-oxo-4-ene structure, while analysis of fraction-B reveals native 3-oxo-4-ene compounds only. Deconvolution of data from A and B allows quantification of 3β-hydroxy-5-ene compounds while data in B allows quantification of native 3-oxo-4-ene compounds (and 7-oxo-5-ene).

significance was determined by the Mann-Whitney Test and confounding variables of sex and age were not considered.

Study 2

CSF samples from a separate cohort of PD patients and controls were analysed for oxysterols, including cholestenoic acids, and their relationship with a range of standard clinical measures was investigated (**Table 1**) in a cross-sectional study. Statistical analysis was performed using Stata software (Stata Statistical Software: Release 14. StataCorp LP, College Station, TX). Pairwise correlations with oxysterol data were performed for continuous demographic and clinical variables. Those correlations with $P < 0.05$ were entered into multiple regression analyses with the oxysterol as the dependent variable and inclusion of relevant confounding variables. For motor scores and BDI, these confounding variables were age, gender and years from onset of disease. For cognitive variables BDI score was also included as a potential confounder. For categorical variables ANOVA was performed, again adjusting for potential confounding variables as above. For clinical scores, data was only used if it had been generated within1 year of the lumbar puncture.

RESULTS

Study 1—Identification of Oxysterol and Cholestenoic Acids in CSF

Initial studies were performed on 18 CSF samples from early-mid stage PD patients [72% male, mean (standard deviation, SD) age = 69 (7) years, disease duration = 4 (4) years, MDS-UPDRS motor score on treatment = 31(12), ACE-R = 89 (8), BDI = 6 (6)] with the aim of identifying non-esterified oxysterols present in the CSF. The oxysterols identified in this first study are listed in **Table 2**. In addition to the expected monohydroxycholesterols, 24S-HC, 25-hydroxycholesterol (25-HC) and 26-HC, we identified (but did not quantify) the dihydroxycholesterols 7α,25-diHC and 7α,26-diHC and their dihydroxycholest-4-en-3-ones, i.e., 7α,25-dihydroxycholest-4-en-3-one (7α,25-diHCO) and 7α,(25R)26-dihydroxycholest-4-en-3-one (7α,26-diHCO, **Figures 1**, **3**). In addition, we identified and approximately quantified the cholestenoic acids, 3β-hydroxycholest-5-en-(25R)26-oic acid (3β-HCA), and the 25R- and 25S-diastereoisomers of 3β,7β-dihydroxycholest-5-en-26-oic (3β,7β-diHCA), of 3β,7α-dihydroxycholest-5-en-26-oic (3β,7α-diHCA) and of 7αH,3O-CA (**Figures 1**, **3**, **4A,B**), as well

TABLE 1 | Study 2 participant demographics.

Factor (Mean ± SD)	Patients (n = 37)	Controls (n = 5)
Age (y)	65.10 ± 8.24	63.60 ± 8.08
Gender (% Male)	45.94	40.00
Years from disease onset	3.98 ± 5.67	
MDS-UPDRS motor score (in the "ON" state)	32.82 ± 11.78	
ACE-R	90.70 ± 9.46	
Semantic fluency	24.8 ± 7.40	
BDI	9.62 ± 7.02	

TABLE 2 | Oxysterols in CSF of PD patients and controls.

Fraction A	Fraction B	Sterol systematic name (common name)	Abbreviation	Study 1					Study 2					Note
m/z	*m/z*			ng/mL					ng/mL					
				PD		Control		Significance	PD		Control		Significance	
				Mean	SD	Mean	SD	PD vs Control	Mean	SD	Mean	SD	PD vs Control	
527.3640	522.3326	7α-Hydroxy-3-oxochol-4-en-24-oic acid	7αH,3O-Δ^4-BA	**0.672**	0.246	**0.708**	0.237	NS	**0.675**	0.167	**0.551**	0.081	NS	
539.4004	534.369	7α-Hydroxy-27-nor-cholest-4-ene-3,24-dione	7αH-27-nor-C-3,24-diO	**0.387**	0.162	**0.245**	0.143	**	**0.698**	0.194	**0.638**	0.025	NS	1
539.4368	NA	Cholest-5-ene-3β,24S-diol (24S-hydroxycholesterol)	24S-HC	**0.050**	0.022	**0.045**	0.019	NS	**0.015**	0.009	**0.008**	0.004	NS	
539.4368	NA	Cholest-5-ene-3β,25-diol (25-hydroxycholesterol)	25-HC	**0.028**	0.028	**0.030**	0.019	NS	**0.016**	0.015	**0.012**	0.005	NS	
539.4368	NA	Cholest-5-ene-3β,(25R)26-diol ((25R),26-Hydroxycholesterol)	26-HC	**0.113**	0.064	**0.100**	0.028	NS	**0.093**	0.053	**0.064**	0.017	NS	
539.4368	NA	Cholest-5-ene-3β,7β-diol (7β-Hydroxycholesterol)	7β-HC	**0.056**	0.066	**0.036**	0.027	NS	**0.181**	0.474	**0.082**	0.034	NS	2
539.4368	534.4054	3β-Hydroxycholest-5-en-7-one (7-Oxocholesterol)	7-OC	**0.601**	0.513	**0.378**	0.225	NS	**0.671**	0.671	**0.731**	0.540	NS	2
539.4368	NA	Cholest-5-ene-3β,7α-diol (7α-Hydroxycholesterol)	7α-HC	**0.063**	0.067	**0.039**	0.032	NS	**0.091**	0.118	**0.056**	0.027	NS	2
539.4368	NA	Cholest-5-ene-3β,6β-diol (6β-Hydroxycholesterol)	6β-HC	**0.345**	0.234	**0.280**	0.312	NS	**0.918**	1.289	**0.593**	0.141	NS	3
537.4212	NA	9,10-Secocholesta-5,7,10-triene-3β,25-diol (25-hydroxyvitamin D_3)	25-D_3	NM	NM	NM	NM	NA	**0.171**	0.095	**0.140**	0.057	NS	
551.4004	546.369	3-Oxocholesta-4,6-dien-26-oic acid	–	**2.654**	2.426	**1.546**	0.468	NS	**1.461**	0.496	**1.154**	0.335	NS	4
551.4004	NA	3β-Hydroxycholesta-5,7-dien-26-oic acid	–	**0.318**	0.334	**0.079**	0.099	**	**0.142**	0.143	**0.043**	0.038	NS	5
553.4161	NA	3β,x-Dihydroxycholest-5-en-y-one	3β,x-diHC-yO	NM	NM	NM	NM	NA	**0.050**	0.036	**0.066**	0.028	NS	6
553.4161	NA	3β-Hydroxycholest-5-en-(25R)26-oic acid	3β-HCA	**1.073**	0.793	**0.959**	0.416	NS	**1.210**	0.557	**0.899**	0.287	NS	
555.4317	550.4003	7α,25-Dihydroxycholest-4-en-3-one	7α,25-diHCO	NM	NM	NM	NM	NA	**0.009**	0.005	**0.006**	0.001	*	
555.4317	NA	Cholest-5-ene-3β,7α,25-triol (7α,25-Dihydroxycholesterol)	7α,25-diHC	NM	NM	NM	NM	NA	**0.006**	0.005	**0.006**	0.004	NS	
555.4317	550.4003	7α,(25R)26-Dihydroxycholest-4-en-3-one	7α,26-diHCO	NM	NM	NM	NM	NA	**0.009**	0.004	**0.005**	0.001	**	
555.4317	NA	Cholest-5-ene-3β,7α,(25R)26-triol (7α,(25R)26-Dihydroxycholesterol)	7α,26-diHC	NM	NM	NM	NM	NA	**0.005**	0.002	**0.002**	0.002	*	
567.3953	562.3639	x-Hydroxy-3-oxocholesta-4,6-dien-26-oic acid	–	**0.190**	0.169	**0.112**	0.041	NS	**0.453**	0.143	**0.361**	0.119	NS	
567.3953	562.3639	x-Hydroxy-3-oxocholesta-4,6-dien-26-oic acid	–	**0.100**	0.090	**0.069**	0.036	NS	NM	NM	NM	NM	NA	
569.4110	NA	3β,7β-Dihydroxycholest-5-en-26-oic acid	3β,7β-diHCA	**0.455**	0.212	**0.403**	0.190	NS	**0.506**	0.169	**0.406**	0.104	NS	7
569.4110	NA	3β,x,y-Trihydroxycholest-5-en-z-one	3β,x,y-triHC-zO	**0.228**	0.122	**0.147**	0.067	*	**0.172**	0.061	**0.127**	0.036	NS	8
569.4110	564.3796	7α-Hydroxy-3-oxocholest-4-en-26-oic acid	7αH,3O-CA	**22.728**	11.445	**15.851**	4.305	*	**21.198**	6.292	**17.731**	3.983	NS	7,9
569.4110	NA	3β,7α-Dihydroxycholest-5-en-26-oic acid	3β,7α-diHCA	**3.235**	3.308	**2.042**	1.577	NS	**3.808**	2.258	**1.785**	1.575	NS	7,10
571.4266	566.3952	7α,x,y-Trihydroxycholest-4-en-3-one	7α,x,y-triHCO	**0.198**	0.258	**0.286**	0.116	NS	**0.116**	0.062	**0.068**	0.013	*	11
583.3903	578.3589	7α-Hydroxy-3,24-*bis*oxocholest-4-en-26-oic acid	7αH,3,24-diO-CA	**0.236**	0.082	**0.208**	0.057	NS	**0.285**	0.094	**0.227**	0.065	NS	12
585.4059	580.3745	7α,24-Dihydroxy-3-oxocholest-4-en-26-oic acid	7α,24-diH,3O-CA	NM	NM	NM	NM	NA	**0.312**	0.080	**0.251**	0.021	NS	
585.4059	580.3745	7α,x-Dihydroxy-3-oxocholest-4-en-26-oic acid	7α,x-diH,3O-CA	**5.212**	1.737	**2.938**	0.887	***	**5.938**	1.522	**5.038**	1.314	NS	13
585.4059	580.3745	7α,25-Dihydroxy-3-oxocholest-4-en-26-oic acid	7α,25-diH,3O-CA	**1.306**	0.472	**0.715**	0.224	***	**1.634**	0.458	**1.353**	0.213	NS	
585.4059	580.3745	7α,12α-Dihydroxy-3-oxocholest-4-en-26-oic acid	7α,12α-diH,3O-CA	NM	NM	NM	NM	NA	**1.100**	1.176	**1.157**	0.600	NS	
601.4008	596.3694	Trihydroxy-3-oxocholest-4-en-26-oic acid	triH,3O-CA	**0.021**	0.063	**0.077**	0.041	*	NM	NM	NM	NM	NA	
		TOTAL 7α-Hydroxy-3-oxocholest-4-en-26-oic acid	7αH,3O-CA	**25.383**	13.206	**17.396**	4.628	*	**22.659**	6.745	**18.886**	4.312	NS	14
		TOTAL 3β,7α-Dihydroxycholest-5-en-26-oic acid	3β,7α-diHCA	**3.553**	3.477	**2.121**	1.648	NS	**3.950**	2.384	**1.828**	1.606	NS	15

**P < 0.05, **P < 0.01, ***P < 0.001 determined using Mann–Whitney Test. NA, not applicable.*

As a visual aid concentrations are written in bold.

1. Decarboxylation product of 7α-hydroxy-3,24-bisoxocholest-4-en-26-oic acid.

2. May be formed enzymatically or by in vivo or ex vivo autoxidation.

3. 6β-HC is the dehydration product of cholestane-3β,5α,6β-triol, formed from 5,6-epoxycholesterol.

4. Dehydration product of 7αH,3O-CA.

5. Dehydration product of 3β,7-diHCA.

6. x and y probably correspond to 22 and 24 or 20 and 22, based on MS^3 spectra.

7. 25R and 25S epimers measured in combination.

8. x, y and z probably 22, 25 and 24.

9. Some dehydration of 7αH,3O-CA (see 4).

10. Some dehydration of 3β,7-diHCA (see 5).

11. x any y probably 24,25, 24,26, or 25,26.

12. Undergoes decarboxylation to 7α-hydroxy-27-nor-cholest-4-ene-3,24-dione (see 1).

13. x is probably on the side-chain.

14. Total 7α-hydroxy-3-oxocholest-4-en-26-oic acid is a combination of molecule and its dehydrated analogue.

15. Total 3β,7α-dihydroxycholest-5-en-26-oic acid is a combination of molecule and its dehydrated analogue.

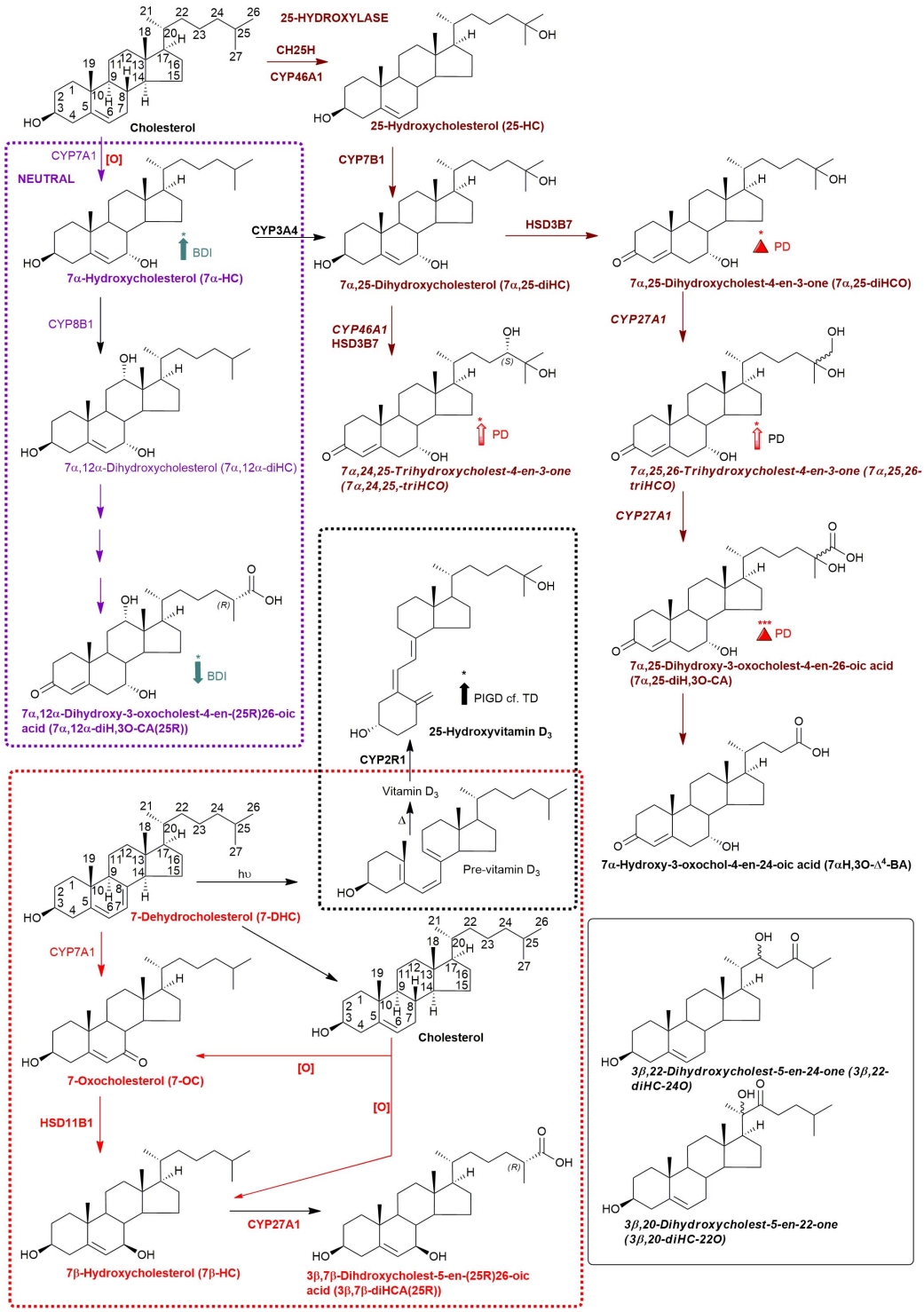

FIGURE 3 | Abbreviated versions of the early steps in the neutral (left, in purple) and the cerebral 25-hydroxylase (right, in brown) pathways of bile acid biosynthesis. Pathway from 7-dehydrocholesterol and cholesterol to 3β,7β-diHCA(25R) are also shown as is the path to 25-hydroxyvitamin D_3 in red and black dashed boxes, respectively. Enzymes, metabolites, and reactions of the neutral pathway are in purple, those of the 25-hydroxylase pathway are in brown, while those generating 3β,7β-diHCA(25R) are in red. Enzymes/genes expressed in brain, and metabolites observed in CSF are in bold. *Italics* indicate that the named structure is one of a number of possibilities. Enzymes in *italics* are postulated catalysts. [O] indicates oxidation *via* non-enzymatic mechanism. Thick coloured arrows pointing upwards or downwards indicate significant positive or negative correlations even when the confounding variables are considered. Red triangles indicate significance ignoring confounding variables, in at least one of the two studies. The full stereochemistry and numbering system for cholesterol and 7-DHC is indicated. Abbreviated structures are shown for other sterols ignoring ring-stereochemistry.

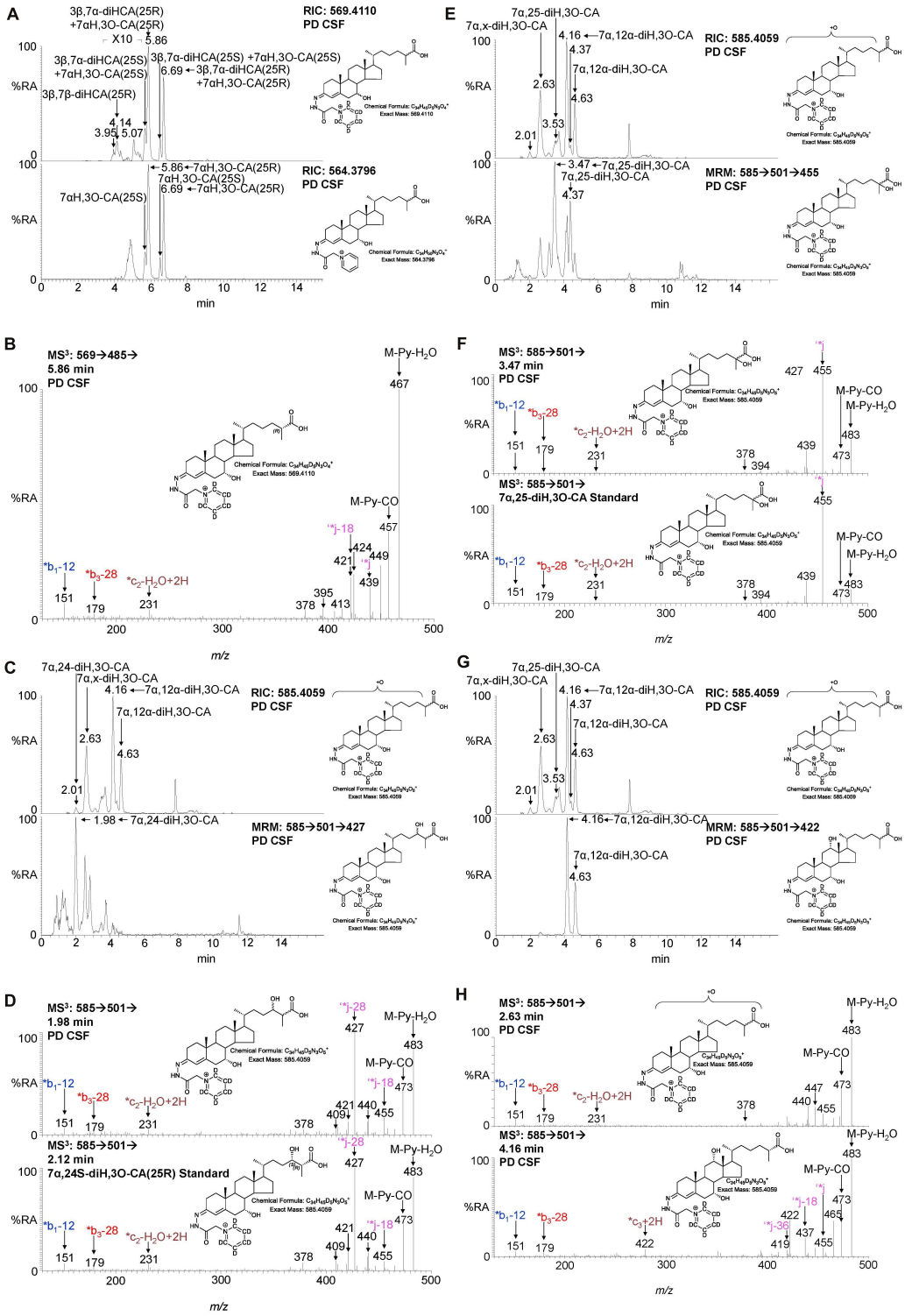

FIGURE 4 | LC-MS(MS^n) analysis of cholestenoic acids found in CSF. **(A)** RIC of *m/z* 569.4110 ± 5 ppm revealing 3β,7β-diHCA and 3β,7α-diHCA (upper panel) and of 564.3796 ± 5 ppm revealing 7αH,3O-CA (lower panel). Each acid is present as two epimers, and each epimer gives *syn* and *anti* conformers of the GP-derivative. **(B)** MS^3 ($M^+ \rightarrow M^+$-Py →) spectrum of 3β,7α-diHCA(25R). **(C,E,G)** RIC of *m/z* 585.4059 ± 5 ppm revealing dihydroxy-3-oxocholestenoic acids (upper panels) and MRM-like chromatogram 585.4 → 501.3 → 427 highlighting 7α,24-diH,3O-CA (lower panel C), 585.4 → 501.3 → 455 highlighting 7α,25-diH,3O-CA (lower panel E) and 585.4 → 501.3 → 422 highlighting 7α,12α-diH,3O-CA (lower panel G). MS^3 ($M^+ \rightarrow M^+$-Py →) spectra of **(D)** 7α,24-diH,3O-CA in CSF (upper panel) and 7α,24S-diH,3O-CA(25R) authentic standard (lower panel), **(F)** 7α,25-diH,3O-CA in CSF (upper panel) and 7α,25-diH,3O-CA authentic standard (lower panel), and **(H)** 7α,x-diH,3O-CA (upper panel) and 7α,12α-diH,3O-CA (lower panel) in CSF.

as uncovering a series of dihydroxy-3-oxocholest-4-enoic acids (diH,3O-CA, **Figures 4C–H**). For this initial study, we did not have access to CSF samples from controls but compared the data from our PD patients to control data generated in a prior study (Crick et al., 2017).

We have previously shown that the acidic pathway of bile acid biosynthesis is at least partially active in the brain (Ogundare et al., 2010). This pathway has two branches which start with (25R)26-hydroxylation and (25R)26-carboxylation of cholesterol by CYP27A1 to give 26-HC and 3β-HCA, respectively (**Figure 1**). 26-HC may be derived from cholesterol in the brain or imported from the circulation (Heverin et al., 2005). These two metabolites are 7α-hydroxylated by CYP7B1 to give 7α,26-diHC and 3β,7α-diHCA(25R), respectively (**Figure 1**) and after oxidation at C-3 and Δ^5 to Δ^4 isomerisation the branches converge at 7αH,3O-CA(25R). We observed each of these metabolites in the CSF and notably the concentration of 7αH,3O-CA was specifically elevated in PD CSF ($P < 0.05$, **Table 2**). It should be noted that both 25R- and 25S-diastereoisomers of 3β,7α-diHCA and 7αH,3O-CA are present in CSF, where the 25R-epimer dominates, however, as the epimers are not fully resolved chromatographically we have measured the two in combination (**Figure 4A**). In the next steps of the acidic pathway 7αH,3O-CA(25R) becomes converted to the CoA thioester and through multiple steps to 7α,24R-dihydroxy-3-oxocholest-4-en-(25R)26-oyl-CoA (7α,24R-diH,3O-CA(25R)-CoA, **Figure 1**; Ferdinandusse et al., 2009; Autio et al., 2014; Griffiths and Wang, 2020), and by generating the appropriate reconstructed ion chromatogram (RIC), we were able to identify a number of chromatographic peaks potentially corresponding to the acid form of this structure (**Figure 4C**). Notably, in CSF and plasma we do not find CoA thioesters but rather the free acids. The CoA thioester of 7α,24R-diH,3O-CA(25R) is a key intermediate in side-chain shortening of C_{27} to C_{24} bile acids, becoming oxidised to 7α-hydroxy-3,24-*bis*oxocholest-4-en-(25R)26-oyl-CoA (7αH,3,24-diO-CA(25R)-CoA, **Figure 5F**). This metabolite is not fully stable in our methodology partially eliminating the C-26 group to give 7α-hydroxy-27-*nor*cholest-4-ene-3,24-dione (7αH-27-nor-C-3,24-diO, see **Supplementary Figure 1**) (Ogundare et al., 2010). We found 7αH-27-nor-C-3,24-diO to be elevated significantly in the CSF from PD patients ($P < 0.01$). In combination, this initial study suggests the acidic pathway is upregulated in the CNS of PD patients.

We were also able to partially identify a number of other oxysterols in the CSF based on retention time, accurate mass and MS^3 spectra, but in the absence of authentic standards, definitive identifications were not made. These partial identifications include 3β,x-dihydroxycholest-5-en-y-one (3β,x-diHC-yO) where x and y may be 22 and 24, or 20 and 22, and 7α,x,y-trihydroxycholest-4-en-3-one (7α,x,y-triHCO, **Figure 5D**) where x and y may be 24, 25, or 26 (*italic* compound names in **Figures 1**, **3**).

We next performed multivariate analysis on the data from Study 1 using SIMCA software and an orthogonal projection to latent structures discriminant analysis (OPLS-DA) and this yielded a robust model separating PD from controls (**Supplementary Figure 2**, $Q2 = 0.68$, ANOVA = 3.2e-7 for cross-validated model), suggesting a cluster of cholesterol metabolites as candidate biomarkers for PD. This data should be treated with caution as the patient and control data were reordered at different times and for samples collected from different hospitals in different countries. Nevertheless, metabolites significant in the univariate analysis (**Table 2**) were important in driving the separation in the multivariate model.

Study 2—CSF Oxysterols, Disease Status, and Clinical Measures of Disease

In this second study, data from 37 PD cases was compared to 5 age-matched controls. Relevant demographic and clinical variables are shown in **Table 1**. Internal standards were also included allowing for the quantification of 7α,26-diHC and 7α,26-diHCO (**Figure 5**) of the acidic pathway and also 7α,25-diHC and 7α,25-diHCO. The availability of samples from matched controls collected from the same geographical area (albeit in lower numbers than the patients) and the recording of LC-MS data in a single study allowed us to perform a deeper interrogation of the data than in Study 1. However, the number of control samples was limited and therefore PD vs. control comparisons need to be interpreted with caution.

7α,26-diHC Is Elevated in PD CSF

Following adjustment for the confounding variables of age and sex, 7α,26-diHC and a second oxysterol 7α,x,y-triHCO whose exact structure is unknown were found to be significantly elevated in PD CSF (**Figures 6A,B**). Based on accurate mass measurement, MS^3 fragmentation and retention time 7α,x,y-triHCO is likely to be 7α,24,25-triHCO, 7α,24,26-triHCO or 7α,25,26-triHCO (the uncertainty of structure is indicated by italicised nomenclature in **Figures 1**, **3**). Notably, 7α,26-diHC is an intermediate of the acidic pathway of bile acid biosynthesis (**Figure 1**). It was identified in Study 1 but not quantified due to an absence of an appropriate internal standard. Numerically, as in Study 1, 7αH,3O-CA (**Figure 6C**), 7αH-27-nor-C-3,24-diO (and its chemically unstable precursor 7αH,3,24-diO-CA) were elevated in PD CSF in Study 2, but not to a level of statistical significance (**Table 2**).

During the intervening period between conducting Study 1 and 2, we were able to purchase the trihydroxycholestenoic acids 3β,7α,24S-trihydroxycholest-5-en-(25R)26-oic (3β,7α,24S-triHCA(25R)) and 3β,7α,25-trihydroxycholest-5-en-26-oic (3β,7α,25-triHCA) acids from Avanti Polar Lipids Inc., which are easily converted in the laboratory to 7α,24S-dihydroxy-3-oxocholest-4-en-(25R)26-oic (7α,24S-diH,3O-CA(25R)) and 7α,25-dihydroxy-3-oxocholest-4-en-26-oic (7α,25-diH,3O-CA) acids, respectively, by treatment with cholesterol oxidase enzyme (Abdel-Khalik et al., 2018). This allowed us to identify and approximately quantify both acids in the CSF from PD patients and controls (**Figures 4C–F**). In the absence of 24S,25S, 24R,25R and 24R,25S diastereoisomers, it was not possible to define the exact stereochemistry for 7α,24-diH,3O-CA, and it may be 24S,25R, 24R,25R, 24S,25S or a mixture of all depending on the pathway(s) of biosynthesis (**Figure 1**; Autio et al., 2014). We were able to presumptively identify two other

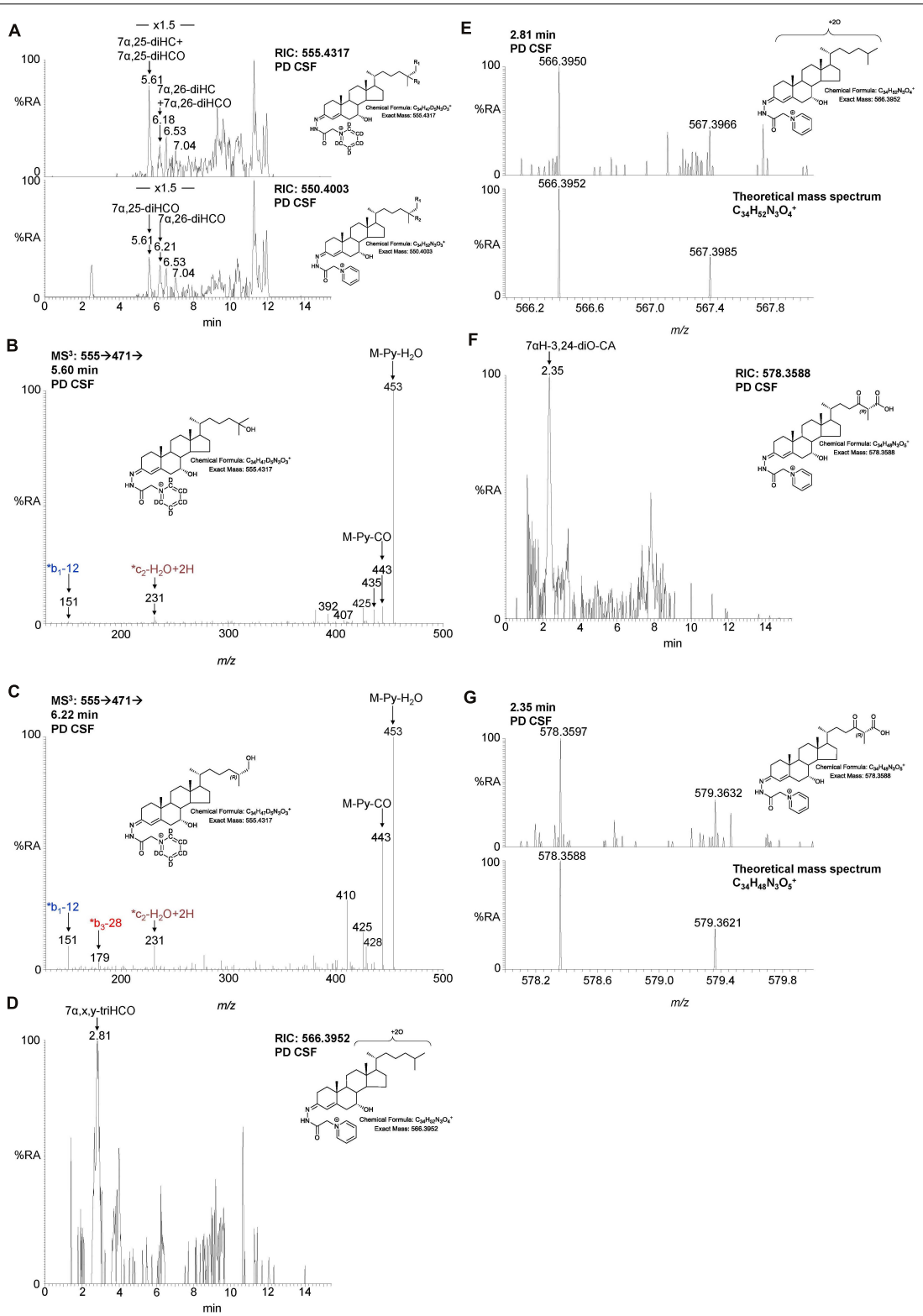

FIGURE 5 | LC-MS(MS^n) analysis of dihydroxycholesterols, dihydroxycholestenones, trihydroxycholestenones, and hydroxy*bis*oxocholestenoic acid in CSF. **(A)** RIC of 555.4317 ± 5 ppm (upper panel) and 550.4003 ± 5 ppm (lower panel) revealing 7α,25-diHC, 7α,26-diHC, 7α,25-diHCO, and 7α,26-diHCO. MS^3 ($M^+ \rightarrow M^+$-Py→) spectra revealing **(B)** 7α,25-diHC and **(C)** 7α,26-diHC. **(D)** RIC of 566.3952 ± 3 ppm corresponding to 7α,x,y-triHCO. **(E)** High resolution (100,000) mass spectrum (upper panel) and theoretical mass spectrum (lower panel) of 7α,x,y-triHCO. **(F)** RIC of 578.3588 ± 3 ppm corresponding to 7αH-3,24-diO-CA. **(G)** High resolution (100,000) mass spectrum (upper panel) and theoretical mass spectrum (lower panel) of 7αH,3,24-diO-CA.

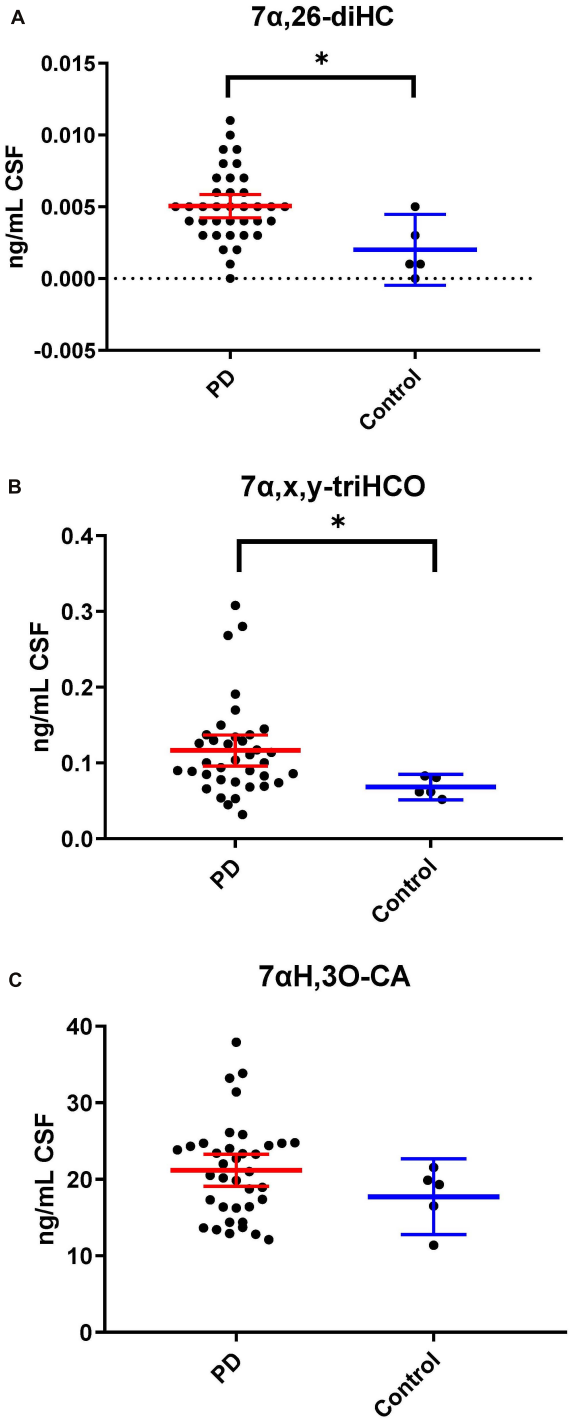

FIGURE 6 | 7α,26-diHC and 7α,x,y-triHCO are elevated in CSF from PD patients. **(A)** 7α,26-diHC. **(B)** 7α,x,y-triHCO. **(C)** 7αH,3O-CA is also elevated numerically but the significance is lost when confounding variables of age and sex are considered. Data from Study 2.

acids, as 7α,12α-dihydroxy-3-oxocholest-4-en-(25R)26-oic acid (7α,12α-diH,3O-CA) and 7α,x-dihydroxy-3-oxocholest-4-en-26-oic acid (7α,x-diH,3O-CA) based on retention time, accurate mass and MS^3 spectra (**Figures 4G,H**). The location of the second hydroxy group in 7α,x-diH,3O-CA is probably on the side-chain.

Combining data from Study 1 and Study 2, we have found that the acidic pathway of bile acid biosynthesis is upregulated in the CNS of PD patients (**Figure 1**).

Correlations With Clinical Data

Bivariate correlation analyses between each PD CSF oxysterol profile and relevant demographic and clinical variables (age, gender, disease duration, MDS-UPDRS motor score, ACE-R score, BDI score) were performed. Correlations of significance (at a level of $P < 0.05$) were found between PD CSF 24S-HC and disease duration ($r = 0.354$, $P = 0.032$), 7α-HC and BDI ($r = 0.436$, $P = 0.023$), 7αH-3,24-diO-CA and BDI ($r = -0.527$, $P = 0.005$) and 7α,12α-diH,3O-CA and BDI ($r = -0.418$, $P = 0.030$). Multivariate regression analysis with 24S-HC as the dependent variable and age and gender as relevant covariates did not confirm the relationship between 24S-HC and disease duration (Beta coefficient 0.313, $P = 0.060$). However, multivariate analyses did confirm the relationships between 7α-HC, 7αH-3,24-diO-CA, 7α,12α-diH,3O-CA, and BDI, with age, gender, and disease duration as relevant confounding covariates (7α-HC: Beta coefficient 0.449, $P = 0.031$; 7αH-3,24-diO-CA: Beta coefficient −0.510, $P = 0.010$; 7α,12α-diH,3O-CA: Beta coefficient −0.414, $p = 0.042$, see **Figure 7**). There were no statistically significant associations between any of the CSF oxysterols and motor measures [MDS-UPDRS motor score, motor phenotype (tremor dominant vs. postural instability subtype)] or cognitive measures (ACE-R, semantic fluency). However, 25-hydroxyvitamin D_3, the precursor of bioactive 1α,25-dihydroxyvitamin D_3, is elevated in CSF of patients with postural instability and gait disturbance (PIGD) compared to tremor dominant patients (TD, $P = 0.04$). Although the reason for this is not known, it may be the case that PIGD patients are more likely to be given calcium/vitamin D supplements because they are at risk of falls. Vitamin D_3 is converted to 25-hydroxyvitamin D_3 in the liver and is transported in the blood stream to the kidney where 1α,25-dihydroxyvitamin D_3 is formed.

DISCUSSION

In an early study looking at total oxysterols (where esterified and non-esterified molecules were measured in combination) in the CSF of PD patients and controls, concentrations of 24S-HC and 26-HC were found to be elevated in about 10% of PD samples above a cut off defined as the control mean + 3 standard deviations (SD) (Bjorkhem et al., 2013). However, when considering all samples, statistically significant differences were lost. In a follow-on study, Bjorkhem et al. (2018) found a small (about 1.75 ng/mL cf. 1.4 ng/mL) but statistically significant ($p < 0.05$) increase in 24S-HC in PD CSF. In this second study the CSF concentration of 24S-HC was found to correlate with disease progression. These results were suggested to relate to the release of 24S-HC from a subtype of dying neurons in PD, leading to an increase in 24S-HC concentration in the CSF during disease progression (Bjorkhem et al., 2013, 2018). The

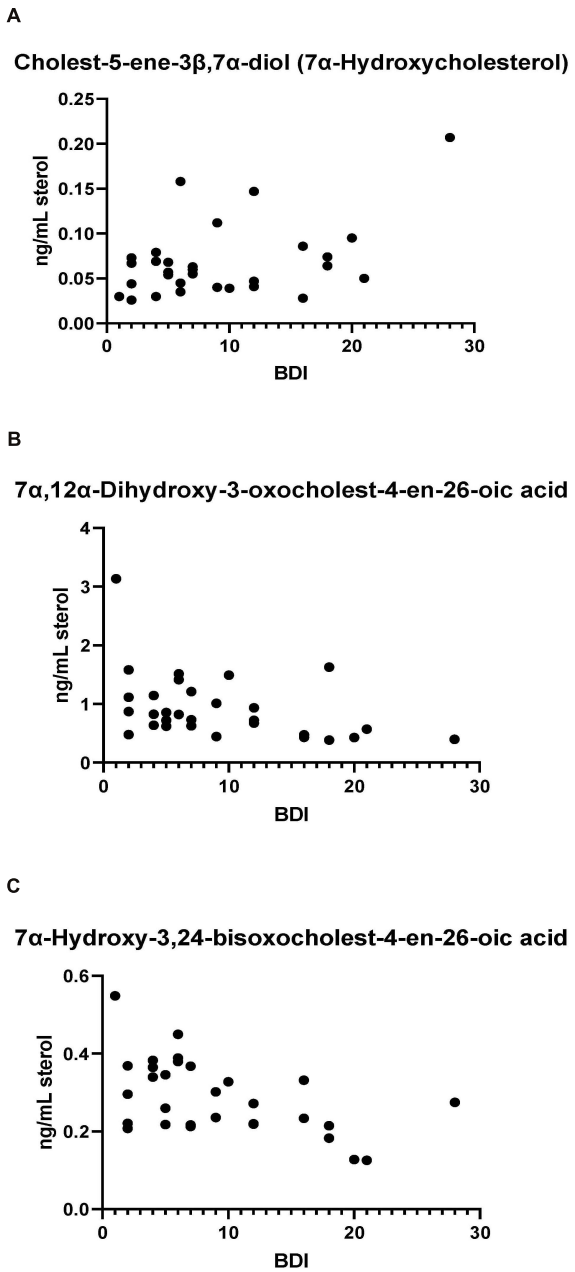

FIGURE 7 | (A) 7α-Hydroxycholesterol correlates positively with Beck Depression Inventory (BDI) scores while presumptively identified acids, **(B)** 7α,12α-dihydroxy-3-oxochelest-4-en-26-oic, and **(C)** 7α-hydroxy-3,24-*bis*oxocholest-4-en-26-oic acids show negative correlations with the BDI score.

explanation for the increase in the CSF content of 26-HC in a sub-set of PD patients was suggested to be a consequence of a defective BBB and excessive import of 26-HC from the circulation (Bjorkhem et al., 2013, 2018).

In our current studies, we have measured the biologically more relevant non-esterified molecules. We did not find a statistically significant increase in 24S-HC in CSF from PD patients in either study. 7α,26-diHC, one of the immediate down-stream metabolites of 26-HC (**Figure 1**), was increased in PD CSF following correction for age and sex (**Figure 6A**). Closer evaluation of the data sets in both Study 1 and Study 2 show that although not statistically significant when confounding variables are adjusted for, early metabolites in the acidic pathway of bile acid biosynthesis are elevated in the CSF from PD patients (**Figure 1**). This supports the suggestion of Bjorkhem et al. (2013, 2018) that a defective BBB may be responsible for distorting the oxysterol pattern in CSF of PD patients. An alternative explanation is that cholesterol released by dying cells in the PD brain is metabolised by CYP27A1, CYP7B1 and HSD3B7 and shunted into the bile acid biosynthesis pathway (**Figure 1**). Interestingly, a recent study has found an upregulation of bacteria responsible for secondary bile acid synthesis in the gastrointestinal tract of PD patients (Li et al., 2021), although how this may relate to CSF changes is not clear.

In brain, the origin of 26-HC may be cerebral or *via* import across the BBB (Heverin et al., 2005), however, there is strong evidence for its conversion to 7αH,3O-CA(25R) in the brain itself (Meaney et al., 2007; Ogundare et al., 2010). Importantly, the necessary enzymes, or their transcripts, for the conversion of 7αH,3O-CA(25R) to the C_{24} bile acid 7α-hydroxy-3-oxochol-4-en-24-oic acid (7αH,3O-Δ^4-BA) are all expressed in human brain (see **Figure 1**; Uhlen et al., 2015; Baloni et al., 2020).

A major route for 24S-HC metabolism is by CYP39A1 catalyzed 7α-hydroxylation to 7α,24S-dihydroxycholesterol (7α,24S-diHC, **Figure 1**) in the liver and onward to bile acids (Russell, 2003; Griffiths and Wang, 2020). CYP39A1 is, however, also expressed in the cerebellum and at low levels in the midbrain (Uhlen et al., 2015), providing a potential route to bile acid biosynthesis from 24S-HC in the brain. Although we did not identify 7α,24S-diHC in human CSF we did find the down-stream metabolic product 7αH,3,24-diO-CA, and its decarboxylation product 7αH-27-nor-C-3,24-diO. It should, however, be noted that 7αH,3,24-diO-CA is also a member of the acidic pathway (**Figure 1**). Interestingly, 7α,x,y-triHCO, is elevated in the CSF of PD patients (**Figure 6B**), and if x and y are 24S- and 26-hydroxy groups, respectively, then this metabolite falls into the metabolic pathway originating from 24S-HC.

Cholesterol 7α-hydroxylase (CYP7A1) is not expressed in brain (Uhlen et al., 2015; Baloni et al., 2020), hence the presence of 7α-HC in CSF must be *via* the circulation or *via* non-enzymatic oxidation of cholesterol. 7α-HC represents the first member of the neutral pathway of bile acid biosynthesis (Russell, 2003), one of the branches of this pathway proceeds through 7α,12α-diH,3O-CA which is one of the acids we presumptively identify in CSF. CYP8B1 is the necessary sterol 12α-hydroxylase but has not been found in human brain (Uhlen et al., 2015; Baloni et al., 2020), suggesting that the origin of 7α,12α-diH,3O-CA is from the circulation. While the 7α,24- and 7α,25-dihydroxy acids found in CSF are barely detected in plasma, 7α,12α-diH,3O-CA is present at the ng/mL level (Abdel-Khalik et al., 2017). In combination this data argues for an extracerebral origin for 7α,12α-diH,3O-CA and its import into CSF from the circulation. In future studies we recommend that wherever possible plasma and CSF from the same PD patient should be analysed in parallel.

This will support or refute the hypothesis that the origin of some oxysterols and cholestenoic acids found in CSF is from the circulation. Assessing the correlations for each analyte between the two media should give a good indication if the origin of the metabolite is extra- or intra-CNS. To investigate the possibility of blood contamination confounding the CSF data, a simple extension to the experimental protocol would be to record a direct infusion mass spectrum from a few μL of CSF to identify the presence or absence of haemoglobin. In the present study we did not perform such an analysis, but any contamination by blood can only be minimal as in all CSF samples 3β-HCA was only a minor oxysterol while it is the most abundant free oxysterol in plasma (Abdel-Khalik et al., 2017).

The levels of the oxysterols 7α-HC, 7αH-3,24-diO-CA, 7α,12α-diH,3O-CA were found to correlate with BDI score (**Figure 7**) in PD cases but not with other clinical measures. No previous studies have identified associations between oxysterols and depression in general. As these oxysterols are predominantly considered to originate from the circulation, this may suggest the involvement of biological processes of systemic origin in PD depression. Depression is known to be associated with markers of systemic inflammation (Miller and Raison, 2016), including in PD (Lindqvist et al., 2013), while oxysterols are known to contribute to inflammatory processes (Duc et al., 2019). Thus, systemic immune modulatory processes may be a potential linking factor mediating the observed relationship between oxysterol levels and depression. However, further studies in larger PD and matched control cohorts will be required to confirm and extend this association and its biological basis, as will measurement of these metabolites in PD plasma. A caveat to the link between oxysterols, inflammation and depression, is the lack of correlation between the major immunoregulatory oxysterols 25-HC and 7α,25-diHC with BDI score.

Interestingly, intermediates in the acidic pathway of bile acid biosynthesis have also been found to be elevated in people suffering from multiple sclerosis but not in those suffering from amyotrophic lateral sclerosis or Alzheimer's disease (Abdel-Khalik et al., 2017; Crick et al., 2017; Griffiths et al., 2019), arguing against a link between a general mechanism for neurodegeneration and cerebral bile acid biosynthesis. Nevertheless, this work points to the potential value of measuring bile acid precursors in CSF in the clinical chemistry laboratory. Further studies with much greater numbers are required to assess the potential of CSF bile acid precursors as prognostic biomarkers or as lead compounds towards a PD therapeutic.

CONCLUSION

In conclusion, despite the limitations mentioned around our control CSF sample collection, a number of interesting and novel observations have been made in our study. Our data suggests a cerebral upregulation of the acidic pathway of bile acid biosynthesis in PD. We have also identified a number of cholesterol metabolites whose CSF levels correlate with depression in PD. Further studies are planned utilising greater sample numbers to confirm or refute the current findings.

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 studies involving human participants were reviewed and approved by the Cambridgeshire 2 Research Ethics Committee. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

WG, ST, EA, RB, and YW designed the study. JA-K, PC, EY, SM, RW, DB, and KF performed the study. CW-G, SM, and MT supervised and performed statistical analysis. All authors contributed to writing of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnagi.2021.685594/full#supplementary-material

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frontiers in Aging Neuroscience

REVIEW
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Neurodegenerative Diseases and Cholesterol: Seeing the Field Through the Players

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Neurodegenerative diseases, namely Alzheimer's (AD), Parkinson's (PD), and Huntington's disease (HD) together with amyotrophic lateral sclerosis (ALS) and multiple sclerosis (MS), devastate millions of lives per year worldwide and impose an increasing socio-economic burden across nations. Consequently, these diseases occupy a considerable portion of biomedical research aiming to understand mechanisms of neurodegeneration and to develop efficient treatments. A potential culprit is cholesterol serving as an essential component of cellular membranes, as a cofactor of signaling pathways, and as a precursor for oxysterols and hormones. This article uncovers the workforce studying research on neurodegeneration and cholesterol using the TeamTree analysis. This new bibliometric approach reveals the history and dynamics of the teams and exposes key players based on citation-independent metrics. The team-centered view reveals the players on an important field of biomedical research.

Keywords: neurologic disease, bibliometric analyses, scientific impact, sterol, research evaluation, informetric, scientometric, key opinion leader

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INTRODUCTION

Neurodegenerative disorders devastate millions of lives worldwide and impose an increasing socio-economic burden (Kalia and Lang, 2015; Feigin et al., 2017; Erkkinen et al., 2018; El-Hayek et al., 2019). Research within the last decades has helped to clarify the mechanisms underlying each disease and suggested new therapeutic approaches (Fu et al., 2018; Ga et al., 2018; Jucker and Walker, 2018; Reich et al., 2018; Lassmann, 2019; Savelieff et al., 2019; Schwartz et al., 2021). A decisive step is the identification of molecular culprits that provoke or contribute to the dysfunction and degeneration of neurons. In the case of AD, research focused on three targets: hyperphosphorylated forms of tau protein, proteolytic fragments of amyloid precursor protein, and specific variants of apolipoprotein E (Long and Holtzman, 2019). A prime target for PD-related research has been alpha synuclein (Rocha et al., 2018), but other genes, as well as environmental factors, have come under scrutiny (Deng et al., 2018; Bandres-Ciga et al., 2020; Blauwendraat et al., 2020). In the case of amyotrophic lateral sclerosis (ALS), superoxide dismutase 1 has been investigated intensely as it was the first gene shown to be mutated in familial forms of the disease (Rosen et al., 1993).

TABLE 1 | Query terms used for the literature search in PubMed/MEDLINE.

Query term*	Article count
(Q1 AND Q2) NOT Q3	4,775
(Alzheimer*[tiab]) AND Q2) NOT Q3	2,514
(Multiple sclerosis[tiab] AND Q2) NOT Q3	570
(Parkinson*[tiab] AND Q2) NOT Q3	459
((Lou Gehrig* disease[tiab] OR amyotrophic lateral sclerosis[tiab]) AND Q2) NOT Q3	132
(Huntington*[tiab] AND Q2) NOT Q3	116

Query term 1 (Q1): ((Pick's disease[tiab] OR progressive supranuclear palsy[tiab] OR tauopathy[tiab] OR tauopathies[tiab] OR neuronal ceroid lipofuscinosis[tiab] OR hereditary spastic paraplegia[tiab] OR ataxia telangiectasia[tiab] OR creutzfeldt-jacob[tiab] OR prion disease[tiab] OR frontotemporal dementia[tiab] OR fronto-temporal dementia[tiab] OR polyglutamine disease[tiab] OR spinocerebellar ataxia[tiab] OR spino-cerebellar ataxia*[tiab] OR motor neurone disease[tiab] OR motor neuron disease[tiab] OR motoneuron disease[tiab] OR Lou Gehrig* disease[tiab] OR amyotrophic lateral sclerosis[tiab] OR huntington*[tiab] OR parkinson*[tiab] OR alzheimer*[tiab] OR neurodegenerative[tiab] OR neurodegeneration[tiab] OR spinal muscular atrophy[tiab] OR multiple system atrophy[tiab] OR multiple sclerosis[tiab] OR dementia[tiab]). Query term 2 (Q2): (sterol OR cholesterol OR hydroxycholesterol OR hydroxy-cholesterol OR oxysterol). Query term 3 (Q3): (review[pt] OR niemann-pick disease type c2[tiab] OR niemann-pick type c2[tiab] OR niemann-pick disease type c1[tiab] OR niemann-pick type c1[tiab] OR niemann-pick type c[tiab] OR niemann-pick disease type c[tiab]).

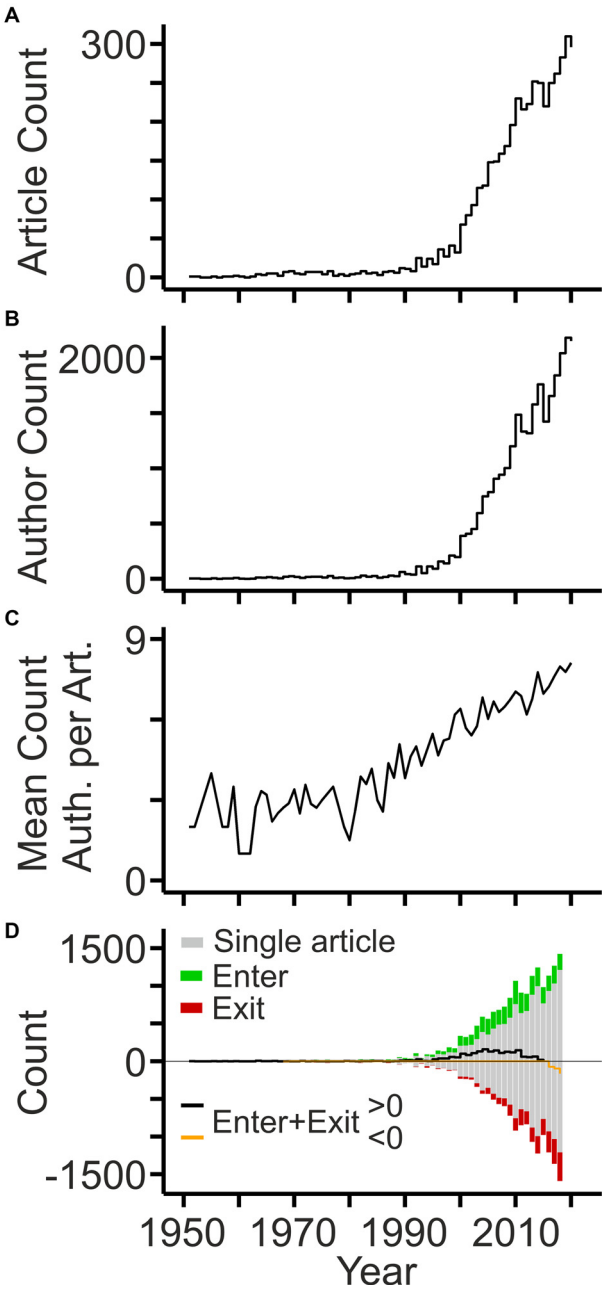

FIGURE 1 | Development of the workforce. **(A)** Annual counts of original articles related to cholesterol and neurodegeneration (PubMed query shown in **Table 1**). **(B)** Annual counts of authors contributing to the field per year. **(C)** Mean number of authors listed on article bylines per year. **(D)** Annual counts of authors entering (green bars) and exiting (red bars) the field per year based on the first and last year of publication, respectively. Black and orange lines indicate the sum of annual author counts. Gray bars indicate the number of authors contributing single articles to the field (shown as negative and positive values).

TAR DNA binding protein-43 (TDP-43) has become a target for ALS- and frontotemporal dementia-related research, as it was identified as a major component of ubiquitin-positive inclusions (Neumann et al., 2006). Since then, other genes have come under study as disease-causing alleles were identified in familial forms of ALS (Chia et al., 2018; Mejzini et al., 2019). Huntingtin has been at the center of attention as the long-sought gene bearing Huntington's disease (HD)-causing mutations (The Huntington's Disease Collaborative Research Group, 1993). Repeat expansions similar to those induced by the Huntingtin alleles cause neurodegeneration in numerous diseases including ALS and frontotemporal dementia by combinations of distinct molecular mechanisms (Malik et al., 2021; Schwartz et al., 2021). Research on multiple sclerosis (MS) has focused on immune and glial cells since chronic inflammation and demyelination are known pathologic changes preceding neurodegeneration (Faissner et al., 2019; Lassmann, 2019; Voet et al., 2019).

Why should cholesterol play a role in these diseases? Cholesterol is one of the most widely known and most studied biological molecules due to its involvement in cardiovascular and other diseases (Goldstein and Brown, 2015; Tall and Yvan-Charvet, 2015; Gliozzi et al., 2021) and due to its functions as a component of membranes in eukaryotic cells (Yeagle, 1985), as a cofactor of signaling pathways and as a precursor for steroid hormones (Miller and Auchus, 2011; Prabhu et al., 2016). Notably, cholesterol is also converted to biologically active oxysterols by specific enzymes or by autoxidation (Mutemberezi et al., 2016; Wang et al., 2021). Given the diverse functions of cholesterol, its cellular homeostasis relies on a multitude of proteins and mechanisms (Ikonen, 2008; Luo et al., 2020). In the brain, cholesterol represents a major building block due to the diversity and sheer mass of membraneous structures. This includes highly branched axons and dendrites of neurons (Elston and Fujita, 2014), fine perisynaptic processes of astrocytes (Oberheim et al., 2009), countless synaptic vesicles (Binotti et al., 2021), and the multi-layered myelin sheaths surrounding axons (Schmitt et al., 2015). Based on these considerations, disturbances of cholesterol homeostasis seem likely to cause neuronal dysfunction and

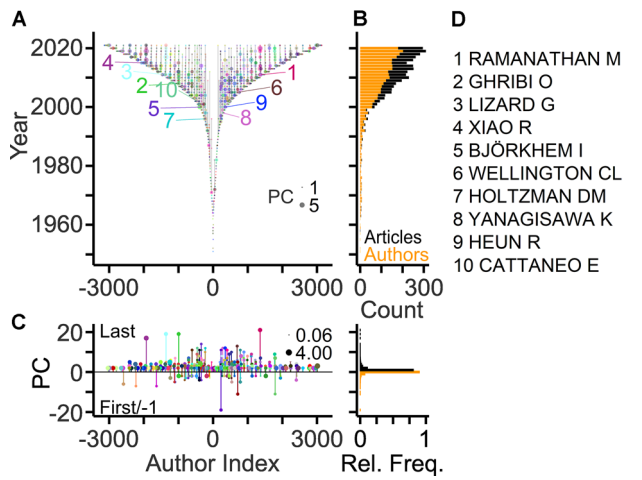

FIGURE 2 | Publication records of last authors. **(A)** TeamTree graph showing the publication records of the last authors contributing to the field. Circles connected by vertical gray lines represent for each author the years of publications as the last author plotted against a chronologic author index with alternating signs and author-specific colors to enhance visibility. Circle area indicates publication count (PC) per year. Numbers indicate authors with 10 largest PCs (names indicated in panel **D**). **(B)** Number of authors entering the field per year (orange) and of articles (black) published per year. **(C)** Left, PCs per author indicating last and first author articles by positive and negative values, respectively. Circle area indicates the average number of publications per year. Right, relative frequency distributions of PC values shown on the left. **(D)** Names of authors with largest PCs in the field.

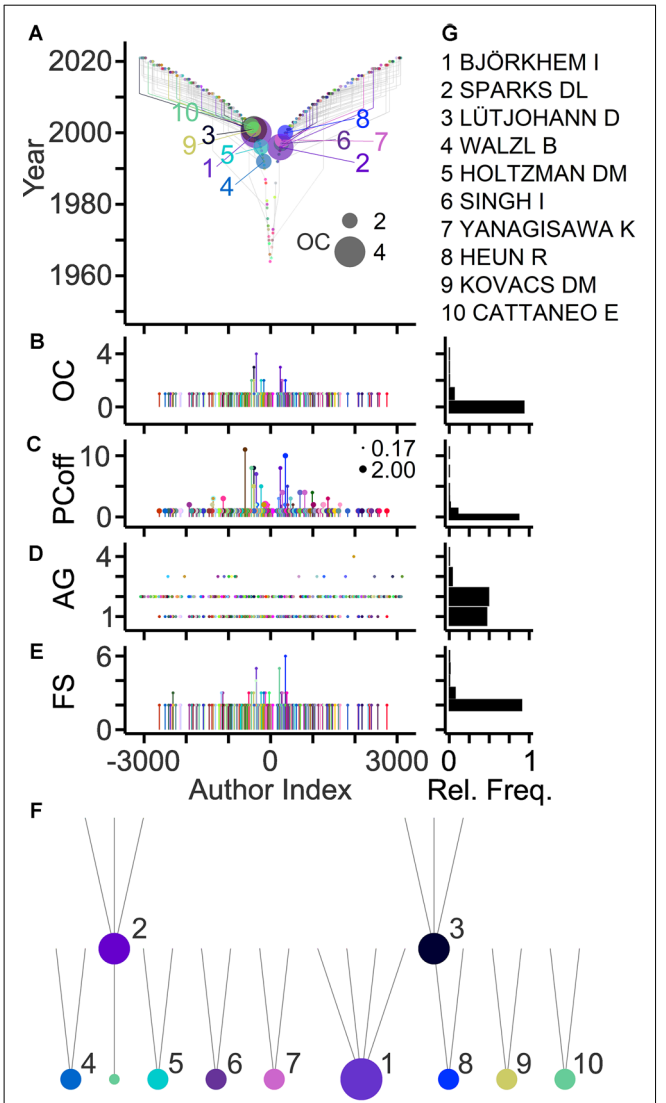

FIGURE 3 | Genealogic relations in the field. **(A)** TeamTree graph showing genealogic relations among authors. Circles and gray lines indicate ancestor-offspring connections based on first author-last author pairs on article bylines. Connections of authors with the 10 largest offspring count (OC) values are shown in color (names indicated in panel **G**). Circle area indicates OC value. The signs of author indices of offspring and of ancestors were adjusted to the first-generation ancestor. **(B–E)** Quantitative data showing for individual last authors, the number of offspring **(B)** the number of articles published together with offspring (PCoff; circle area indicates average PC per year) **(C)**, the generation of authors (AG) starting with AG = 1 for first ancestors **(D)** and the family size (FS) of individual first-generation ancestors comprising all offspring across subsequent generations **(E)**. **(F,G)** Family trees **(F)** and names **(G)** of authors with 10 largest OC values (indicated by circle area).

degeneration. The mechanisms of cholesterol homeostasis in brain cells are probably distinct from those operating in the rest of the body (Dietschy, 2009; Pfrieger and Ungerer, 2011; Zhang and Liu, 2015; Mahley, 2016; Moutinho et al., 2016; Yoon et al., 2016; Hussain et al., 2019). Possible implications of cholesterol and derived molecules in neurodegenerative diseases have been reviewed elsewhere (Martín et al., 2014; Zarrouk et al., 2014; Leoni and Caccia, 2015; Doria et al., 2016; Arenas et al., 2017; Chang et al., 2017; Testa et al., 2018; Zarrouk et al., 2018; Adorni et al., 2019; Griffiths and Wang, 2019; Hussain et al., 2019; Jeong et al., 2019; Jin et al., 2019; Loera-Valencia et al., 2019; Petrov and Pikuleva, 2019; Segatto et al., 2019; Blauwendraat et al., 2020; González-Guevara et al., 2020; McFarlane and Kędziora-Kornatowska, 2020; Sáiz-Vazquez et al., 2020; Dai et al., 2021; Duong et al., 2021; Feringa and van der Kant, 2021; García-Sanz et al., 2021; Pikuleva and Cartier, 2021; Samant and Gupta, 2021). This article shows the workforce driving research in the field using original research articles obtained from MEDLINE (**Table 1**) and a new bibliometric approach (Pfrieger, 2021; https://github.com/fw-pfrieger/TeamTree). Bibliometric analyses of other aspects can be found elsewhere (Guido et al., 2015; Barboza and Ghisi, 2018; Zhang et al., 2020; Du et al., 2021; Li et al., 2021; Rizzi et al., 2021). Articles related to Niemann-Pick type C disease were excluded from the analysis as this rare lysosomal storage disorder is directly linked to perturbed cholesterol transport (Loftus et al., 1997; Naureckiene et al., 2000; Vanier, 2010).

DEVELOPMENT OF THE WORKFORCE CONTRIBUTING TO THE FIELD

The earliest publications date back to the 1950s when three groups investigated the cholesterol content in tissues and body fluids of patients with dementia (Mori and Barucci, 1951;

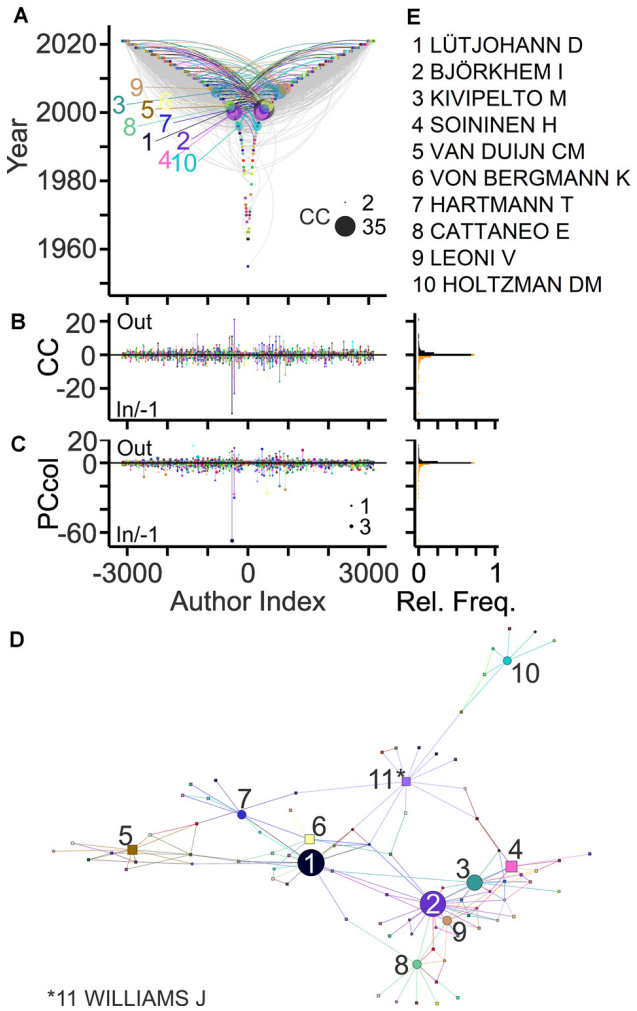

FIGURE 4 | Collaborative connections in the field. **(A)** TeamTree graph showing collaborations between last authors (out; negative author index) and (non-first) co-authors (in; positive author index) on article bylines. For out- and in-degree connections an author lists other authors as co-authors and an author is listed as a co-author, respectively. Connections of authors with 10 highest connection count (CC) values (in+out) are shown in color (names indicated in panel **E**). Circle areas indicate CCout and CCin values of these authors. **(B,C)** Left, counts of collaborators **(B)** and of collaborative articles **(C)** per author. Circle area indicates PCannu. Right, relative frequency distributions of parameters shown on the left. **(D,E)** Network **(D)** and names **(E)** of authors with 10 largest CC values. Symbol areas **(D)** indicate CC values normalized to the maximum. Circles and rectangles represent family and non-family authors, respectively.

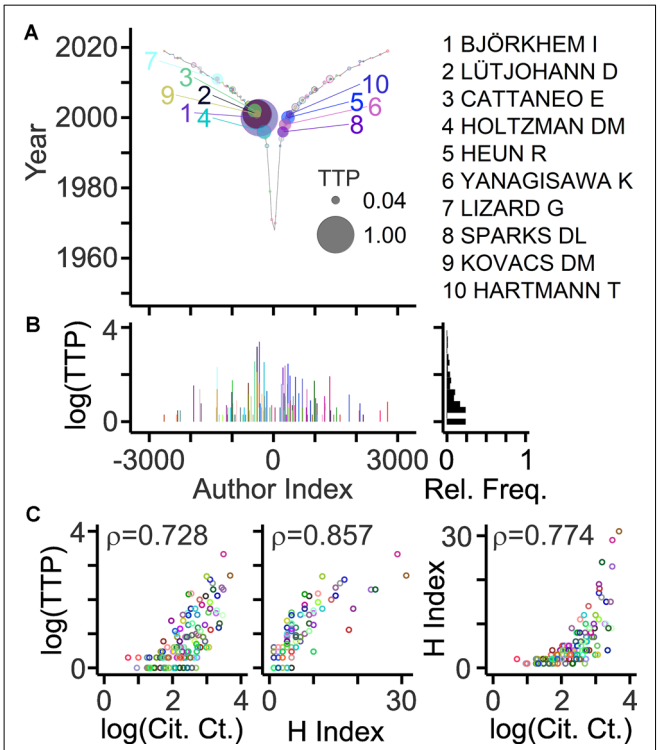

FIGURE 5 | Author ranking based on the TeamTree product. **(A)** Graph showing the TeamTree product (TTP) of individual last authors in the field represented by their author indices. This new metric takes into account publication records, offspring training and mentorship, and collaborative connections. Numerically, it represents the product of PC (last author articles) × OC × CC. Circle sizes indicate TTP values normalized to the maximum. Colored circles and numbers indicate authors with 10 highest values. Their names are shown on the right. Gray circles with colored border indicate authors with TTP values above zero. **(B)** Log10(TTP) values and their relative frequency distribution. **(C)** Scatterplots with circles representing individual authors (indicated by color; different from panels **A,B**) with their TTP values (log10) plotted against the total number of citing articles (left; Cit. Ct.; log10 values) and their H indices (middle) and with their H indices plotted against the total number of citing articles (right; log10 values). Numbers represent correlation coefficients [Spearman's rho values; two-sided test; $n = 126$; $S = 90{,}803$ (left)/47,558 (middle)/75,414 (right); $p < 10^{-10}$]. Citation-related parameters were calculated from bibliographic records obtained by a Web of Science query (Clarivate Analytics).

Scanu et al., 1955) and MS (Chiavacci and Sperry, 1952; Poser and Curran, 1958). The number of articles published per year remained relatively low until the 1990s and increased thereafter. Since 2000, the annual count of articles has grown linearly reaching around 300 articles per year in 2020 (**Figure 1A**). The number of authors listed on the article byline grew in parallel, however at a much stronger pace reaching more than 2,000 per year within the last years (**Figure 1B**). The strong expansion of the workforce was due to an increasing number of authors per article (**Figure 1C**). Notably, the expansion of the field was mainly driven by authors contributing single articles, as their number grew steadily. The balance of authors publishing in the field for more than 1 year has become negative within the last years, but the number of authors leaving the field within the last years is inherently inaccurate (**Figure 1D**).

PUBLICATION RECORDS, FAMILY RELATIONS, AND COLLABORATIVE CONNECTIONS IN THE FIELD

More information about the workforce can be drawn by analyzing the authors on specific positions of the article byline, which indicate the roles and contributions of authors (Claxton, 2005; Marušić et al., 2011). A total of ∼3,100 authors was listed on the last byline position of articles identifying these

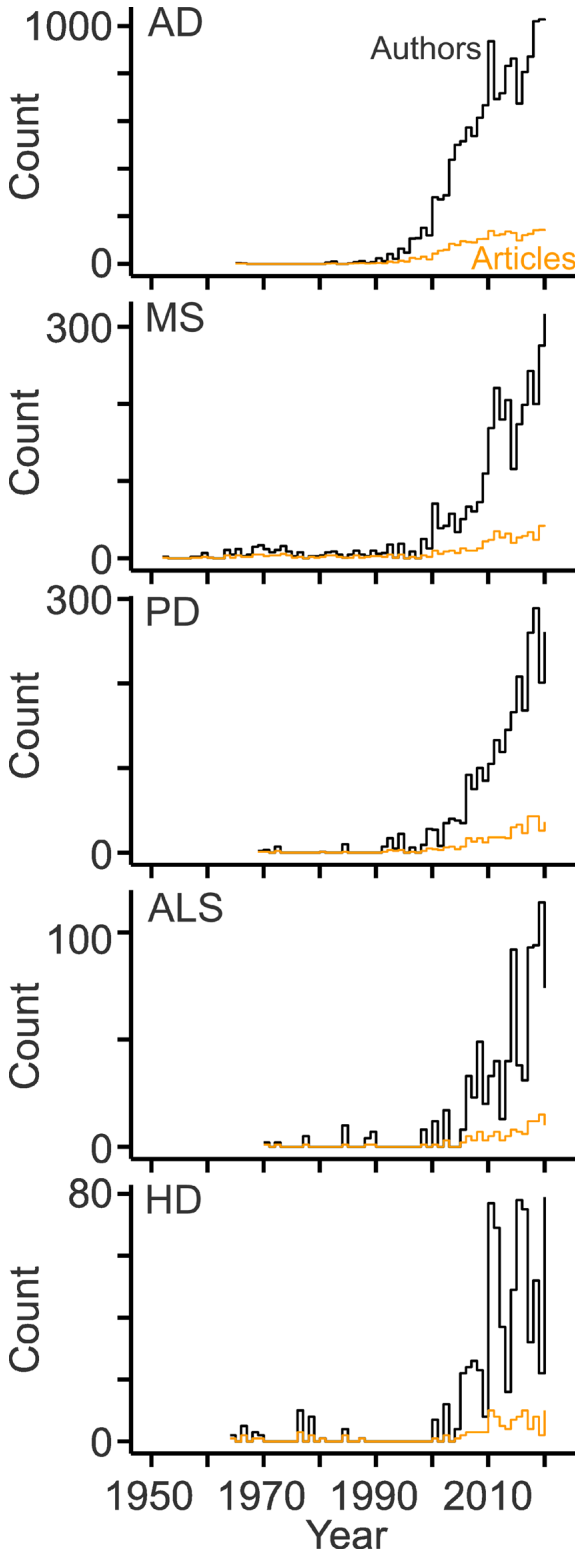

FIGURE 6 | Development of the disease-specific workforce. Line plots showing counts of original articles (orange) and of the contributing authors (black) per year related to cholesterol and the indicated diseases. AD, Alzheimer's disease; MS, multiple sclerosis; PD, Parkinson's disease; ALS, amyotrophic lateral sclerosis; HD, Huntington's disease.

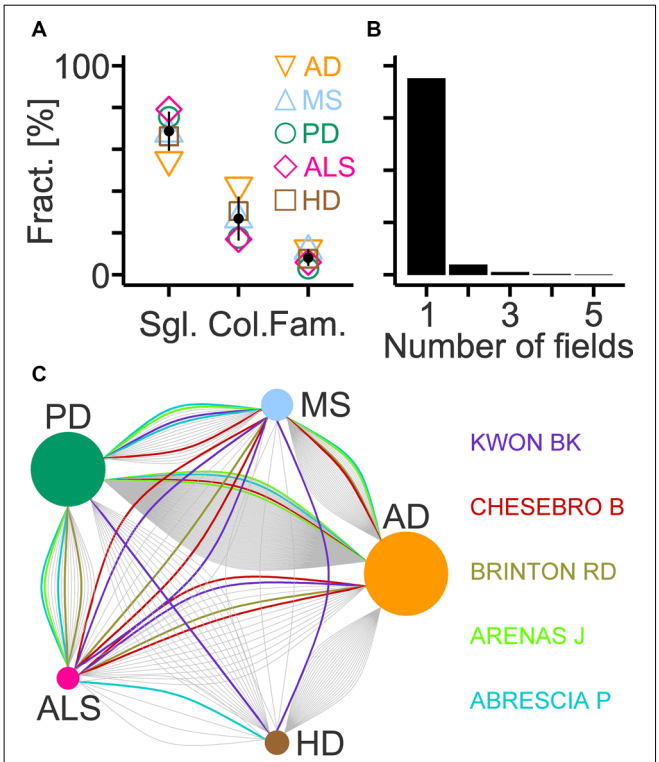

FIGURE 7 | Workforce composition and overlap across selected diseases. **(A)** Fractions of authors contributing single articles compared to the total workforce (Sgl.), of collaborating authors among last authors (Col.) and of authors with family ties among last authors (Fam.) in indicated fields (AD, Alzheimer's disease; MS, multiple sclerosis; PD, Parkinson's disease; ALS, amyotrophic lateral sclerosis; HD, Huntington's disease). Black circles and lines indicate mean and standard deviation (n = 5), respectively. **(B)** Histogram showing the fraction of last authors that contributed articles to the indicated number of fields. **(C)** Diagram showing connections between two diseases that are established by last authors contributing to both fields. Names and colored lines indicate the last authors with the highest number of connections (n = 6). Circle size represents the number of connections normalized to the maximum (AD; 160 links).

authors as principal investigators in the field. This corresponds to 10% of the total workforce. The development of the field with respect to these contributors is shown in **Figure 2A** using TeamTree graphs. In this type of scatterplot, the years of publication are plotted against a chronologic index assigned to each author (Pfrieger, 2021). The number of last authors entering the field per year has grown steadily during the last two decades (**Figure 2B**). The total publication counts of individual last authors reached up to 21 articles, but the large majority (81%) contributed single articles (**Figure 2C**) as observed for the entire workforce (**Figure 1D**). Ranking authors by PCs identified the top contributors among the last authors (**Figure 2D**).

Genealogical relations in a field can be derived from the last and first authors on article bylines representing ancestor and offspring, respectively (Pfrieger, 2021). **Figure 3A** shows family relations among authors highlighting those with the largest offspring counts. About 10% of last authors published previously as first authors thus qualifying as offspring, and 7% of last authors

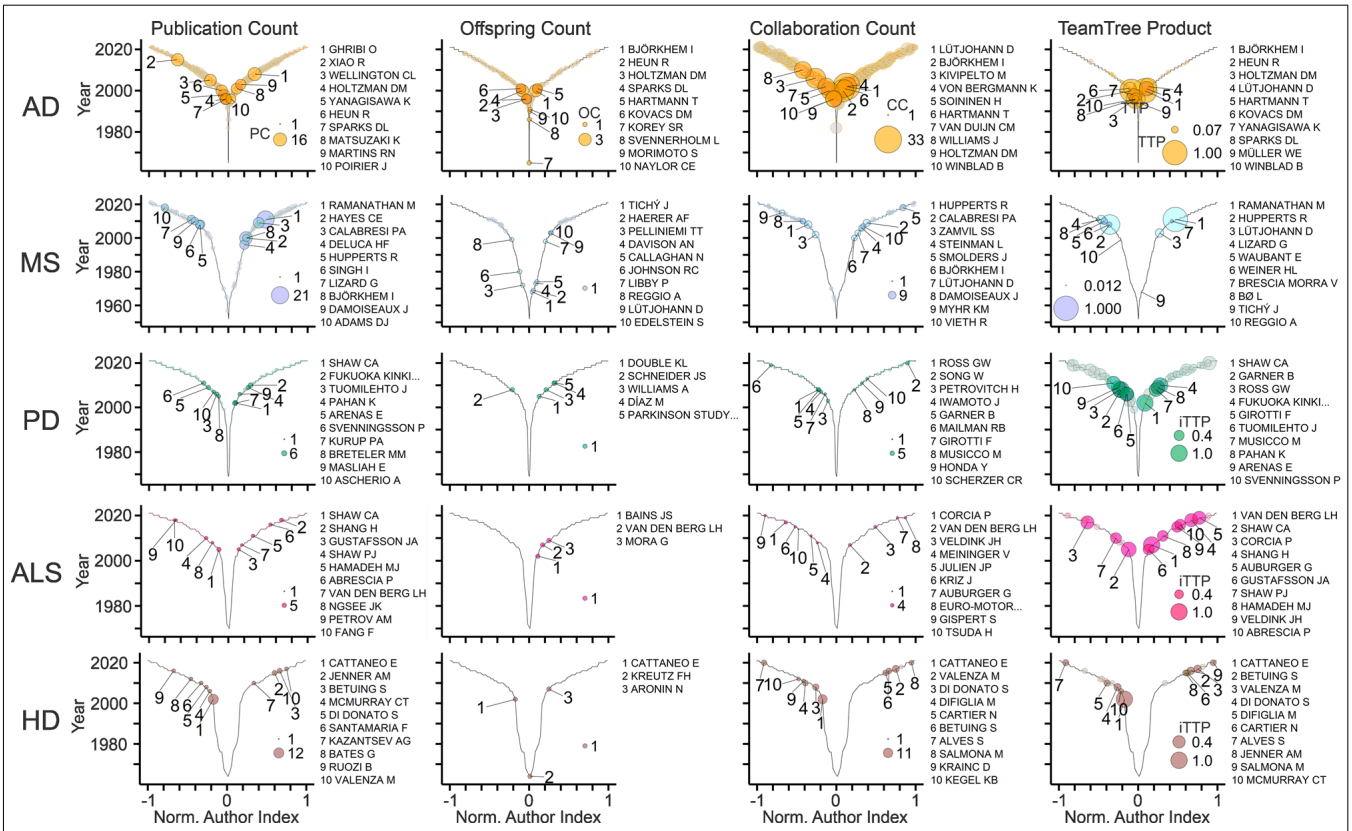

FIGURE 8 | In-depth view on the field-specific workforce. TeamTree graphs showing counts of publications (PC), offspring (OC), collaborative connections (CC), and the TeamTree product (TTP) in the indicated fields (AD, Alzheimer's disease; MS, multiple sclerosis; PD, Parkinson's disease; ALS, amyotrophic lateral sclerosis; HD, Huntington's disease) together with names of authors with the 10 largest values for each parameter. Note that for PD, ALS and HD, TTP values were replaced by an inclusive version of this measure (iTTP). For iTTP, zero counts of OC or CC values are set to one to include authors without offspring or lacking collaborators in the TTP-based ranking.

qualified as ancestors (**Figure 3B**). These ancestors generated up to four offspring authors and published up to 10 articles with their offspring (**Figure 3C**). Overall, the field comprised 192 families with up to six members spanning maximally four generations (**Figures 3D,E**). The large majority of families (91%) had only two members. Ranking by OCs revealed the most prolific authors and their families in the field (**Figures 3F,G**).

Collaborative connections can be delineated based on middle and last byline positions (Newman, 2001; Pfrieger, 2021). **Figure 4** exposes collaborations between authors contributing to the field. In total, 43% of the authors established collaborations with maximally 46 other authors and published up to 77 collaborative articles as last and co-author, respectively (**Figures 4B,C**). Ranking authors based on collaboration counts revealed the most strongly connected teams in the field and their networks (**Figures 4D,E**).

IDENTIFICATION OF MAJOR CONTRIBUTORS TO THE FIELD

An important goal of bibliometric analyses is to estimate the contribution of individual authors. The "key players" may serve as experts, key opinion leaders, referees, and collaborators. Different indicators of scientific production have been explored including PCs, citations, invitations, grants, and honors (Hicks et al., 2015; Schimanski and Alperin, 2018; Braithwaite et al., 2019). Original articles represent an accessible primary basis to estimate the contribution of an author. A new approach takes into account publication record, offspring generation, and collaborative connections, and delivers a new citation-independent parameter named TeamTree product (TTP; Pfrieger, 2021). Based on this parameter, key players studying neurodegenerative diseases and cholesterol are exposed in **Figure 5**. Due to the high selectivity, only a small fraction of authors (5%) reached TTP values above zero. Notably, TTP values of authors were strongly correlated with citation-dependent measures such as the total number of citations or the H index (**Figure 5C**).

DISEASE-SPECIFIC WORKFORCE ANALYSES

To gain deeper insight, diseases with the largest numbers of publications were analyzed separately (**Table 1**). Notably, AD-related research produced half of the articles published in

the field (**Table 1**). Overall, the fields showed marked differences with respect to length and growth pattern: MS has the longest and most continuous publication record (**Figure 6**). Except for two articles published in the 1960s, research on AD and cholesterol started in the 1980s. The subsequent growth of this field was probably triggered by discoveries that the epsilon allele of apolipoprotein E (Corder et al., 1993; Poirier et al., 1993; Rebeck et al., 1993; Saunders et al., 1993; Strittmatter et al., 1993) and high blood levels of cholesterol raise the risk of sporadic AD (Kivipelto et al., 2001). Parallel studies revealed connections between cholesterol and beta amyloid (Hartmann et al., 1994; Bodovitz and Klein, 1996; Avdulov et al., 1997; Howland et al., 1998; Simons et al., 1998; Refolo et al., 2000; Fassbender et al., 2001; Kojro et al., 2001; Puglielli et al., 2001; Runz et al., 2002; Wahrle et al., 2002) and between statins and AD (Wolozin et al., 2000; Refolo et al., 2001). The other disease fields are characterized by intermittent publication activity starting in the 1960s (HD) and 1970 (PD, ALS) and a more continuous development since 2000 (**Figure 6**). In the case of HD, pioneering studies showing links to cholesterol synthesis were published at the beginning of the 2000s (Sipione et al., 2002; Valenza et al., 2005). In all fields, the workforce grew more strongly than the number of publications (**Figure 6**) due to the increasing number of authors per article (**Figure 1C**). The ratios of author counts to publication counts were very similar across fields (6.6 ± 0.5; mean $\pm$ standard deviation; $n = 5$).

In each field, most authors contributed single articles with their fractions ranging from the lowest value in AD to the highest in ALS (**Figure 7A**). Inversely, the AD and ALS fields showed the highest and lowest fraction of authors involved in collaborations, respectively (**Figure 7A**). Authors with family ties represented a minority of the workforce with disease-specific fractions between 3% and 13% (**Figure 7A**). The analysis also revealed relatively little overlap among the workforce of each disease. Only 6% of authors (146 out of 2,379) contributed articles to more than one field (**Figure 7B**) and established up to six connections among them with AD and PD showing the largest workforce overlap (**Figure 7C**).

TeamTree graphs illustrate the workforce that studies links between cholesterol and the selected diseases (**Table 1**; **Figure 8**). Not surprisingly key players of the AD field dominate the global rankings (**Figures 2–5**, **8**). The analysis shows further that OCs are particularly sensitive to the size of the field. In those with the lowest number of articles and the smallest workforce (PD, ALS, HD), authors produced maximally one offspring indicating that this parameter requires a critical mass of authors (**Figure 8**). The TTP values reveal distinct disease-specific origins of the top 10 contributors. Notably, in the AD field, these authors entered the field within one decade starting in the 1990s, whereas, in other fields, these contributors entered after the year 2000 (**Figure 8**).

CONCLUSIONS

The new bibliometric analysis provides a detailed view of the development and structure of the workforce driving research on cholesterol and neurodegenerative diseases and complements content-specific summaries. The analysis revealed that the field started in the 1950s and remained relatively small until the 1990s. Except for MS, all fields showed intermittent publications, but a strong growth since 2000. The continuous expansion of the workforce during this period was mainly driven by authors contributing single articles although their contribution varied among the diseases analyzed. More than half of the articles are related to AD, therefore, the family ties, collaborative connections, and key players of this field dominate the overall picture. The analysis has caveats. A key challenge for this and other bibliometric approaches are ambiguous author names, as distinct authors can share the same name precluding correct evaluation (Smalheiser and Torvik, 2009). Evaluation of contributions based on single metrics such as TTP values is context-dependent, unsuited to evaluate junior scientists, and insensitive to ground-breaking contributions from small teams or from teams that contribute only briefly to a field.

AUTHOR CONTRIBUTIONS

FWP designed the review, performed literature queries, wrote and validated the code, analyzed the bibliographic records, prepared figures, and wrote the manuscript.

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REVIEW
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Altered Cholesterol Homeostasis in Huntington's Disease

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Huntington's disease (HD) is an autosomal dominant genetic disorder caused by an expansion of the CAG repeat in the first exon of Huntingtin's gene. The associated neurodegeneration mainly affects the striatum and the cortex at early stages and progressively spreads to other brain structures. Targeting HD at its earlier stages is under intense investigation. Numerous drugs were tested, with a rate of success of only 3.5% approved molecules used as symptomatic treatment. The restoration of cholesterol metabolism, which is central to the brain homeostasis and strongly altered in HD, could be an interesting disease-modifying strategy. Cholesterol is an essential membrane component in the central nervous system (CNS); alterations of its homeostasis have deleterious consequences on neuronal functions. The levels of several sterols, upstream of cholesterol, are markedly decreased within the striatum of HD mouse model. Transcription of cholesterol biosynthetic genes is reduced in HD cell and mouse models as well as post-mortem striatal and cortical tissues from HD patients. Since the dynamic of brain cholesterol metabolism is complex, it is essential to establish the best method to target it in HD. Cholesterol, which does not cross the blood-brain-barrier, is locally synthesized and renewed within the brain. All cell types in the CNS synthesize cholesterol during development but as they progress through adulthood, neurons down-regulate their cholesterol synthesis and turn to astrocytes for their full supply. Cellular levels of cholesterol reflect the dynamic balance between synthesis, uptake and export, all integrated into the context of the cross talk between neurons and glial cells. In this review, we describe the latest advances regarding the role of cholesterol deregulation in neuronal functions and how this could be a determinant factor in neuronal degeneration and HD progression. The pathways and major mechanisms by which cholesterol and sterols are regulated in the CNS will be described. From this overview, we discuss the main clinical strategies for manipulating cholesterol metabolism in the CNS, and how to reinstate a proper balance in HD.

Keywords: CYP46A1, cholesterol 24-hydroxylase, cholesterol, astrocytes, neurons, therapy

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INTRODUCTION

Huntington's disease (HD) is a hereditary neurodegenerative disease, one of the most frequent genetic brain disorder with a triad of symptoms: movement disorders, cognitive and psychiatric manifestations. Movement disorders, characterized by extrapyramidal signs of chorea, dystonia and bradykinesia, occur early in the course of the disease, and it is preceded by subtle cognitive or behavioral disturbance before the onset of these motor signs (Ross and Tabrizi, 2011). Later, motor impairments include bradykinesia, rigidity and incoordination. HD has a single genetic cause and its transmission is autosomal-dominant with a complete penetrance. The prevalence of HD is about 10 per 100,000 births, with a higher prevalence in some regions of the world (up to 700 per 100,000) (Harper, 1992; Paradisi et al., 2008). The age of onset is usually mid-life, with about 15% of patients showing symptoms before 30 years of age (referred to as juvenile HD). Once the first symptoms have appeared, they progress inexorably and irreversibly over 15–20 years. The availability of informative pre-manifest genetic and neuropsychological testing, along with peripheral and neuroimaging biomarkers of HD progression, offer a window of therapeutic intervention at early stages of the disease, with the perspective to delay the onset of the disease, slow its progression and even prevent HD.

There is no available drug therapy, or gene therapy for slowing disease progression. Over the past two decades, 99 clinical trials were performed in HD investigating 41 different compounds. However, the success rate is low with only 3.5% of trials progressing to the next stage (Travessa et al., 2017). Currently there are 23 active clinical trials in HD registered with ClinicalTrials.gov. Targeting the genetic cause of HD by lowering the product of huntingtin gene (*HTT)* or specifically the harmful *HTT* is still promising but preclinical findings are still essential to open new windows to treat HD by focusing on the cellular consequences of mutant HTT (mHTT) expression. In this regard, several neuronal dysfunctions have been described and could be targeted in clinical assays: excitotoxicity, transcriptional deregulation, dopaminergic alteration, autophagy, loss of neurotrophic support, mitochondrial dysfunction, oxidative stress, and neuroinflammation (Saudou and Humbert, 2016). Studies in humans and mouse models deeply described changes in cholesterol metabolism (Trushina et al., 2006; del Toro et al., 2010; Valenza et al., 2010; Leoni and Caccia, 2015; Boussicault et al., 2016; Kacher et al., 2019) that appear to be a seminal and early event in HD. Hence, targeting cholesterol homeostasis has emerged as an interesting therapeutic approach in HD. In this review, we summarize informative research on cholesterol metabolism in HD before going through the specific role of astrocytes and microglia in cholesterol metabolism deregulation. We address the main clinical strategies to manipulate cholesterol metabolism and we list the pressing questions to optimize the success of a cholesterol-based therapy in HD.

Abbreviations: 7-DHC, 7-dehydrocholesterol; 24S-OHC, 24S-hydroxycholesterol; AD, Alzheimer's disease, APOE apolipoprotein E; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors; BBB, blood brain barrier; BDNF, Brain Derived Neurotrophic Factor; CNS, central nervous system; CRAC, cholesterol-interacting sequence; CYP46A1, cholesterol 24-hydroxylase; CYP51, lanosterol C14 demethylase; DHCR7, -dehydrocholesterol reductase; DHCR24, 24-dehydrocholesterol reductase; ER, endoplasmic reticulum; Fdft1, farnesyl-diphosphate farnesyltransferase 1; GFAP, Glial Fibrillary Acidic Protein; HMG-CoA, 3-hydroxyl-3-methylglutaryl-coenzyme A; HMGCoAR, 3-hydroxy-3-methylglutaryl-coenzyme-A reductase; HD, Huntington's disease; HTT, Huntingtin; INSIG, insulin-induced protein; LXR, Liver X Receptor; LTP, long-term potentiation; mHTT, mutant Huntingtin; MSNs, medium Spiny Neurons; NMDAR, *N*-methyl-D-aspartate receptor; NPC, Niemann-Pick disease; PAT, palmitoyl-acyl transferases; PD, Parkinson's disease; polyQ, polyglutamine; PGC-1, peroxisome proliferator-activated receptor gamma coactivator-1; PTM, post-translational modifications; SDH, succinate dehydrogenase; SRE, sterol regulatory elements; SREBP, sterol regulatory element-binding protein.

HUNTINGTON'S DISEASE

The mutation responsible for HD consists of an unstable CAG repeat located at the 5′ end of *HTT* gene on chromosome 4p16.3 (The Huntington's Disease Collaborative Research Group, 1993). *HTT* gene is non-pathogenic when it contains less than 27 CAG repeats. Between 27 and 35 CAG, repeats do not cause HD but may expand in successive generations. Intermediate allele repetitions (36–39) are associated with late-onset and may express a variable penetrance and different clinical presentation. Individuals with 40 CAG repeats or more will develop HD, with nearly full penetrance by age 65 years (Langbehn et al., 2004). The age of onset inversely correlates with the number of repeats (Bates et al., 2015; Paulson, 2018). However, a large genome-wide association study, showed that genetic factors might influence the onset of HD; specifically these factors could explain the variability of disease onset for a given repeat size (Genetic Modifiers of Huntington's Disease (GeM-HD) Consortium, 2015, 2019).

The mutation in *HTT* results in an abnormal expansion of polyglutamine (polyQ) in the N-terminal region of the Huntingtin protein (HTT). This mutation causes neuronal dysfunction and death, particularly in the striatum and cortex, despite similar expression of the protein in other brain areas (Ross and Tabrizi, 2011). As neurodegeneration progresses in the striatum, the severity of symptoms increases, and imaging biomarkers of HD progression indicates that striatal atrophy begins up to 15 years before predicted onset (Aylward et al., 2011). Thinning of the cortex, which contains neurons projecting to the striatum, occurs in early symptomatic patients (Rosas et al., 2008), probably as a consequence of neurodevelopmental abnormalities (Barnat et al., 2020). In addition to the gray matter atrophy, several functional magnetic resonance imaging studies showed widespread atrophy of the white matter including from striatal projection fibers and corpus callosum in premanifest HD patients (Dumas et al., 2012; Gregory et al., 2020).

Macroscopic and microscopic criteria have defined a gradation, ranging from grades 0–4, showing a close correlation with the extent of clinical disability and a progressive atrophy of striatal neurons (Vonsattel's grade) (Vonsattel et al., 1985). The most dramatic degeneration occurs in the medium spiny neurons (MSNs) of the striato-pallidal pathway, which express dopamine receptors of the D2 subtype (indirect pathway; grade 2). This initial loss leads to a hyperkinetic phenotype. Then striato-nigral MSNs, which express dopaminergic D1 receptors

(direct pathway), degenerate (grade 3), resulting in bradykinesia. The striatal interneurons, aspiny striatal cholinergic, and somatostatine containing neurons are relatively spared (Roze et al., 2011). Dopamine D2 receptors, which are selectively expressed by indirect but not direct MSNs may be a key factor in the early and selective vulnerability of the indirect pathway and have been implicated in HD pathogenesis (Charvin et al., 2005; Deyts et al., 2009).

Whether neuronal degeneration in HD is due to the loss of one normal *HTT* allele or gain of toxic functions due to *mHTT* on this allele, or both, is not fully elucidated. Knockout of *HTT* in mice is lethal (embryonic day 8.5) (Duyao et al., 1995; Zeitlin et al., 1995), while selective knockout of *HTT* in neuronal cells and testis produces apoptosis in these tissues (Dragatsis et al., 2000), indicating that the HTT protein is required for normal cell development and survival. HTT is a ubiquitous protein, present in all tissues, and within cells, in virtually all cellular compartments. HTT is a large protein of 3,144 amino acids (348-kDa) with numerous intracellular interactors (more than 350), which implicates HTT in diverse cellular functions, including transcription, intracellular transport, metabolism, and homeostasis (Saudou and Humbert, 2016). The interaction with these partner proteins is altered by the mutation, with either enhanced or decreased functions.

Multiple post-translational modifications (PTM) can also affect HTT and/or mHTT functions (Saudou and Humbert, 2016). These include phosphorylation, acetylation, palmitoylation, ubiquitylation, and sumoylation. In the context of mHTT, PTM modulate the toxicity of the protein. For example, acetylation increases clearance by the autophagic-lysosomal pathway and decreases toxicity. Phosphorylation on diverse amino acids within the protein regulates the nuclear transport of mHTT, thus reducing its local toxic functions; this PTM can also decrease the proteolysis of mHTT, or regulates microtubule-dependent transport of organelles. In addition, recent evidence indicate that HTT can regulate the activity of palmitoyl-acyl transferases (PATs), via protein-protein interactions, and increasing brain palmitoylation restores neuropathology, locomotor deficits, and anxio-depressive behaviors in an HD knock-in mouse model (Virlogeux et al., 2021).

Huntingtin is subjected to proteolysis at multiple sites by a variety of proteases, including caspases, calpain, cathepsins, and the metalloproteinase MMP10 (Saudou and Humbert, 2016). The activity of these proteases is increased in the brain of HD patients, resulting in enhanced proteolysis of mHTT. Consequently, small N-terminal fragments containing the polyQ stretch accumulates. These cleaved versions give rise to insoluble aggregates and accumulate in the brain of HD patients and in various HD mouse models (DiFiglia et al., 1997; Scherzinger et al., 1997). Of interest, they first appear in striatal neurons, more abundantly in the neuronal processes and axonal terminals, where they induce altered synaptic functions and neuritic degeneration (Li et al., 2000), thereby eliciting a microglial reaction and inflammatory cytokines production (Yang et al., 2017). In other cellular compartments, mHTT sequester the wild-type HTT or associated proteins involved in normal cellular functions, including transcription, transport, or the ubiquitin-proteasome machinery (Ross and Tabrizi, 2011; Roze et al., 2011). The machineries of unfolded protein clearance, including the ubiquitin-proteasome and autophagy machineries, are altered by the mutation. As such, the aggregates cannot be properly cleared from the cells. It is noteworthy that inhibiting the aggregation of mHTT can alleviate the symptoms in various models of HD. It is noteworthy that inhibiting the aggregation of mHTT can alleviate the symptoms in various models of HD. Although these results support that mHTT aggregates participate to toxicity, other studies indicate that they may initially play a protective role in cells (Saudou et al., 1998; Arrasate et al., 2004). Indeed, smaller oligomeric aggregates could be more toxic than larger ones (Gong et al., 2008), at least in non-neuritic compartments. *mHTT* can also be mis-spliced to generate a short mRNA, which is translated into a highly toxic N-terminal fragment (Bates et al., 2015).

Due to its polyQ expansion, mHTT also alters protein-protein interactions and cellular functions including transcription (Moumné et al., 2013), axonal transport (Vitet et al., 2020), synaptic transmission (Roze et al., 2011), mitochondrial ATP production (Duan et al., 2014), the ubiquitin-proteasome system, and autophagy (Ortega et al., 2010; Li and Li, 2011; Martin et al., 2015). In the last decade, alteration of cholesterol homeostasis was demonstrated in HD (see below for further details; Karasinska and Hayden, 2011; Valenza and Cattaneo, 2011).

Transcriptional dysregulation is a pivotal feature of HD. In HD patients and experimental (cellular, mouse) models, early and progressive changes in transcriptional profiles occur in the prodromal period and affect multiple genes involved in cell survival, plasticity, neurotransmission, metabolism, and homeostasis. These alterations occur principally in the striatum and cerebral cortex, the most affected brain areas in HD, and result from altered nuclear localization and interactions of mHTT (under its soluble or aggregated form) with transcription factors or co-activators/co-repressors (Moumné et al., 2013). Altered cytoplasmic interaction of mHTT with the transcriptional repressor R element-1 silencing transcription factor (REST), results in a nuclear translocation of REST and its inhibitory role on transcription of survival genes, including Brain Derived Neurotrophic Factor (BDNF) (Zuccato and Cattaneo, 2009). Transcriptional dysregulation also occurs in astrocytes, where downregulation of the glutamate transporter GLT1 occurs because of mHTT expression. This in turn leads to a reduction of glial glutamate uptake, resulting in increased extracellular levels of glutamate and excitotoxicity within the striatum (Roze et al., 2011). With regard to cholesterol metabolism, mHTT reduces nuclear translocation of the sterol regulatory element-binding protein 2 (SREBP2), a master transcriptional regulator of genes involved in the cholesterol biosynthesis pathway in astrocytes (Moumné et al., 2013). It also interferes with the DNA binding of nuclear receptors, liver X receptors (LXR), which participate in the cholesterol metabolism and transport (see below). Besides these transcriptional dysregulations, mHTT also affects the epigenetic status, via altered methylation of DNA, histone PTMs, and non-coding RNAs (Moumné et al., 2013).

Alteration of axonal transport is also an important hallmark in HD. Altered interaction of mHTT with motor proteins has a strong impact on vesicular transport and therefore synaptic dysfunction. More specifically, the transport and release of BDNF, from cortical to striatal neurons, is impaired in HD, thus participating in striatal vulnerability (Vitet et al., 2020).

Clinical, biochemical, and neuroimaging studies in HD brain patients provided evidence for deficits in energy metabolism, reduced glucose consumption, and increased lactate concentrations in the basal ganglia and the cortex (Mochel et al., 2007). Furthermore, 3-Nitropropionic Acid (3-NP), an irreversible inhibitor of the respiratory chain complex II, Succinate Dehydrogenase (SDH), induces clinical and neuropathological features that resemble those described in HD, in patients and in rodent models (Brouillet et al., 2005). The mechanisms underlying the deficit energy in HD include impaired oxidative phosphorylation, oxidative stress, impaired mitochondrial handling and trafficking, along with transcription dysregulation (Johnson et al., 2021). Altogether, these data highlight the molecular and pathological consequences of HTT mutation, thereby offering therapeutic opportunities for treatments.

CHOLESTEROL IN CENTRAL NERVOUS SYSTEM AND AGING

In the human brain, cholesterol accounts for 23% of the total body cholesterol, when the brain volume accounts for about 2.1% of the body mass (Dietschy, 2009). Brain cholesterol is mainly unesterified; the larger pool being found in oligodendrocytes myelin sheaths (70% of the brain cholesterol), with a very slow turnover (5 years half-life). The remaining 30% is found in cell membranes, with a faster turnover and a half-life of 5–10 months (Davison, 1965; Andersson et al., 1990). Cholesterol cannot cross the blood-brain barrier (BBB), due to its association with lipoproteins, so it is synthesized locally in the CNS (Jeske and Dietschy, 1980). Newly synthesized cholesterol comes mainly from neurons during embryogenesis then from oligodendrocytes during postnatal myelination, and from astrocytes in the adult brain (Saher and Stumpf, 2015). Cholesterol synthesis in mammals' CNS involves a complex series of reactions, catalyzed by over 30 enzymes, and requires energy and oxygen (**Figure 1**; Gaylor, 2002). The first step is the conversion of acetyl-CoA into 3-hydroxyl-3-methylglutaryl-coenzyme A (HMG-CoA) via the reaction catalyzed by HMG-CoA synthetase and then by HMG-CoA reductase (HMGCR) into mevalonate, an irreversible and rate-limiting step in cholesterol synthesis (Rodwell et al., 1976). This is followed by a sequence of enzymatic reactions converting mevalonate into 3-isopenenyl pyrophosphate, farnesyl pyrophosphate, squalene, and lanosterol, followed by 19 steps involving two related pathways. These two pathways for cholesterol production in the brain seemingly have preferential cell expression: neurons preferentially go through the Kandutsch-Russel pathway via synthesis of 7-dehydrocholesterol (7-DHC), and astrocytes preferentially go through the Bloch pathway via synthesis of desmosterol. In the adult brain, the Bloch pathway seems to be preferred (Pfrieger and Ungerer, 2011). Cholesterol is synthesized *via* reduction of 7-DHC by 7-dehydrocholesterol reductase (DHCR7) in the Kandutsch-Russel pathway, and reduction of desmosterol by 24-dehydrocholesterol reductase (DHCR24) in the Bloch pathway (**Figure 1**). The machinery for cholesterol synthesis resides in the endoplasmic reticulum (ER), where one of its main regulators is SREBP2 (**Figure 2**), a transcription factor functioning as a sensor of cholesterol in the cell. When inactive, SREBP2 is anchored to the ER membrane and binds to SREBP cleavage activating protein (SCAP). When cholesterol concentration is high in the cell, SCAP, which has a cholesterol-sensing domain, binds to an insulin-induced protein (INSIG), maintaining the SREBP-2/SCAP complex in the ER membrane. When cholesterol levels decrease, SREBP-2/SCAP complex is dissociated from INSIG, allowing SCAP to escort SREBP-2 to the Golgi, where SREBP-2 is cleaved by SCAP. The resulting N-terminus domain of SREBP-2 then translocates into the nucleus and binds to the sterol regulatory elements (SRE) in the promoter region of target genes involved in the synthesis and uptake of cholesterol, fatty acids, triglycerides, and phospholipids (Horton, 2002). Cholesterol biosynthesis has an important feedback control on HMGCR activity and degradation (Goldstein and Brown, 1990). Accumulation of sterols in the ER membranes triggers the proteasome-mediated degradation of HMGCR through an INSIG/GRP78 dependent ubiquitination (Tsai et al., 2012). Thus, cholesterol synthesis is regulated by a negative feedback loop dependent on sterol levels.

The cholesterol brain turn-over is ensured by the cholesterol 24-hydroxylase or CYP46A1, a neuronal-enriched enzyme that converts, in a reaction requiring NADPH, cholesterol into 24S-hydroxycholesterol (24S-OHC), by addition of a hydroxyl group to the lateral hydrocarbon chain of cholesterol (Dhar et al., 1973; Dzeletovic et al., 1995). This conversion allows the crossing of 24S-OHC through the BBB, thus the elimination of cholesterol from the brain. *In vivo* formation of 24S-OHC was measured in the blood in humans (Lütjohann et al., 1996), rats (Breuer and Björkhem, 1995), and mice (Meaney et al., 2000). CYP46A1 is responsible for 99% of 24S-OHC in the brain, which account for 60–80% of 24S-OHC in the serum (Lund et al., 2003). According to the continuous flux of 24S-OHC in the serum (Lütjohann et al., 1996), cholesterol turnover by CYP46A1 in the brain appears to be a daily mechanism, which could account for a daily turnover of 20% in certain neurons (Dietschy and Turley, 2004). Within the brain, 24S-OHC is also a key modulator of the nuclear receptors: Liver X Receptor (LXR), which regulate the transcription of cholesterol transport proteins (see below). To a lesser extent, CYP46A1 can also catalyze the formation of 25-hydroxycholesterol, 24,25-dihydroxycholesterol and 24,27-dihydroxycholesterol (Lund et al., 1999; Mast et al., 2003).

In the CNS, cholesterol has many essential functions in the cells, and particularly in neurons. Due to its rigid apolar ring structure, cholesterol is deeply immersed in membranes reducing membrane dynamic and fluidity (Sankaram and Thompson, 1990a,b). The increase of cholesterol in membranes slows down the lateral diffusion of lipids and proteins (Rubenstein et al., 1979) and therefore can influence the conformation of

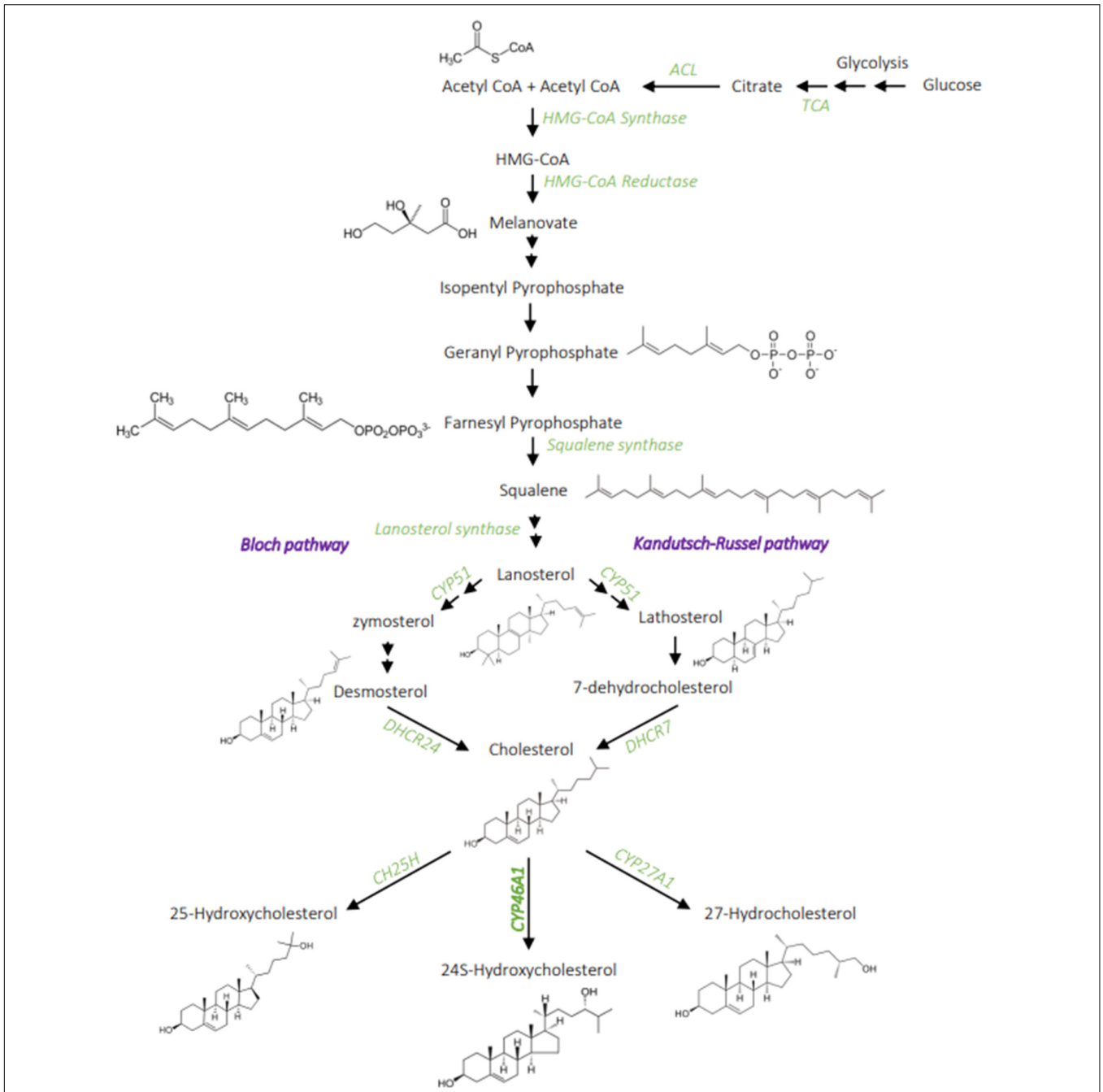

FIGURE 1 | Cholesterol metabolism. Cholesterol synthesis involves a complex chain of enzymatic reaction, from acetyl-CoA to the melanovate pathway that then divides into two possible pathways: the Bloch and Kandutch-Russel. The main product of cholesterol hydroxylation in the brain is the 24S-hydroxycholesterol, it can also be hydroxylated into 27-hydroxycholesterol and 25-hydroxycholesterol to a lesser extent. ACL, ATP citrate lyase; TCA, tricarboxylic acid cycle; CH25H, Cholesterol 25 hydroxylase; CYP51, lanosterol 14 alpha-demethylase; DHCR7, 7-dehydrocholesterol reductase; DHCR24, 24-dehydrocholesterol reductase; CY46A1, cholesterol 24-hydroxylase. Created with BioRender.com.

membrane proteins, changing for instance the stability and binding properties of neurotransmitter receptors with their ligands (Gimpl et al., 1997; Saxena and Chattopadhyay, 2012). Cholesterol has also an essential role in vesicle dynamics and constitutes 40% of total lipids in synaptic vesicle membranes (Takamori et al., 2006). It can modulate the vesicular transport likely through the recruitment of motor proteins (van der Kant et al., 2013), and influence fusion pore formation and stability during exocytosis (Koseoglu et al., 2011), by directly reducing the fusion pore-bending energy (Stratton et al., 2016). In this way, cholesterol depletion by β-cyclodextrin increases the spontaneous neurotransmission

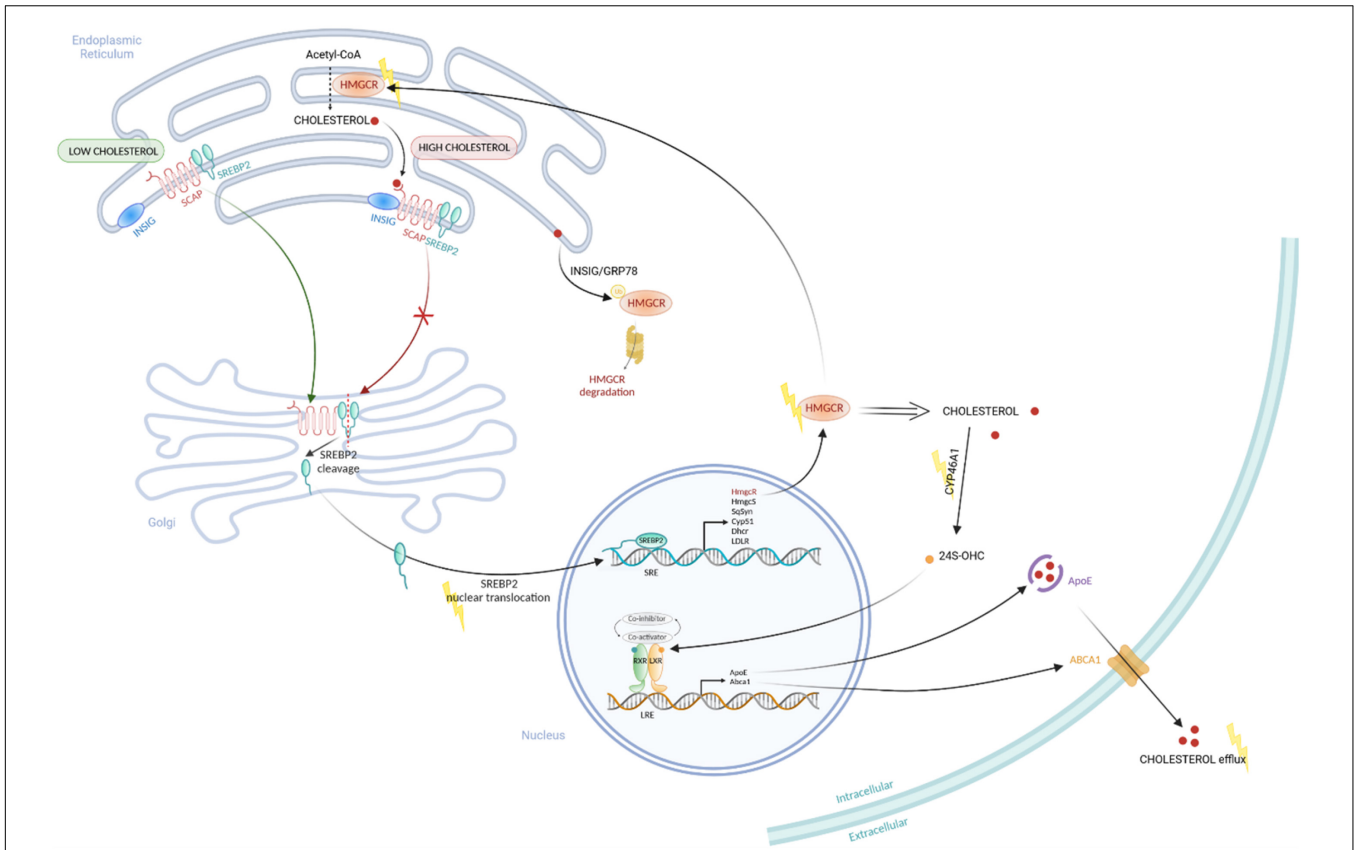

FIGURE 2 | Regulation of Cholesterol metabolism pathways. Cholesterol is synthesized in the endoplasmic reticulum (ER) from Acetyl-CoA through several steps using different enzymes, including the rate limiting enzyme HMGCOA-reductase (HMGCR). SREBP2 forms a complex with SCAP, a sensor of cholesterol, which is anchored at the ER membrane. When cholesterol is high, the sensor SCAP interact to INSIG protein, maintaining the SREBP2/SCAP complex at the ER membrane. When cholesterol level is low, SREBP2/SCAP complex can translocate to the Golgi where SREBP2 is activated by a cleavage. The activated SREBP2 can then translocate to the nucleus to induce the transcription of genes coding cholesterol synthesis enzymes, such as HMGCOA-reductase (HmcR), HMGCO-synthase (HmgcS), Squalen Synthas (SqSyn), Cyp51, Dhcr. This is associated to a negative feedback loop on cholesterol synthesis pathway. Sterol accumulation in the ER membrane triggers proteasome-mediated degradation of HMGCR through INSIG/GRP78-mediated mechanism. In parallel, the enzyme CYP46A1 can catalyze the cholesterol into 24S-OHC, a ligand of the nuclear receptor LXR. When the 24S-OHC binds to LXR, a co-activator is recruited and induce the transcription of genes involved in cholesterol efflux (Abca1, ApoE), leading to a decrease of intracellular cholesterol level. Yellow lightning points out altered functions in cholesterol metabolism in HD. Created with BioRender.com.

in hippocampal cultures, due to an enhanced spontaneous vesicle recycling (Wasser et al., 2007). In a recent study, cholesterol was shown to be an important endogenous regulator of synaptic transmission by acting on the post-synapse (Korinek et al., 2020). Within the bilayer membrane, cholesterol is enriched in lipid rafts, which participate in the compartmentation of proteins involved in the spatial and temporal organization of cell signaling, and as such have a critical role in the signal integration at the synapse; they are likely associated with the organization of post-synaptic density (Suzuki et al., 2011). Acute cholesterol depletion decreases both *N*-methyl-D-aspartate receptor (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPA)/kainate receptor-mediated evoked excitatory postsynaptic currents and impairs NMDA-dependent long-term potentiation (LTP) (Korinek et al., 2020). Moreover, alteration of lipid rafts by cholesterol depletion suppresses AMPA receptor exocytosis, reducing its cell surface expression (Hou et al., 2008), and produces a gradual loss of synapses and dendritic spines (Hering et al., 2003).

Besides its role at post-synaptic membranes, cholesterol accumulation at lysosomal membranes reduces the lysosome ability to fuse with endocytic and autophagic vesicles, thus affecting autophagy, *via* a process involving abnormal sequestration of SNARE proteins in cholesterol-enriched regions of endolysosomal membranes (Fraldi et al., 2010). Cholesterol distribution and regionalization within synaptic vesicles modulate SNARE protein conformations and complex assembly (Wang et al., 2020). An essential role of cholesterol in vesicle trafficking comes from studies on Niemann-Pick disease (NPC), a lysosomal storage disease characterized by lysosomal accumulation of unesterified cholesterol and glycosphingolipids, which results in alteration of axonal lysosome trafficking and positioning in neurons (Roney et al., 2021). In a cellular model of NPC, lowering lysosomal cholesterol levels with cyclodextrin reduces lysosome transport into NPC axons and reduces axonal

autophagic stress. Therefore, cholesterol is an essential structural component for the integration of intracellular signaling through the organization of the plasma membrane in micro-domains like lipid rafts, it regulates the dynamic of synaptic vesicle biogenesis and vesicle fusion, thus participating to the proper transport of vesicles along microtubules.

Because of its critical role in membrane dynamics and cell signaling, alterations of cholesterol content and homeostasis can have deleterious impacts on cell survival. The binding of BDNF to its cognate TrkB receptor triggers activation of two major survival intracellular signaling pathways: the Extracellular-signal Regulated Kinase (ERK) and Akt pathways. Stimulation of cultured neurons and hippocampal slices by BDNF induces an increase of TrkB receptor location in lipid rafts, showing a potential need for selective receptor location for efficient signaling (Suzuki et al., 2011). Cholesterol overload can decrease TrkB signaling and cell survival (Huang et al., 2016), and, in aging neurons, loss of cholesterol is associated with increased TrkB activity (Martin et al., 2008) while knockdown of CYP46A1 in neurons triggers low TrkB activity and increased apoptotic levels (Martin et al., 2011). Identification of cholesterol-interacting sequences (CRAC and CARC) was reported in TrkB sequence and their mutation can interfere with plasticity-related BDNF signaling (Cannarozzo et al., 2021; Casarotto et al., 2021). Because of their high need for energy production, neurons progressively accumulate reactive species of oxygen (ROS). In conditions of oxidative stress-related production of ROS, loss of membrane cholesterol is associated to the activation of TrkA, which binds Nerve Growth Factor (NGF) another prosurvival Growth Factor. Addition of cholesterol in non-stressed condition inhibits activation of TrkA and favors stress (Iannilli et al., 2011). Thus, changes in cholesterol content might be a way to regulate receptor activity toward cell surviving pathways, acting as a sensor of the cell state. Altogether, cholesterol level seems to be precisely regulated to favor cell survival, within a physiological range of concentration.

To understand pathological alterations of cholesterol metabolism, it is necessary to understand its evolution during normal aging. Alteration of cholesterol content is highly variable during aging in the different brain regions, ranging from negligible changes in the hippocampus and pons to a 40% decrease in the caudate at 90 years old (Söderberg et al., 1990). On average, cholesterol decreases by 47% in women and 53% in men, with a significant loss of myelin lipids (Svennerholm et al., 1997). Cholesterol loss is more pronounced within the white matter than in the cerebral cortex (Svennerholm et al., 1991). Cholesterol synthesis rate declines with aging in the human hippocampus, with a lower concentration of lanosterol and desmosterol precursors in elderly subjects (Thelen et al., 2006). A decreased synthesis with altered expression of genes related to cholesterol synthesis also occurs in the aging rat brain (Blalock et al., 2003). In the aging rat hippocampus, lanosterol, desmosterol, and lathosterol levels decrease, whereas in the cortex, only desmosterol levels decrease over time (Smiljanic et al., 2013). The mouse hippocampus shows an age-dependent loss of cholesterol (Martin et al., 2008; Sodero et al., 2011) which is associated with a cognitive decline (Martin et al., 2014). Plasma levels of 24S-OHC are very high in children, then decreases to become constant during adult life and tends to increase with age (Bretillon et al., 2000). The exact causes of this loss are not yet clear, but some mechanisms were proposed. Among them, an increase of transcriptional activation (Ohyama et al., 2006) and membrane mobilization (Sodero et al., 2012) of CYP46A1 could participate in cholesterol loss during aging. Particularly, CYP46A1 increases in high-stress situations, accumulation of cellular stress over time could favor cholesterol loss in aging. The age-dependent lowering of cholesterol may also be due to reduced synthesis or impaired delivery from glial cells.

IMPAIRMENT OF CHOLESTEROL IN HUNTINGTON'S DISEASE

Deregulation of Cholesterol Metabolism in Huntington's Disease

An increasing number of studies have implicated dysregulations of cholesterol metabolism in HD (see **Table 1** for references). Studies on rodent models, cell lines, and data from patient samples have shown a global downregulation of cholesterol metabolism. The amount of lathosterol and lanosterol decreases in multiple rodent models and patient plasma. This global alteration of the cholesterol biosynthesis pathway could be due to a decreased translocation of the transcription factor SREBP2 to the nucleus and a decreased expression and activity of synthesis enzymes. In the YAC128 HD mouse model, while SREBP2 processing (i.e., cleavage) is not affected, the mature cleaved SREBP2 accumulates in the cytoplasm, due to increased binding of mHTT to the SREBP2/importin β complex required for its nuclear import (Di Pardo et al., 2020). This could explain the reduction of the expression levels of HMGCR, CYP51, DHCR7, and DHCR24 found in HD whole brain extracts and in astrocyte primary cultures (**Table 1**).

Studies of cholesterol content gave rise to differential results, with decreased, increased, or no changes in cholesterol levels in the brain of HD animal models; whereas studies in human post-mortem caudate and putamen showed increased cholesterol levels (**Table 1**). Studies on cell cultures showed an accumulation of cholesterol in membranes due to the altered trafficking of cholesterol by caveolin-1 (**Table 1** for reference). The methodology used to measure cholesterol levels is critical to have reproducible data. A comparative study demonstrated that biochemical and mass spectrometry methods showed reduced cholesterol levels whereas colorimetric and enzymatic methods showed increased cholesterol levels. Colorimetric and enzymatic methods appear to have a lower sensitivity, giving more variable results than analytical methods like gas chromatography-mass spectrometry. The method for sample preparation is also critical for sensitive and reliable measures (Marullo et al., 2012).

The level of inactive esterified cholesterol, cholesterol esters, is increased in HD mouse models and post-mortem brain tissues (**Table 1**). This may be a compensatory mechanism to reduce cholesterol accumulation since the conversion in this inactive form facilitates cholesterol transport by increasing the amount of

TABLE 1 | Impairment of cholesterol metabolism in HD models and patients.

Alteration of cholesterol homeostasis	Experimental model	References
Decreased cholesterol synthesis		
Decreased levels of cholesterol biosynthesis enzymes	Striatal derived cells (67Q, 105Q, 118Q), YAC128, HdhQ140/7, cell line ST14A, R6/2 mice Human striatum, cortex and fibroblasts	Sipione et al., 2002; Valenza et al., 2005, 2010, 2015b; Samara et al., 2014
Decreased SREBP nuclear translocation	mHTT cell line ST14A R6/2, zQ175 mice, YAC128	Valenza et al., 2005; Kacher et al., 2019; Di Pardo et al., 2020
Reduced levels of lathosterol, lanosterol	R6/2, R6/1, YAC128 mice Human plasma	Valenza et al., 2007a,b; Leoni et al., 2011; Kreilaus et al., 2015; Boussicault et al., 2016
Reduced sterol content (myelin, synaptosomes)	R6/2 mice	Valenza et al., 2010
Reduced brain levels of desmosterol	YAC128 mice	Valenza et al., 2007a
textcolorredReduced lathosterol	HD transgenic rat YAC46, YAC72, YAC128, zQ175, Q80, Q111, Q175, HdhQ150 mice	Valenza et al., 2010; Trushina et al., 2014; Shankaran et al., 2017; Kacher et al., 2019
Reduced capacity to activate cholesterol biosynthesis	Human fibroblasts	Valenza et al., 2005
Increased lathosterol	Human post mortem putamen	Kreilaus et al., 2016b
Increased desmosterol (late stages)	R6/1 mice Human post mortem putamen	Kreilaus et al., 2015, 2016b
Impairment of cholesterol levels		
Reduced total cholesterol content	mHTT cell line ST14A HdhQ140 primary neurons	Valenza et al., 2005; Ritch et al., 2012
Reduced levels of cholesterol	YAC72, YAC128, HdhQ111, HdhQ150 mice, Q80, zQ175, R6/2 mice, HD transgenic rat Human plasma	Valenza et al., 2005, 2007a, 2010; Leoni et al., 2011; Trushina et al., 2014; Shankaran et al., 2017
No changes of cholesterol levels	R6/2 mice Human plasma	Valenza et al., 2007b; Leoni et al., 2008
Increased cholesterol levels	R6/2, YAC72 mice Primary striatal neurons and cell lines from HdhQ111 mice Primary striatal neurons from HdhQ150 and YAC72 mice Human caudate and putamen	Trushina et al., 2006, 2014; del Toro et al., 2010; Boussicault et al., 2016; Kreilaus et al., 2016b
Increased cholesterol levels and esters	Striatal neurons infected with lentiviral Htt171-82Q	Luthi-Carter et al., 2010
Increased levels of cholesterol ester	zQ175 (striatum, synaptic fraction) Human (caudate-putamen, cerebellum)	Phillips et al., 2020; Iuliano et al., 2021
Decreased cholesterol degradation		
Decreased CYP46A1 expression	R6/2, zQ175 mice STHdhQ111/Q111 cells Human post mortem brain	Boussicault et al., 2016; Kreilaus et al., 2016b; Kacher et al., 2019
Impairment of oxysterols levels		
No changes of 24S-OHC brain levels	R6/2, zQ175 mice	Valenza et al., 2007b; Kacher et al., 2019
Reduced levels of 24S-OHC	HD transgenic rat zQ175, R6/1, YAC46, YAC72, YAC128, HdhQ111 mice Human post-mortem brain	Valenza et al., 2007a, 2010; Kreilaus et al., 2015, 2016b; Shankaran et al., 2017
Reduction of plasma 24S-OHC	Q80, Q111, zQ175 mice Human plasma	Leoni et al., 2008, 2011, 2013; Shankaran et al., 2017
Decreased levels of 27-OHC	Human plasma, R6/1 mice, zQ175	Leoni et al., 2011; Kreilaus et al., 2015; Kacher et al., 2019
Increased levels of 27-OHC, 7β-OH, 7-keto cholesterol	Human post-mortem brain	Kreilaus et al., 2016b
Impairment of cholesterol transport		
Reduced ApoE production and/or release	HdhQ140/7, HdhQ50/7, HdhQ111/7, R6/2, Primary astrocytes (YAC128) YAC128 mice	Valenza et al., 2010, 2015b

Rodent models in purple, cell cultures in orange and human samples in blue. Decreased levels are in red, increased levels in green.

cholesterol packaged in lipoproteins. The product of cholesterol degradation in the brain, 24S-OHC is decreased in the brain of several HD models and patient tissues. Interestingly, 24S-OHC levels decrease in patient plasma at early stages and correlate with motor impairment and caudate atrophy (measured by MRI) (**Table 1**). 24S-OHC seems to be an interesting biomarker for HD progression, although other peripheral factors have to be considered to measure its accurate levels in the blood, such as plasma lipoproteins turnover and the rate of excretion of oxysterols by the liver (Leoni and Caccia, 2013).

Cholesterol transport by astrocytes is less efficient in HD, with decreased expression of apolipoprotein E (ApoE) and ABCA1. Astrocytes expressing mHTT produce and release less ApoE, impacting cholesterol transport to neurons (**Table 1**). Reduced transport might be associated with a decreased activity of LXR in the context of HD. Indeed, LXR are nuclear receptors, which bind oxysterols (including 24S-OHC) and desmosterol, and regulate the transcription and expression of protein involved in cholesterol transport like ApoE and ABCA1 (Abildayeva et al., 2006). LXR activation is also important for the expression of the cholesterol synthesis enzymes FDFT1 (Farnesyl-Diphosphate Farnesyltransferase 1) and CYP51 (lanosterol C14 demethylase) (Wang et al., 2008), as well as for oligodendrocyte differentiation and myelination (Xu et al., 2014; Meffre et al., 2015), and modulation of inflammation (Zelcer et al., 2007; Morales et al., 2008). In normal conditions, HTT acts as a co-activator for LXR transcription factors, and HTT mutation can potentially lead to a loss of this function and thus a decreased LXR activity (Futter et al., 2009). The Peroxisome proliferator-activated receptor gamma coactivator-1 alpha (PGC-1 α) is a key protein that regulates energy metabolism and in particular mitochondrial biogenesis. Of interest in HD, mHTT decreased PGC1α expression and knockout of PGC1α causes a reduction of cholesterol synthesis and degradation and is associated with a defect of myelination (Xiang et al., 2011). Especially, a knockout of PGC1α in oligodendrocytes affects cholesterol biosynthetic pathway, by decreasing expression of HMGCS1 and HMGCR (Xiang et al., 2011).

The whole body cholesterol metabolism seems to be altered in HD. Cholesterol metabolism changes may also results of dysfunction related to food digestion and absorption. In this regard, increased intestinal permeability and dysbiosis in the R6/2 mouse model of HD was observed (Kong et al., 2020; Stan et al., 2020). Gut dysbiosis was also shown in HD patients with associations among gut bacteria, cognitive performance and clinical outcomes (Wasser et al., 2020). As the gut-brain axis seems to be important for brain function (Dinan and Cryan, 2017), the involvement of gut dysbiosis in brain cholesterol metabolism dysfunction in HD cannot be excluded.

Cellular Consequences of Altered Cholesterol Metabolism in Huntington's Disease

Altered cholesterol metabolism can have deleterious impacts at several levels. Modification of cholesterol content and dynamics can influence membrane fluidity and the distribution of micro-domains. The study of peripheral cells from HD patients showed an alteration of membrane properties and fluidity related to differences in cholesterol and phospholipid content (Muratore, 2013). The number of ordered domains was higher in mHTT expressing neurons, suggesting that cholesterol accumulation is associated with an increased amount of lipid rafts. This was accompanied by an increased localization of NMDA-R in the cholesterol-enriched domains, thus altering NMDA-R distribution, and potentially contributing to NMDA-mediated excitotoxicity. Moreover, administration of cholesterol lowering drugs such as simvastatin and β-cyclodextrin protected against NMDA mediated excitotoxicity (del Toro et al., 2010). Cholesterol can also influence HTT binding and aggregation to membranes, with decreased HTT insertion as cholesterol content increases (Gao et al., 2016).

More recently, analysis of mouse model striatum and post-mortem putamen showed a disrupted localization of synaptic proteins and lipids important for synaptic function, dependent of age, with an altered integrity of synaptic compartments in HD mice (Iuliano et al., 2021). Aberrant interaction between mHTT and caveolin-1 impairs the intracellular trafficking of cholesterol, leading to cholesterol accumulation in the Golgi and lysosomes. This accumulation could affect the normal function of the Golgi apparatus and lysosomes, contributing to cellular toxicity. Interestingly, loss of caveolin-1 in HdhQ150 mouse model was able to rescue cholesterol trafficking and motor decline in these mice (Trushina et al., 2006, 2014).

A proper supply of cholesterol is critical for neurite outgrowth, synapses and dendrites formation, along with axonal guidance (Goritz et al., 2005; Fester et al., 2009). A global alteration of cholesterol metabolism can affect neurotransmission, and cholesterol depletion leads to a decreased synaptic plasticity and neurite degeneration caused by a neurodegenerative break of neurofilaments integrity (Koudinov and Koudinova, 2005). Moreover, targeting cholesterol synthesis by inhibition of HMGCR causes neurite loss by interfering with the melanovate pathway (Schulz et al., 2004). At late stages in the transgenic R6/2 HD mouse model, cholesterol content decreases in myelin, highlighting a potential link between cholesterol impairment and myelin defects in HD (Valenza et al., 2010). Since cholesterol in myelin is critical for efficient transmission of the action potential, this defect could impair neuronal transmission. Thus, a global alteration of cholesterol metabolism can affect neurotransmission.

Studies showed that mHTT decreased mitochondrial membrane fluidity in *STHdh* cells, in isolated mitochondria from HD knock-in mice and BACHD rats, while mitochondrial cholesterol levels only decreased in BACHD rats (Eckmann et al., 2014). The authors concluded that cholesterol levels might not be the only determinant of membrane fluidity changes found in mitochondria isolated from different HD models.

Several strategies can be used to manipulate and modulate the cholesterol metabolism pathway and cholesterol content in order to improve cell survival in HD. For example, the addition of cholesterol rescued the cell death induced by mHTT in human HD cell lines (Valenza et al., 2005) and, when supplemented in nanoparticules *in vivo*, partially alleviated HD phenotype in a mouse model (Valenza et al., 2015a). In HD primary cultures, where mHTT increased cholesterol level, inhibition of sirtuin 2 decreased nuclear trafficking of SREBP-2, cholesterol synthesis and protected from mHTT toxicity (Luthi-Carter et al., 2010). Of interest, addition of cholesterol precursors lanosterol and desmosterol are neuroprotective *in vitro* on primary cultures of striatal neurons expressing mHTT (Boussicault et al., 2016; Kacher et al., 2019), showing a global need

to compensate for impaired cholesterol metabolism through cholesterol precursors.

ROLE OF ASTROCYTES AND MICROGLIA IN HUNTINGTON'S DISEASE AND CONSEQUENCES IN CHOLESTEROL DYSREGULATION

Cellular Dysfunctions in Huntington's Disease Astrocytes

Astrocytes are critical component of the CNS, where they play an important role in the maintenance of brain homeostasis and neuronal function. They are involved in the neurovascular coupling to ensure the supply of neurons, in maintaining extracellular ion balance, supporting synaptogenesis, supplying nutrients to neurons and have an important role in neurotransmission. Astrocytes regulate the levels of neurotransmitters (Glutamate, GABA) and ion homeostasis at the synapse, they also release gliotransmitters to influence synapse functions (Wilton and Stevens, 2020). Therefore, the alteration of their normal function can affect the global CNS homeostasis and functions. Although neurodegeneration in HD is classically associated with cellular dysfunctions in striatal MSN, the contribution of glial cells in this neuronal pathogenicity must be taken into consideration. Studying glial alteration could also help to understand the region-specific susceptibility observed in HD.

In HD, mHTT aggregates accumulate less in astrocytes than in neurons (Jansen et al., 2017), possibly because of a faster degradation of mHTT in astrocytes (Zhao et al., 2016). However, several cellular alterations are found in HD astrocytes, such as a reduction of astrocyte surface area, alteration of differentiation, reduced association with neuronal connexions, altered regulation of neurotransmitter release and uptake from synapse, and reduction of intracellular calcium signaling pathway (Wilton and Stevens, 2020). mHTT-induced transcriptional dysregulations can also occur within astrocytes, including a downregulation of mRNA levels coding the glutamate uptake transporter GLT1 and the potassium channel Kir4.1. GLT1 mRNA and protein levels are decreased early in the putamen of HD patients and correlates with disease severity (Arzberger et al., 1997; Behrens et al., 2002). The decrease of astrocytic GLT1 levels in R6/2 mice induces a decrease of glutamate re-uptake, associated with an increased level of extracellular glutamate, which contribute to the excitotoxicity described in striatal neurons. When mHTT is only expressed in astrocytes, GLT1 expression is affected, glutamate uptake is altered, dysfunction of striatal neurons and motor abnormalities are observed (Bradford et al., 2009; Faideau et al., 2010), arguing for an astrocyte specific effect of mHTT in the HD pathogenesis. Interestingly, treating symptomatic R6/2 mice with ceftriaxone, an antibiotic known to increase GLT1 expression, prevents the decrease of glutamate uptake as well as the motor performance deficits (Miller et al., 2008). However, the GLT1 ablation does not worsen motor deficits and weight loss in R6/2 mice (Petr et al., 2013). Protein levels of the glutamate transporter GLAST are also decreased in the striatum and cortex of R6/2 mice (Estrada-Sánchez et al., 2009), associated with neuronal vulnerability, and the selective inhibition of both GLT1 and GLAST in R6/2 brain slice worsen electrophysiological properties of HD cortical neurons (Estrada-Sánchez et al., 2019). The astrocytic Kir4.1 channel has a role in the influx of potassium and therefore it is essential to support MSNs electrophysiological properties (Nwaobi et al., 2016). The reduced expression of Kir4.1 in astrocytes in R6/2 and zQ175 mice induces an increase of striatal extracellular K^+ level, which might underlie the hyperexcitability of MSNs in HD. The restoration of Kir4.1 channel expression in R6/2 mice, restores normal extracellular K^+ level, rescues MSN excitability and improves motor phenotype (Tong et al., 2014).

Another important feature in HD is astrocyte reactivity. In response to a homeostatic dysregulation, astrocytes become reactive, and present transcriptional, morphological and functional changes. Reactive astrocytes become hypertrophic and present an increased GFAP (Glial Fibrillary Acidic Protein) expression, which is observed in the striatum of HD mice and in post-mortem striatal tissues from HD patients at early stages. The number of GFAP positive cells correlates with the disease severity, and spread latter in the cortex of HD patients (Faideau et al., 2010). A deleterious role of reactive astrocytes has been shown in several neurodegenerative diseases (Ben Haim et al., 2015a), such as Alzheimer's disease (AD), where blocking astrocyte reactivity in mice reduces amyloid deposition, improves synaptic functions and spatial learning (Ceyzériat et al., 2018). By contrast, the prevention of astrocyte reactivity in HD, using an endogenous inhibitor of the JAK/STAT3 pathway, increases the number of mHTT aggregates, but does not affect neuronal death (Ben Haim et al., 2015b). In HD mouse model, mHTT nuclear inclusion are less numerous in GFAP-positive astrocytes (4–10%), as compared with S100β astrocytes (30%) (Jansen et al., 2017), which could be explained by an increased proteasome activity in GFAP-positive astrocytes (Orre et al., 2013). These data suggest a protective role of reactive astrocytes in HD, due to their higher ability to clear mHTT aggregates.

Besides astrocyte reactivity, mHTT expression in astrocytes plays a key role in HD pathogenesis, as demonstrated in several studies, which showed that astrocyte-specific expression of mHTT is sufficient to induce age-dependant HD phenotype, including motor dysfunction, weight loss, shortening of life span (Bradford et al., 2009), along with neuronal excitotoxicity in neurons-astrocytes co-cultures (Shin et al., 2005). mHTT expression in astrocytes also induces a decrease of BDNF transcription and release *in vitro* (Wang et al., 2012) due to an impairment of BDNF-containing vesicles exocytosis (Hong et al., 2016). The striatal engraftment of mHTT-expressing astrocytes in WT mice induces some HD phenotype, with an alteration of motor performance and a MSNs hyper-excitability, compared to control (Benraiss et al., 2016). Conversely, the prominent neuronal alterations exhibited by HD models can be prevented or reversed by WT astrocytes, such as mHTT-mediated neurotoxicity *in vitro* (Shin et al., 2005) and the striatal engraftment of WT astrocytes in R6/2 mice slows disease progression, improves electrophysiological and

behavioral alterations, restores K^+ homeostasis and increases cell survival (Benraiss et al., 2016). Altogether, these results show how astrocyte alterations are deleterious for neuronal functions. Recent studies highlighted the importance of astrocyte regional heterogeneity, which might explain the regional specific vulnerability of striatal MSNs. For example, *in vitro* HD striatal astrocytes release higher amount of the pro-inflammatory mediator TNFα, toxic for neurons, compared to HD cortical astrocytes. BDNF treatment is protective for HD cortical and striatal astrocytes, inducing an increase of GLT1 expression, but conditioned medium from HD striatal astrocytes treated with BDNF is protective for HD striatal neurons only (Saba et al., 2020). HD is also associated with several metabolism alterations, including low glucose levels in HD mouse brain (Grafton et al., 1992). Astrocytes take up glucose from the blood, metabolize it into lactate and redistribute it to neurons, to ensure neuronal activity (Pellerin and Magistretti, 1994). Glucose uptake is decreased in HD mouse striatum and *in vitro* overexpression of mHTT in astrocytes indirectly impairs glucose uptake in neurons and triggers oxidative stress (Boussicault et al., 2014). To counteract the low glucose levels in a HD brain, astrocytes adapt by metabolically reprogramming their mitochondria to use endogenous and non-glycolytic metabolites as a substitute for energy production, depending on metabolic pools available in each CNS regions. Indeed, striatal astrocyte mitochondria switch to fatty acid oxidation for fuel, with the cost of inducing the production of ROS, toxic for neurons, whereas astrocyte mitochondria from cerebellum use amino-acids precursors for glucose, not associated with ROS production (Polyzos et al., 2019). This metabolic reprogramming depending on brain region might be another cue to better understand specific region vulnerability of striatal neurons in HD. According to these studies, astrocyte dysfunctions need to be carefully considered as a contributor of HD pathogenesis.

Cholesterol Metabolism and Astrocytes

As mentioned above, another metabolic pathway ensured by astrocytes is cholesterol metabolism, which is strongly impaired in HD. Cultures of HD astrocytes, from different mouse models, show a downregulation of mRNA levels for cholesterol biosynthesis (HMGCR, CYP51, 7DHCR) and efflux (APOE, ABCA1) genes, associated with a decrease of APOE protein levels and secretion of APOE lipoprotein in the medium. HD astrocyte conditioned medium is detrimental for neurons, and does not support synaptic activity (Valenza et al., 2010, 2015b). Enhancing glial cholesterol biosynthesis or transport, with overexpression in astrocytes of SREBP2 or ABCA1, reverses neuronal dysfunctions in HD (Valenza et al., 2015b), supporting the involvement of astrocytic cholesterol metabolism in neuronal survival. Transcriptional dysregulation occurs in HD astrocytes, and a recent study (Benraiss et al., 2021) investigated the specific cell-type changes in gene expression associated to mHTT in R6/2 and zQ175 mice using fluorescence-activated cell sorting (FACS) followed by RNAseq analysis. Astrocytes from R6/2 mice -which overexpress the cleaved version of mHTT corresponding to the exon 1 of the gene- displayed a downregulation of cholesterol biosynthesis genes (SREBF2, INSIG1, CYP51A1, HMGCR, IDI1, HMGCS2, DHCR24, MVD), cholesterol sensors (INSIG1, INSIG2), and cholesterol uptake (LDLR), while cholesterol efflux (ABCA1) was increased, suggesting an abnormal response of HD astrocytes to cholesterol levels. Noteworthy, the cholesterol pathway genes were not altered in isolated astrocytes from zQ175 mice – which express the full-length mHTT. Comparison of these results to *in vitro* human striatal astrocytes expressing either full-length mHTT or exon1-mHTT showed a similar pattern of genes deregulation, with cholesterol pathway deregulation only in exon1-mHTT human astrocytes. Although these data support the implication of astrocytic cholesterol metabolism dysregulation in HD.

Other cross talks between neurons and astrocytes are necessary for the maintenance of cholesterol homeostasis, and for regulating cholesterol synthesis in neurons. APOE particles from astrocytes to neurons contain, besides cholesterol, non-coding microRNAs that will silence neuronal genes encoding cholesterol biosynthesis enzymes (HMGCR, HMGCS1, CYP51) resulting in a downregulation of *de novo* cholesterol synthesis in neurons (Li et al., 2021). Exploring a link between cholesterol metabolism in astrocytes and BDNF in HD could also help to better understand astrocyte alterations and their involvement in neuronal pathogenesis. Indeed, BDNF stimulates cholesterol biosynthesis and efflux, through ABCA1 and APOE expression in WT astrocytes, and BDNF levels are reduced in HD striatum, due to a decrease of supply from cortical neurons and astrocytes. Altogether, these studies underlie the importance of maintaining a correct cholesterol homeostasis in astrocytes and an efficient neuron-astrocyte cross-talk.

Cellular Dysfunctions in Huntington's Disease Microglia

Microglia are the resident immune cells in the brain, they monitor the environment and become activated in response to a stimulus to maintain brain homeostasis. In the resting state, they also play an important role in brain development, neurogenesis, synapse maturation and plasticity, they are able to contact neurons with highly motile processes. When microglia become activated, they ensure a phagocytic role, and are involved in synaptic plasticity, neuronal growth and survival (Wilton and Stevens, 2020). Activated microglia have been largely described for their inflammatory function; they can either be neurotoxic or protective depending on the factors released, the duration of activation and the state of activation: pro-inflammatory M1 microglia or pro-repair anti-inflammatory M2 microglia (Yang et al., 2017).

Huntington's disease is associated with several microglial dysfunctions. mHTT inclusions are present in a very few proportion of microglia from the striatum and the frontal cortex in R6/2 and zQ175 mice (Jansen et al., 2017). However, microglia activation is observed in HD patient post-mortem brains (Singhrao et al., 1999), increasing with disease severity, and correlates with neuronal loss suggesting an involvement of microglia in HD pathogenesis (Sapp et al., 2001). A significant increase of microglial activation was observed by Positron

Emission Tomography (PET) in the striatum of HD patients, correlating with HD severity and striatal D2 MSNs dysfunction (Pavese et al., 2006). However, this signature in brain patients seems to be dependent on the PET methodology used. Indeed, microglia activation was detected by PET in pre-symptomatic gene carriers patients, prior to symptom onset (Tai et al., 2007; Politis et al., 2010, 2015), suggesting that it is an early event in HD. Conversely, in a recent PET study using another type of radio-ligand (Rocha et al., 2021), microglia activation in basal ganglia of HD patients occurred later in the course of the disease. HD microglia display morphology changes and reduced motility, which is essential for their monitoring functions (Wilton and Stevens, 2020). Differences in the disease stages of HD microglia alteration are also described between various HD mouse models. FACS isolated microglia from R6/2 mice display transcriptional alterations early in the disease that increase with age, while transcriptional perturbations in zQ175 mice appear latter. These transcriptional modifications are associated to different cellular pathways, with an alteration of inflammation genes pathway in R6/2 microglia, and an alteration of genes associated to cell–cell contact and morphology in zQ175 microglia (Benraiss et al., 2021).

Huntington's disease microglia is highly associated with a pro-inflammatory phenotype, deleterious for neurons. The levels of pro-inflammatory cytokines IL-6, IL-8, TNFα increase with disease progression in the plasma and cerebrospinal fluid of pre-manifest carrier gene patients (mean of 16 years before symptom onset) (Björkqvist et al., 2008), and are considered as an early event in HD. In the plasma of HD patients, the levels of two anti-inflammatory cytokine (IL-4, IL-10) increase significantly later in the disease, i.e., in moderate stages, suggesting an adaptive response to the early pro-inflammatory response (Björkqvist et al., 2008). Pro-inflammatory mediator levels follow a brain specificity, with an up-regulation of IL-6, IL-8, TNFα, CCL2, IL-10, MMP9 in port-mortem HD human striatal tissue, but only IL-6, IL-8 and MMP9 in the cortex and cerebellum, CCL2 (Björkqvist et al., 2008; Silvestroni et al., 2009). The upregulation of pro-inflammatory cytokines has been also reported in the serum of several HD mouse models (IL-6, IL-10, IL-1β, IL-12p70) (Björkqvist et al., 2008), in primary cultures of HD microglia isolated from R6/2 mice (IL-6, TNFα) (Crotti et al., 2014), and in microglia derived from HD human pluripotent stem cells (PSC) (IL-6, TFNα) (O'Regan et al., 2021). This pro-inflammatory phenotype of HD microglia is linked to a cell-autonomous mechanism as specific expression of mHTT in microglia induces pro-inflammatory transcriptional activation (Crotti et al., 2014). A strategy to reduce pro-inflammatory cytokines in HD mice prevents motor impairment, restores neuronal DARPP32 levels and expands life span (Siew et al., 2019).

Overall morphology, transcriptional profile, function alteration, and pro-inflammatory phenotype of microglia might underlie neuronal degeneration in HD. Since microglia has a crucial role in synaptic pruning, maturation, maintenance, their dysfunctions could be linked to HD synaptic alteration, including electrophysiological modifications in MSN and a decreased dendritic spine density. Neuronal damages in the striatum of R6/2 mice are associated with an alteration of microglia-synapse interaction. HD microglia display a more mature morphological phenotype, increase phagocytosis across the age and make fewer contact with synaptic structure, as compared with control mice. This is related with a disruption of synaptic contact localization and synaptic density in R6/2 mice (Savage et al., 2020). Altogether, these data suggest that, like astrocytes, microglia and their interaction with other cell types in the CNS need to be considered as a contributor of HD. Indeed, the expression of mHTT in microglia has a toxic effect when co-cultured with WT neurons. Moreover, the lipopolysaccharide- induced-microglia activation leads to a higher neuronal death in HD mice as compared with control, which might be due to an over-inflammatory response in mHTT-expressing microglia (Crotti et al., 2014). Reactive astrocytes can also produce pro-inflammatory and/or anti-inflammatory molecules, and activated microglia can induce the activation of pro-inflammatory astrocytes, favoring cell death (Liddelow et al., 2017). Microglia release exosome containing RNA identified to be part of hub genes and protein networks known to have a role in neurodegenerative diseases, including HD, Parkinson's disease (PD), AD, through immune inflammation and oxidative stress pathways (Xie et al., 2022). In parallel to the deleterious effect of mHTT in microglia for brain homeostasis, several studies focused on the consequences of the depletion of either mHTT in microglia, or HD microglia. The depletion of mHTT selectively in microglia does not rescue behavioral or neuropathological phenotype in BACHD mouse model, whereas mHTT depletion in all other cell type except microglia rescue behavioral phenotype and striatal volume (Petkau et al., 2019). Conversely, the depletion of microglia in R6/2 mice, prevents some motor and cognitive deficits, mHTT aggregates accumulation, astrogliosis, and striatal volume loss (Crapser et al., 2020).

Microglia can affect neuronal functions and survival in HD, but a bidirectional interaction and the impact of HD neurons on microglia also need to be explored. Indeed dynamic of microglia processes can be regulated by neuronal activity (Liu et al., 2019), and conditioned medium from human striatal neurons expressing mHTT is toxic for HD human pluripotent stem cell-derived microglia (O'Regan et al., 2021). The neuro-inflammation observed in HD most probably arise from a combination of cell-autonomous and non-cell autonomous activation of microglia, with a communication between damaged neurons, reactive astrocytes and microglia. The cross talk between these cellular populations is a key point to understand CNS alterations in HD.

Cholesterol Metabolism and Microglia

So far, there are no studies demonstrating a direct link between cholesterol metabolism dysregulation and microglia alterations in HD. However, some directions might be of interest to explore such as a link between microglia and myelin deficits. Indeed, myelin contain 70% of total CNS cholesterol, and HD is associated with a decrease of myelinisation process and an age-dependent demyelination (Huang B. et al., 2015). Following myelin impairment, microglia induces the phagocytosis of myelin debris (Reichert and Rotshenker, 2003), associated

with a production of pro-inflammatory cytokines. Therefore, microglia have an important role in cholesterol clearance, and is essential for maintaining cholesterol homeostasis in the brain, thus avoiding its neurotoxic accumulation. Cholesterol debris clearance by microglia could be linked to the altered activity of the nuclear receptor LXR. Indeed, HD is associated to a downregulation of LXR target genes, and a decreased level of LXR ligand (desmosterol, 24S-OHC), suggesting a reduced LXR activity. In demyelinated regions, desmosterol is synthetized in microglia during myelin debris phagocytosis, inducing LXR activation, followed by inflammation resolution, stimulation of oligodendrocyte differentiation and increased cholesterol efflux from microglia for supporting re-myelinization process (Berghoff et al., 2021). Therefore cholesterol homeostasis is important for microglia phagocytosis functions, and *in vitro* treatment of microglia with simvastatin, a cholesterol lowering drug, alters microglia phagocytosis, BDNF and inflammatory factors (IL1-β, TNFα) release (Churchward and Todd, 2014), described to be downregulated in HD (Björkqvist et al., 2008). Cholesterol is also important for microglia survival (Bohlen et al., 2017). Therefore, exploring microglial phagocytosis of myelin debris in HD could provide new insight into understanding cholesterol metabolism in HD and its involvement brain homeostasis.

CLINICAL STRATEGIES TO MANIPULATE CHOLESTEROL METABOLISM IN CENTRAL NERVOUS SYSTEM

Brain cholesterol content must be tightly regulated to ensure normal brain function and its dysregulation is linked to neurodegenerative diseases for not only HD but also AD and PD (Dai et al., 2021). Based on these reports, cholesterol metabolism is a potential therapeutic target for neurodegenerative diseases (**Figure 3**). Statins are a class of molecules used to lower cholesterol by inhibiting HMGCoA-R, the rate-limiting enzyme in cholesterol pathway synthesis. These molecules, which are capable of crossing the blood-brain barrier, confer suitable drugs to regulate brain cholesterol. Over the past decade, preclinical and clinical studies using statins in AD and PD have been reported with controversial findings. In AD, several studies showed that statins can reduce the risk of the disease (Wolozin et al., 2000; Rea et al., 2005; Sparks et al., 2005; Feldman et al., 2010; Lin et al., 2015) whereas no therapeutic effect was described in other studies (Rea et al., 2005; Feldman et al., 2010). The clinical studies in PD have shown that the use of statins can reduce the risk of the disease (Bai et al., 2016; Sheng et al., 2016), whereas other studies describe no effect of these molecules (Huang X. et al., 2015; Liu et al., 2017; Rozani et al., 2017; Lin et al., 2021). If statins are primary used to lower cholesterol content, these molecules also show anti-inflammatory, anti-oxidative, and anti-excitotoxic properties that need to be considered in therapeutic options for neurodegenerative diseases, especially in HD. Lowering sterol levels by statin treatment is not intuitive in HD clinical strategy as the content of cholesterol precursors and enzyme activity are decreased within the brain. However, statin assay can be relevant to reduce the inflammatory response, oxidative stress and excitotoxicity that occur in HD. In HD cellular models, administration of simvastatin protected against NMDA mediated excitotoxicity by reducing the content of lipid rafts domains in the plasma membrane of mHTT cells (del Toro et al., 2010). In quiescent HD fibroblasts an impairment of the ATM-dependent signaling and repair pathways of the DNA double-strand breaks by the non-homologous end-joining (NHEJ) repair process has been described after ionizing radiation (Ferlazzo et al., 2014). In this study, a combination treatment with statin and bisphosphonate, which inhibits membrane farnesylation of the nucleus-, increased the nucleo-shuttling of ATM kinase and improved DNA double-strand repair by NHEJ. In the experimental HD rat models induced by quinolinic and malonic acids or 3-NP, simvastatin showed beneficial effects on HD phenotype (Patassini et al., 2008; Ahmed et al., 2016). In the quinolinic acid HD rat model, simvastatin induced immunoreactivity for Bcl-2, an anti-apoptotic factor, on one hand, and down-regulated immunoreactivity for Bax, a pro-apoptotic factor (Patassini et al., 2008). Atorvastatin and simvastatin treatment alleviated malonic acid induced HD like symptoms and related cognitive dysfunctions (Kumar et al., 2013). In particular, the motor behavior was improved, with reduced oxidative stress, restoration of mitochondrial dysfunction, increased mitochondrial complex I, II, III, and IV activities, and reduced neuro-inflammation accompanied by a decrease of TNF-α and IL-6 levels. In the 3-NP HD model, simastatin can decrease neurotoxicity, mitochondrial dysfunction through SDH activity regulation and inflammatory response by modulating the nitric oxide synthases eNOS and iNOS activity (Ahmed et al., 2016). Electrophysiological studies addressed the effects of simvastatin on striatal activity of MSNs from symptomatic R6/2 mice and showed an increased frequency of spontaneous inhibitory postsynaptic currents compared with controls. Simvastatin treatment decreased sIPSCs of R6/2 slices through a mechanism that needs to be determined (Chen et al., 2016).

So far, no clinical trials have been conducted to evaluate the effect of statin in HD. One study used the Enroll-HD database to investigate if statin use among patients with premotor HD can be associated with beneficial effects (Schultz et al., 2019). In patients with premotor HD, statin use was associated with a delayed motor diagnosis of HD suggesting that statin may provide a neuroprotective benefit in the early course of HD. It seems reasonable to encourage clinical trials of statins in HD because of their effects on modulating the inflammatory response and oxidative stress rather than their ability to regulate cholesterol metabolism.

As mentioned above, reduced synthesis of brain cholesterol precursors is well described in the literature whereas the cholesterol content is the subject of controversy: studies reported an increased accumulation of free cholesterol whereas a decrease of cholesterol content is also described (**Table 1**). The temporal alterations of cholesterol metabolism needs to be considered as HD progresses. Dietary supplementation to regulate cholesterol metabolism has been performed using anthocyanin and Manganese (Kreilaus et al., 2016a; Pfalzer et al., 2019). Anthocyanin is highly concentrated in berry extracts

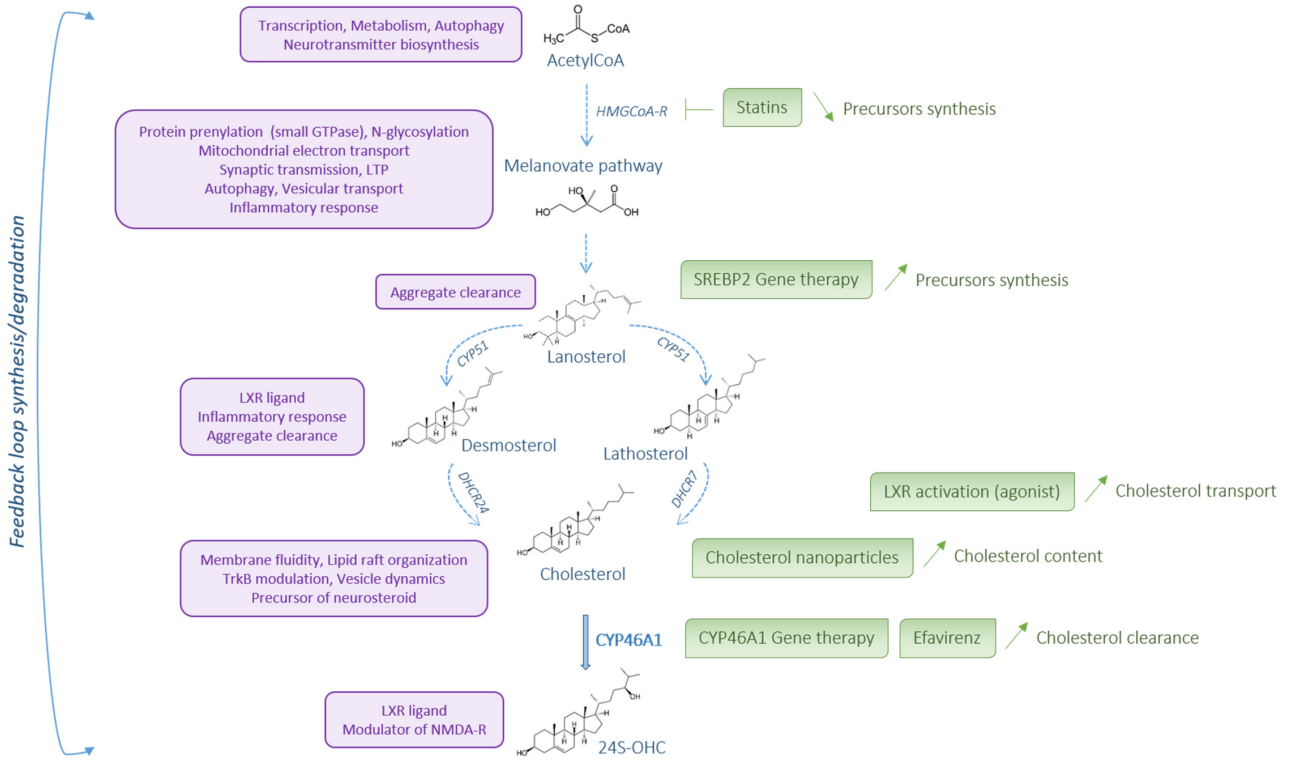

FIGURE 3 | Cholesterol metabolism: a potential therapeutic target. Cholesterol metabolism steps are represented in blue. The role and action of the different metabolites are described on the left, in purple. On the right, in green, are listed some options for therapeutical intervention to restore cholesterol metabolism in Huntington's disease. Created with BioRender.com.

which improved cognitive function in rodents during aging (Galli et al., 2006; Duffy et al., 2008). However, anthocyanin supplementation did not influence disturbances to cholesterol synthesis whereas it improves motor performance in R6/2 mice (Kreilaus et al., 2016a). Manganese is one variable which may impact cholesterol biosyntheis but acute systemic exposure of pre-manifest and manifest YAC128 to manganese did not regulate cholesterol biosynthesis (Pfalzer et al., 2019). The team of Prof E. Cattaneo provided consistent studies to demonstrate the beneficial effect of striatal cholesterol infusion in HD mouse models. The first proof of concept was achieved using brain-permeable polymeric nanoparticles loaded with cholesterol, which can cross the blood-brain barrier to reach glial and neuronal cells in different brain regions. After repeated systemic administration of the cholesterol-loaded nanoparticles in R6/2 mice, synaptic and cognitive defects were rescued whereas global activity was partially improved and no effect on neuropathology was noticed (Valenza et al., 2015a). Furthermore, beneficial effects of cholesterol supply were confirmed using novel hybrid polymeric nanoparticles to favor BBB transit (Birolini et al., 2021a). A deeper characterization of cholesterol supply was later provided using osmotic mini pumps that can directly infuse cholesterol within the striatum (Birolini et al., 2020). In R6/2 mice, cholesterol infusion prevented cognitive defects, alleviated motor phenotype and improved synaptic transmission. Interestingly, cholesterol precursors such as lanosterol, lathosterol, desmosterol and the product of cholesterol catabolism 24S-OHC were enhanced following striatal cholesterol infusion. Another way of brain cholesterol delivery was achieved using an intranasal dose of liposomes loaded with deuterium-labeled cholesterol in WT mice (Passoni et al., 2020). If the benefits of cholesterol loading are well demonstrated, further studies are necessary to determine the long-term effect of cholesterol infusion using a chronic HD mouse model. The different ways of cholesterol administration need also to be compared in terms of safety, invasive procedure, and cholesterol dose and long-term beneficial effects before clinical trials.

Cholesterol metabolism in the brain is mainly regulated *via* two key transcription factors: the LXR nuclear receptors and the sterol SREBP2. As dysfunctions of LXR and SREBP2 in HD have been reported, manipulation of these transcription factors could be considered in clinical research for HD. The LXR family includes two isoforms: LXRα, which is mainly expressed in the liver and other tissues critical for peripheral lipid metabolism whereas LXRβ is prominently expressed in the brain (Whitney et al., 2002). LXR regulate the transcription of APOE and its lipidation transporters ABCA1 and ABCG1 (**Figure 2**) as well as the expression of the cholesterol synthesis enzymes Fdft1 (squalene synthase) and Cyp51 (lanosterol C14 demethylase) (Wang et al., 2008). Mouse genetic models generated to invalidate both isoforms showed impairment

of lipid homeostasis in the brain and neurodegeneration, illustrated by nuclear and cytoplasm condensation as well as axonal demyelination (Wang et al., 2002). Specific ablation of LXRβ impaired motor coordination associated with lipid accumulation and loss of motor neurons in the spinal cord (Andersson et al., 2005). Cholesterol transport by astrocytes is less efficient in HD, with decreased expression of APOE and ABCA1. Astrocytes expressing mHTT produce and release less ApoE, affecting cholesterol transport to neurons (Valenza et al., 2010, 2015b). Reduced transport might be associated with a decreased activity of LXR. Indeed, in normal conditions, HTT acts as a co-activator for LXR transcription factors, therefore HTT mutation can potentially lead to a loss of function and thus a decrease of LXR activity (Futter et al., 2009). Manipulating LXR activity is possible using the synthetic agonists T0901317 and GW3965 and beneficial effects of these ligands have been observed on preclinical models of AD (Suon et al., 2010; Vanmierlo et al., 2011). These compounds regulate APOE, ABCA1, ABCG1 expression, decrease cognitive defects and improve brain pathology in AD mouse models. In HD, incubation of R6/2 brain slices with T0901317 reduced the frequency and amplitude of spontaneous inhibitory postsynaptic currents compared with controls (Chen et al., 2016). The same compound can partially rescue the phenotype and the expression of LXR target genes in HTT-deficient zebrafish (Futter et al., 2009). Further studies are necessary to determine LXR agonist effects on HD preclinical model. It should be noted that in addition to their role in cholesterol homeostasis, LXR activation induces an anti-inflammatory response by decreasing the expression of many pro-inflammatory genes (iNos, IL-1β, IL-6, TNFα) (Bensinger et al., 2008). This latter point favors the need to explore LXR therapeutic targets in rodent HD model as inflammation is associated with HD pathogenesis and cholesterol metabolism is linked to neuroinflammation (González-Guevara et al., 2020). Manipulating LXR activity offers new therapeutic strategies in HD but the clinical use of these compounds is limited to the peripheral hypercholesterolemia/hypertriglyceridemia undesirable side effects. To counteract this limitation, new compounds and tools need to be designed with better brain specificity and with limited lipogenic effects attributed to LXRα in peripheral tissues (Stachel et al., 2016; Navas Guimaraes et al., 2021).

SREBP2 regulates cholesterol biosynthesis through the expression and activation of cholesterol biosynthesis genes (**Figure 2**). SREBP2 is expressed in glial cells, especially in astrocytes and oligodendrocytes, where it controls lipid synthesis (Camargo et al., 2009). Mutant HTT decreases the activity of SREBP2 by 50% in cells and mouse brain tissues (Valenza et al., 2005), and overexpression of its active form in astrocytes, *in vitro* and *in vivo* in R6/2 mice, showed beneficial effects in neurons (Valenza et al., 2015b; Birolini et al., 2021b). This suggests an important dialogue between glial and neuronal cells for proper cholesterol homeostasis. In this way, in an elegant study, a gene therapy approach was used to express the transcriptionally active form of human SREBP2 specifically in striatal astrocytes of R6/2 mice (Birolini et al., 2021b). The authors found a re-activation of the cholesterol pathway biosynthesis, associated with restoration of synaptic transmission, clearance of mHTT aggregates, and improvement of motor defects and cognitive decline.

Focus on CYP46A1 as a Therapeutic Option in Huntington's Disease

A critical role of CYP46A1 in cholesterol turnover and neuronal function, through regulation of cholesterol precursors, was well described in *Cyp46a1*$^{-/-}$ mice. In this mouse model, brain cholesterol levels were unchanged but cholesterol synthesis was reduced by 40%, potentially to compensate for the lack of degradation, showing the importance of CYP46A1 in cholesterol turnover (Lund et al., 2003). *Cyp46a1*$^{-/-}$ mice present severe cognitive deficiencies, with impairment of spatial, associative and motor learning, associated with deficiency to establishing LTP in the hippocampus. The effects on LTP were reversed by treatment with geranylgeraniol, a precursor of cholesterol from the melanovate pathway, but not by adding cholesterol, showing the importance of cholesterol turnover, with a specific and quick action of the melanovate pathway (Kotti et al., 2006, 2008). The phosphorylation levels of many proteins was altered in the brain of these mice, including GTPase proteins (RAB8, CDC42, RAC), microtubules associated and neurofilaments proteins (MAP and NEF) along with proteins involved in synaptic vesicles formation and neurotransmission (SLCs, SHANKs). Moreover, ubiquitination is increased in proteins important for cognition, cytoskeleton function and energy production (Mast et al., 2017). By contrast, the brain of transgenic mice overexpressing human CYP46A1 (C46-HA) showed increased production of 24S-OHC and enhanced synthesis of cholesterol synthesis, with higher levels of lanosterol, consistent with increased cholesterol turnover by CYP46A1 (Shafaati et al., 2011). Interestingly, the C46-HA transgenic mice showed improvement of spatial memory with increased levels of proteins involved in neurotransmission: PSD-95, synapsin-1, synaptophysin, GluN1 and phosphorylated GluN2A subunit of NMDA-R (Maioli et al., 2013). CYP46A1 overexpression in neurons *in vitro* increases neuronal dendritic outgrowth and protrusion density, associated to an enhancement of synaptic proteins in synaptosomal fractions (Moutinho et al., 2015). These effects are dependent on geranylgeranyl transferase-I (GGTase-I) activity, which in turn increase HMGCR activity and membrane levels of sGTPase Rac1, Cdc42, Rab8, and RhoA. This increase in membrane sGTPases is also observed *in vivo*, in transgenic C46-HA mice (Moutinho et al., 2015). Reduction of cholesterol content by CYP46A1 is the trigger for increased phosphorylation of TrkB receptor, TrkB interaction with GGTase-I, which allows GGTase-I activity, and consequently dendritic outgrowth. These results were replicated *in vivo*, with an increase of p-TrkB and synaptic proteins in synaptosomal fractions prepared from CYP46A1 transgenic mouse cortex (Moutinho et al., 2016). Overall, CYP46A1 has an essential role in synaptic functions by stimulating cholesterol turnover, particularly through the melanovate pathway.

Overall, these findings indicate that CYP46A1 is an interesting therapeutic target to consider for the treatment of neurological

disorders. Levels of 24S-OHC, which are directly correlated to CYP46A1 activity, are changed in several neurodegenerative diseases such as PD (Björkhem et al., 2013), AD (Bretillon et al., 2000; Lütjohann et al., 2000; Schönknecht et al., 2002), multiple sclerosis (Teunissen et al., 2003), dementia (Kölsch et al., 2004), and HD (Leoni et al., 2008). Because of these links between CYP46A1 and neurodegenerative diseases, therapeutic strategies focusing on CYP46A1 activity have been explored in preclinical studies (Petrov et al., 2019a,b). We refer to the review of Cartier and Pikuleva for further details in AD and PD (Pikuleva and Cartier, 2021). In HD, CYP46A1 expression is decreased in the post-mortem putamen of patients and the striatum of R6/2 and zQ175 mouse models (Kreilaus et al., 2015, 2016b; Boussicault et al., 2016; Kacher et al., 2019). In the WT context, knocking down CYP46A1 expression in the striatum, *via* an adeno-associated virus-mediated delivery of selective shCYP46A1, reproduced the HD phenotype, with spontaneous striatal neuron degeneration and motor deficits, as assessed by rotarod (Boussicault et al., 2016). A recent strategy of gene therapy delivery of CYP46A1 in the striatum of R6/2 and zQ175 mice allowed an improvement of neuronal atrophy, a decrease of mHTT aggregates and improved motor behavior (Boussicault et al., 2016; Kacher et al., 2019). In these mice, cholesterol metabolism was enhanced, with not only an increase of cholesterol degradation (decrease content of cholesterol, and increased production of 24S-OHC), but also an increase of cholesterol precursors content (lanosterol, desmosterol), and expression levels of cholesterogenic enzymes (HMGCR, FDFT1, CYP51, DHCR24, DHCR7) and APOE. Importantly, in HD zQ175 mice, CYP46A1 broadly affects the transcriptomic signature related to major pathways altered in HD, including synaptic transmission, vesicular transport and unfolded protein metabolism. This new transcriptomic signature showed a global compensation of altered functions in HD through stimulation of cholesterol metabolism, causing a global restoration of striatal neurons dysfunctions, including neurotransmission, spine density and axonal transport. Other studies support the role of CYP46A1 overexpression in alleviating HD phenotypes. Indeed, in a neuroblastoma culture model of HD, CYP46A1 over-expression reduces the quantity and size of mHTT aggregates, as well as the levels of mHTT protein, potentially through the activation of autophagy (Nóbrega et al., 2020). Interestingly, CYP46A1 overexpression protects against NMDA-mediated excitotoxicity in a cellular model of HD (Boussicault et al., 2018). Therefore, CYP46A1 appears like an interesting therapeutic target as a neuroprotective strategy for the treatment of HD. Consideration of CYP46A1-based gene therapy in HD are also of interest, especially since vector delivery strategies in patient brain have considerably improved (Piguet et al., 2021).

DISCUSSION

Numerous studies confirm the promising strategy of targeting cholesterol homeostasis in HD. Now it is key to define the most appropriate approach, several being currently under investigation, from gene therapy to pharmacological molecules. Optimization of these approaches is a major challenge to achieve a successful treatment for HD patients and some major pressing questions need to be addressed in this regard:

(1) How cholesterol metabolism is regulated in pathological conditions and what is the consequence on glial cell and neuron metabolism? Given that cholesterol metabolism is impaired in HD, it is of interest to determine whether other metabolic regulations occur to compensate and in which cells. In this way, the role of astrocyte-neuron substrate shuttle in fueling neurons is well described for glucose oxidation (Pellerin and Magistretti, 2012). A clearer view on the interaction between the different cell types and metabolic pathways will facilitate the elaboration of more precise treatment.

(2) What are the consequences of cholesterol metabolism restoration, especially in regards to re-instating cell homeostasis in an HD context? Since mHTT is expressed throughout development and life, compensatory processes will likely occur to mitigate deleterious effects of this mutation. In particular, protein clearance is a major cellular compensatory response to face mHTT-mediated toxicity. For instance, autophagy may be an essential factor for neuronal homeostasis maintenance and its impairment has been reported for the development of HD (Martin et al., 2015). Restoration of cholesterol metabolism through CYP46A1 activity promotes protein clearance likely by induction of autophagy (Kacher et al., 2019; Nóbrega et al., 2019, 2020). Other cell compensatory mechanisms, described in HD, should be considered such as anti-oxidative stress response and DNA damage response to determine whether cholesterol metabolism can also affect these processes.

(3) How cholesterol precursors and cholesterol catabolism products can influence HD pathogenesis? The modulation of cholesterol metabolism can regulate cellular processes at different levels. Indeed, cholesterol precursors from the melanovate pathway are involved in protein prenylation, production of dolichols, heme A and Ubiquinone (Moutinho et al., 2017). Protein prenylation is essential for synaptic transmission and intracellular signaling. Dolichols function as a membrane anchor for glycoproteins. Cholesterol itself participate to membranes fluidity, organization of lipid rafts, vesicles dynamics and decreased cholesterol content by CYP46A1 is involved in TrkB activation (Martin et al., 2008). Cholesterol precursors, including desmosterol and lanosterol are directly involved in the clearance of aggregated proteins (Zhao et al., 2015; Upadhyay et al., 2018; Kacher et al., 2019). The 24S-OHC and desmosterol are ligands for LXR and therefore can modulate gene expression. Finally, 24S-OHC is an allosteric modulator of NMDA receptors mainly involved in neurotransmission. Specific roles of these bioactive molecules in HD pathogenesis need to be studied to advance our knowledge.

(4) How cholesterol metabolism within glial cells can influence HD pathogenesis? The implication of astrocytic cholesterol metabolism dysregulation in HD has been largely described but the role of microglia and oligodendrocytes need to be explored. Astrocytes secrete a range of factors including ATP, BDNF, growth factors which maintain neuronal viability and synaptic plasticity. The specific role of astrocytic cholesterol metabolism dysregulation on neuronal viability and synaptic plasticity would bring further insights in understanding the motor, psychiatric and cognitive manifests in HD.

(5) Can altered cholesterol metabolism be a good indicator of the severity of HD? Plasma cholesterol levels are similar between control, pre-HD and HD patients. However, 24S-OHC concentrations are lower in HD patients as compared with control (Leoni et al., 2008) and parallels striatal volume reduction (Leoni et al., 2013) which make this oxysterol a good biomarker to monitor disease progression. The question is now to evaluate if this oxysterol can be proposed in addition to mHTT quantification and neurofilament light chain as a biomarker to track HD progression. From there, 24S-OHC would be an interesting marker to monitor therapeutical strategies targeting cholesterol metabolism (Sipione et al., 2002; Valenza et al., 2007a,b; Leoni et al., 2011; Ritch et al., 2012; Samara et al., 2014; Shankaran et al., 2017; Phillips et al., 2020).

AUTHOR CONTRIBUTIONS

RK: substantial contributions to the conception and wrote sections. JC: substantial contributions to the conception and wrote sections and introduction. CM: substantial contributions to the conception and wrote one section. SB: substantial contributions to the conception and wrote sections and discussion. All authors contributed to the article and approved the submitted version.

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